Review

mRNA vaccines: Past, present, future

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ARTICLE INFO

ABSTRACT

mRNA vaccines have emerged as promising alternative platforms to conventional vaccines. Their ease of production, low cost, safety profile and high potency render them ideal candidates for prevention and treatment of infectious diseases, especially in the midst of pandemics. The challenges that face *in vitro* transcribed RNA were partially amended by addition of tethered adjuvants or co-delivery of naked mRNA with an adjuvant-tethered RNA. However, it wasn't until recently that the progress made in nanotechnology helped enhance mRNA stability and delivery by entrapment in novel delivery systems of which, lipid nanoparticles. The continuous advancement in the fields of nanotechnology and tissue engineering provided novel carriers for mRNA vaccines such as polymeric nanoparticles and scaffolds. Various studies have shown the advantages of adopting mRNA vaccines for viral diseases and cancer in animal and human studies. Self-amplifying mRNA is considered today the next generation of mRNA vaccines and current studies reveal promising outcomes. This review provides a comprehensive overview of mRNA vaccines used in past and present studies, and discusses future directions and challenges in advancing this vaccine platform to widespread clinical use.

1. Introduction

Vaccines have been used since 1796 for immunization of humans and animals against viral and bacterial diseases. Their worldwide use has enabled the prevention of millions of infections and led to the complete eradication of smallpox and rinderpest diseases [1]. The first vaccines developed were based on live attenuated viruses that had lost their ability to replicate within the host but were still able to induce a strong immune response. Nowadays, they are obtained by serially passaging the virus in cell cultures or in animal embryos to promote accumulation of mutations that mediate attenuation of the virus' virulence, as is the case for vaccines against poliomyelitis, rubella, and influenza among others. Even though these vaccines showed highly

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effective and long-lasting immune response and were able to spread to non-vaccinated individuals, they were associated with the risk of mutating back to their virulent genotype and causing illness in vaccinated people as was observed with the oral poliovirus vaccine [2]. Accordingly, inactivated vaccines were developed. They consist of disrupting the genetic material and/or proteins of the infectious pathogen and hence destroying its ability to replicate by using chemical or physical techniques. While these vaccines were more advantageous regarding their higher thermostability and safety, the immune response they generated was however of shorter duration than that induced by the previously mentioned attenuated vaccines [3]. Moreover, vaccines are also used to provide immunization against bacterial diseases. The latter are often caused by bacterial toxins rather than the pathogen itself such as in tetanus, diphtheria, and pertussis. As such, chemically or physically inactivated toxins termed toxoids are employed as bacterial vaccines and they have been shown to induce moderate adaptive immunity and require as such the addition of adjuvants such as aluminum salts or booster doses to achieve proper immunity [4]. Subunit vaccines are yet another type of vaccines commonly used for prevention of infectious diseases. They consist of only one or few antigens of the infectious pathogen instead of the whole organism. Accordingly, the immune system responds by generating antibodies that target these specific antigens, whether proteins or polysaccharides, and as such, it is vital to identify the most potent antigen or combination of antigens. Similarly to toxoids, subunit vaccines require adjuvants and/or multiple or booster doses [4]. In the past few decades, recombinant viral vectors have emerged as promising vaccine platforms as they can induce potent antigen-specific cellular and humoral immune responses and do not require additional adjuvants. They are based on the delivery of viral antigens inserted in the genome of a viral vector that has maintained or lost its ability to replicate. However, they were also associated with high risks to human health such as potential integration into the host genome or persistent replication of the attenuated vaccine [5].

All these concerns and risks associated with the previously mentioned vaccines not only require additional safety studies but also delay clinical trials especially in the midst of a pandemic. From here arises the need for safer, more effective, scalable, and rapidly designed vaccines.

Messenger ribonucleic acid (mRNA) has been extensively explored since 1989 as a potential therapeutic agent for various diseases [6]. The interest in mRNA stems from its simple and inexpensive production, its transient activity and natural degradation in the human body, and its safety advantages compared to DNA therapeutics as it does not integrate the human genome, avoiding the risk of insertional mutagenesis, and is readily available for translation into protein in the cell cytoplasm. Accordingly, mRNA has been explored for cancer immunotherapy, *in vivo* protein replacement or supplementation, genome engineering and genetic reprogramming, as well as infectious disease vaccines. mRNA vaccines consist of single-stranded mRNA encoding the antigen of interest. They can be delivered as naked mRNA or enveloped in delivery systems to facilitate their internalization into cells. Once in the cytoplasm, mRNA is translated by the cell's natural translation machinery into a protein, which is later subjected to post-translational modifications to produce a fully functional protein. Upon its expression on the cell's surface, it activates immunological responses and generates a protective immunity [7].

In this review, we provide an overview of the history of mRNA vaccines, go over the recent advances in the field for prophylactic and therapeutic applications, discuss the remaining challenges and concerns as well as provide suggestions for future applications.

2. History of mRNA vaccines

2.1. *In vitro transcribed mRNA vaccines*

The pioneers of mRNA vaccines, Katalin Karikó and Drew Weissman, were long interested in mRNA as they both viewed this molecule to be the future of all therapeutic and prophylactic applications. While Karikó's primary interest was to use mRNA technology for the treatment of cystic fibrosis and strokes, Weissman was more interested in developing an mRNA vaccine for the treatment and prevention of acquired immune deficiency syndrome (AIDS), a disease caused by the human immunodeficiency virus type 1 (HIV-1). Their initial work focused on the use of HIV mRNA *ex vivo* to transfect dendritic cells (DCs), promote their maturation, and induce primary T cell responses *in vitro*, which they successfully achieved [8]. However, for more convenient vaccination, direct injection of mRNA was the main focus of research studies. The first mRNA vaccines were developed against the single-stranded RNA influenza viruses as they evolve and modify their antigens seasonally, and the production of their vaccines is still suboptimal. In fact, if a strain not included in the seasonal vaccine unexpectedly came into circulation, it would be difficult to rapidly produce a vaccine against it due to the variable yield of antigenic material from eggs or tissue culture and the long production times. Accordingly, researchers were interested in new technologies that could allow faster production and adaptation of vaccines which is vital during a pandemic.

In 1993, it was found that liposome-entrapped *in vitro* transcribed (IVT) mRNA encoding the influenza virus' nucleoprotein was successful in inducing virus-specific cytotoxic T lymphocytes (CTL) in mice that could efficiently target and lyze cells infected with the nucleoprotein or the wild type (WT) influenza virus [9]. IVT mRNA is a non-replicating synthetic single-stranded mRNA engineered to resemble fully processed mature mRNA molecules as they naturally occur in the cytoplasm of eukaryotic cells. It is produced easily, rapidly, and at large scale, by cloning the target antigen into a DNA plasmid template which is then linearized and used for *in vitro* transcription by a T3, T7, or an Sp6 phage RNA polymerase. After phenol/chloroform extraction, the resulting mRNA vector typically contains an open reading frame (ORF) encoding the antigen of interest flanked by 2 untranslated regions (5′ and 3′ UTR) which increase its half-life and enhance its translation efficiency, a polyadenylate (polyA) tail which helps further increase mRNA stability and translation efficiency, and a 5′ cap
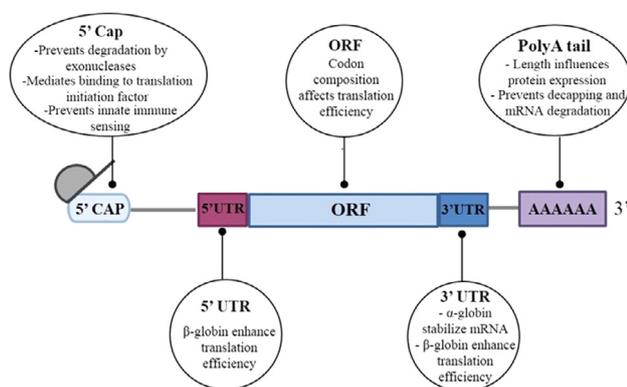
Fig. 1 – Structure of an IVT mRNA and the commonly used modification strategies.

structure which prevents mRNA degradation and innate immune sensing. 5′ cap can take on various structures: cap0 (m7G(5′)pppN1pN2p) is an N7-methylguanosine connected to the 5′ nucleotide via a 5′ to 5′ triphosphate linkage, cap1 (m7G(5′)pppN1mpNp) additionally has a 2′O methylation exists on the first ribose sugar of the 5′ end of the mRNA, and cap2 (m7G(5′)pppN1mpN2mp) has a 2′O methylation exists on the first two ribose sugars of the 5′ end of the mRNA (Fig. 1) [10].

A few studies aimed for the use of naked IVT mRNA vaccines to prevent or treat viral or cancerous diseases. Similarly to the approach adopted by Karikó and Weissman, Van Gulck et al. adopted in 2012 IVT mRNA-based DC vaccination for the treatment of HIV-1 in a phase I/II clinical trial. Six patients with chronic HIV-1 subtype B were involved in this study. Autologous monocytes-derived DCs were electroporated *ex vivo* with an IVT mRNA encoding consensus Gag protein or the chimeric Tat-Rev-Nef proteins. The transfected cells were then administered half intradermally and half subcutaneously at 4 doses every 4 weeks. This therapeutic immunization was found to be safe as only mild adverse events were recorded and successful as antiviral activity was induced. Indeed, it enhanced HIV-1 specific interferon (IFN-γ) response, cytokines release by T helper 1 cells (Th1), and T cell proliferation, which were correlated with increased HIV-1 inhibitory activity of effector CD8$^+$ T cells. These immunostimulatory and antiviral effects of the mRNA vaccine were not however uniformly observed in all patients and were effective against some but not all strains of HIV-1, which leaves room for further improvement [11]. Naked IVT mRNA vaccines were also tested in phase I/II clinical trial as immunotherapy for cancer patients. mRNA molecules encoding 6 different tumor-associated antigens (TAAs) (MUC1, CEA, Her2/neu, telomerase, survivin, MAGE-A1) were tested in 30 patients of metastatic stage IV renal cell carcinoma who were followed up for 10 years. The mRNA vaccine was administered intradermally at 4 doses with a 2-week interval for 14 patients, while the remaining 16 patients received a more intense regimen consisting of daily injections on d0–3 and d7–10 followed by single injections on d28 and d42. Patients in both cohorts were then vaccinated monthly after the induction period until tumor progression.

Immunological analysis showed significant increase in IFN-γ, CD4$^+$ and CD8$^+$ T cell responses to the TAAs. Also, the metastasized disease was stabilized for ≥ 3 months in 50% of patients and 27% of the patients had survived more than 5 years. Survival data also demonstrated a positive correlation between immunological response and long-term survival as all patients had a median survival of 24.5 months which exceeded the predicted survival. It is important to note here that while the vaccine was administered at repeated doses, it was necessary to co-deliver granulocyte macrophage colony-stimulating factor (GM-CSF) as an adjuvant to enhance the immune response to the naked mRNA (Table 1) [12].

2.2. Adjuvant-Tethered IVT mRNA vaccines

As the potential of naked IVT mRNA vaccines to stimulate the innate immune system and hence drive an enhanced adaptive immunity remained unknown, one study aimed to investigate this further. In 2017, Edwards et al. used a novel mRNA-based vaccine encoding influenza A hemagglutinin (HA) of the pandemic strain H1N1pdm09 as a model to study the innate immunostimulatory potential of IVT mRNA vaccines in an *in vitro* human innate immunity model and an *in vivo* C57BL/6 mouse model. IVT mRNA was found to induce a dose-dependent decrease in immune cell recovery, an increase in the activation and maturation of antigen-presenting cells (APCs) and B cells, as well as an increase in Th1 cytokine production of interleukin-12 (IL-12) and tumor necrosis factor (TNF-α). On the transcriptional level, mRNA induced the up-regulation of the genes involved with cellular RNA sensors or pattern recognition receptors (PRRs) in both humans and mice, mainly *ddx58* (RIG-1) and *ifih1* (MDA-5) of RIG-1 like receptors (RLRs); *tlr3*, *tlr7*, and *tlr8*-human only of Toll-like receptors (TLRs); and *clec4gp1*, *clec2d*, and *cledl1* of C-type lectin receptors (CLRs). It was found that IVT mRNA induces its phenotypic and chemokine/cytokine effects in part through IL-1 and c-Jun N-terminal kinase (JNK) pathways but more importantly through the TLR7/8 signaling pathway, implicating the involvement of both myeloid and plasmacytoid DCs. Accordingly, naked IVT mRNA vaccines act through cellular RNA sensors and follow mechanisms of action relatively conserved between the 2 species to stimulate the activation and maturation of immune cells as well as secrete factors that attract and activate key players of the innate and adaptive immune system [13]. While IVT mRNA has self-adjuvanted potential, it is in most cases insufficient to drive complete protective immunity and accordingly naked IVT mRNA vaccines would still have to be administered in intensified repeated/booster regimens, as was observed with the mRNA vaccine against Crimean-Congo Hemorrhagic Fever virus (CCHFV), and/or accompanied with co-delivered adjuvants [14]. As an alternative to co-administration of adjuvants, mRNA vaccines can be tethered to natural adjuvants such as TLR agonists and hence relieving the patient from multiple injections and their related complications. This can be achieved by hybridization of small molecule biotinylated adjuvant to the 3′UTR end of naked IVT mRNA through RNA-targeting antisense 2′O-methyl oligonucleotides with a 5′ NeutrAvidin cap. This can help enhance the immunostimulatory properties of the adjuvant

Table 1 – Naked IVT mRNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Preclinical/ Clinical Setting	Immune Response	Ref.
Naked IVT mRNA	Chronic HIV-1 subtype B	Gag Tat-Rev-Nef	Phase I/II clinical trial	- HIV-1 specific IFN-γ response - HIV-1 inhibitory activity of effector CD8+ T cells	[11]
Naked IVT mRNA + GM-CSF adjuvant co-delivery	Metastatic stage IV renal cell carcinoma	MUC1, CEA, Her2/neu, Telomerase, Survivin, MAGE-A1	Phase I/II clinical trial	- IFN-γ response - CD4+ and CD8+ T cell responses	[12]

itself without impairing its activity or significantly affecting mRNA translatability. Indeed, upon *in vivo* intramuscular injection of tethered IVT mRNA-TLR7 agonists, a more significant increase in local immune response and antigen-specific cellular and humoral responses were observed as compared to IVT mRNA and TLR7 delivered as untethered adjuvant [15]. Another research team attempted in 2018 to enhance the innate immunostimulatory potential of mRNA without addition of any adjuvant but rather by hybridization of the polyA tail of the single-stranded mRNA with a complementary polyU sequence to produce a highly immunogenic double stranded mRNA vaccine. This technique was found to enhance the immune response through TLR3 and RIG1 RNA sensors and increase DCs activation since both antigen expression and immunostimulation occurred simultaneously in the same APCs [16].

The efforts towards making IVT mRNA more immunostimulatory were still ongoing. A new technique was introduced in the year 2000, almost a decade after the first mRNA-based vaccine study, when Hoerr et al. thought of using a natural cell penetrating peptide (CPP) as a mean to render IVT mRNA a self-adjuvanted vaccine. Protamine was their CPP of choice as it is a small naturally-occurring arginine-rich DNA-binding polycationic peptide. It was complexed to the mRNA by polycondensation into nanoparticles (NPs) due to the electrostatic interaction between the negatively charged phosphate groups in mRNA and the positively charged amino acids in protamine.

Protamine was found to protect the RNA from nuclease degradation and prolong its *in vitro* stability. The authors tested 3 different vaccine formulations: the naked IVT mRNA encoding a model antigen (β-galactosidase), the mRNA-protamine complex with or without entrapment in the commercial cationic liposome Unifectin. Their study showed that the use of IVT mRNA for cancer immunotherapy is safer and more effective than tumor-derived mRNA as it does not pose the risk of inducing autoimmunity but was still effective in generating CTL and humoral responses. They also found that protamine-protected mRNA vaccines can be administered without any vehicle such as cationic liposomes which are highly toxic towards macrophages, they are also likely to be directly taken up by DCs, and they do not appear to be immunogenic as no protamine-specific IgG antibodies were detected in sera of mice [17]. Protamine-protected mRNA was deemed to be less sensitive to RNase activity, easy to handle, and thermostable. Indeed protamine-complexed mRNA retained its protective capacity and ability to stimulate synthesis of neutralizing antibodies after prolonged storage

at temperatures ranging from −80 °C to +70 °C for several months. Moreover, to simulate interruptions of the cold chain during vaccine transport, the vaccine was stored at oscillating temperatures between +4 °C and +56 °C for 20 cycles and this did not affect its immunogenicity or protective characteristics. Previous work by the same research group also showed that mice immunized with a lyophilized protamine-mRNA vaccine that was stored for up to 3 weeks at 37 °C were still protected against the viral infection, indicating that the vaccine was resistant to thermal stresses, which further underlines the protective role of protamine [18,19]. Protamine-complexed mRNA vaccines were also found to act as danger signals and elicit the activation of important subpopulations of immune cells, such as Th1, via TLR7/8 pathways, enhancing as such the vaccine's immunogenicity [20]. One way to enhance the uptake of protamine-mRNA vaccine by DCs is to entrap this complex in a drug delivery vehicle. Accordingly, Mai et al. designed cationic liposomes for the entrapment of protamine-complexed mRNA encoding cytokeratin 19 to provoke anti-tumor immunity in Lewis lung cancer murine model. The liposome-protamine-mRNA complex was administered intranasally, for induction of systemic and local mucosal immunity, and was found to transfect DCs and to induce their maturation more efficiently than protamine-mRNA or liposome-entrapped mRNA. This shows on one hand the advantage of using protamine to condense mRNA into NPs and hence facilitate complete entrapment within the liposomes, and on the other hand it demonstrates the benefit of using cationic liposomes for improved uptake by DCs and hence greater antigen expression for more pronounced anti-tumor cellular immunity. It also underlines the limited potential of protamine-complexed mRNA to translate to the clinics without entrapment in any delivery system [21]. A similar approach was adopted for the delivery of a liposome-entrapped protamine-complexed mRNA vaccine encoding pseudorabies virus glycoprotein D in mice models. Significant increase in geometric mean titers (GMT) of neutralizing antibodies and geometric mean concentrations (GMC) of antigen-specific binding antibodies were noted along with enhanced cytokine release and CD4+/CD8+ T cell responses, indicating the protective efficacy of this vaccine (Table 2) [22].

2.3. RNActive vaccines

While the complexation of mRNA with protamine was effective for enhancing the nucleic acid's immune-stimulating potential, it was still associated with the risk of reducing

Table 2 – Adjuvant-tethered IVT mRNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref.
IVT mRNA tethered to TLR7 agonists	N/A	Ovalbumin	N/A	Mice	- Enhanced local immune responses - Stimulation of antigen-specific cell-mediated and humoral responses	[15]
IVT mRNA tethered to polyU tail	N/A	Ovalbumin	N/A	Mice	- Enhanced immunostimulation through TLR3/RIG1 RNA sensors - Increased DCs activation - Enhanced intensity of specific cellular and humoral immune responses	[16]
IVT mRNA tethered to protamine	Cancer	B-galactosidase	Cationic liposome Unifectin	Mice	- Internalization into APCs without liposomal assistance - Induction of CTL and humoral responses	[17]
	Influenza A Rabies	HA RABV-G	N/A	Mice Ferrets Pigs	- Enhanced vaccine's thermostability - Decreased vaccine's RNase sensitivity - Induction of neutralizing antibodies and long-lived B and T cell immunity	[18,19]
	Metastatic melanoma	Melan-A, Tyrosinase, gp100, Mage-A1, Mage-A3, Survivin	N/A	Phase I/II clinical trial (NCT00204607)	- Activation of Th1 cells via TLR7/8 - Decrease of immunosuppressive cells - Increased vaccine-directed T cells	[20]
	Lung cancer	Cytokeratin 19	Cationic liposomes	Mice	- More efficient transfection of DCs - Induction of systemic and local mucosal immunity	[21]
	Pseudorabies	Glycoprotein D	Cationic liposomes	Mice	- Increase in neutralizing and antigen-specific binding antibodies - Enhanced cytokine release - Induction of CD4$^+$/CD8$^+$ T cell responses	[22]

its translatability which decreases the vaccine's prophylactic role. As such, one study aimed to solve this problem by designing a newer and more effective mRNA vaccine comprised of 2 components: a naked IVT mRNA with GC-enrichment at the 5′ and 3′ ends to promote high antigen expression, and a protamine-complexed mRNA to stimulate the innate immune cells via TLR7. This two-component vaccine induced stronger and more balanced adaptive humoral and cellular immune responses, mediated by the activation of antigen-specific CD4$^+$ T helper cells and CD8$^+$ cytotoxic T cells, which upon secretion of cytokines help activate Th1 response. *In vivo*, the vaccine also induced sustained memory responses, mediated by antigen-specific memory T cells. Testing this vaccine in a murine cancer model showed that it can be used for both prophylactic and therapeutic applications [23]. This two-component self-adjuvanted vaccine technology was patented by CureVac and designated as RNActive®. Preclinical studies showed that upon intradermal administration of an RNActive® vaccine, it is taken up in the skin by both non-leukocytic and leukocytic cells, mainly APCs. The vaccine was then transported to the draining lymph nodes by migratory DCs which efficiently expressed the encoded protein on their surface and induced adaptive immunity. As the immunostimulation was limited to the injection site and lymphoid organs, the vaccine was considered to have a favorable safety profile. Moreover, RNActive® was applied as prime and boost vaccine and immune responses were recorded in both cases with an enhanced immunity after the booster dose, indicating that

RNActive® can be used effectively in repetitive immunization schedules [24]. *In vivo* studies were also carried out in small and large animals where an RNActive® vaccine CV7201 encoding the rabies virus glycoprotein was tested in mice and domestic pigs. Humoral response was recorded in mice where high antibody titers were stable and lasted for an entire year, and cellular responses for both antigen-specific CD4$^+$ and CD8$^+$ T cells were also recorded where CD4$^+$ T cells activation was found to be crucial for synthesis of neutralizing antibodies. RNActive® also protected vaccinated mice against lethal intracerebral challenge infection. In newborn and adult domestic pigs, RNActive® vaccine provided protection against the rabies virus by inducing high antibody titers [25]. Following these successful findings, RNActive® vaccines were later used in clinical studies for the prevention or treatment of various cancerous and infectious diseases including prostate cancer, non-small cell lung cancer, and rabies [26]. Two RNActive® vaccines were developed for the treatment of prostate cancer patients with advanced metastatic castration-resistant tumors. CV9103, a self-adjuvanted RNActive® vaccine encoding 4 prostate-specific antigens PSA, PSCA, PSMA, and STEAP1, was tested in a phase I/IIa clinical trial (NCT00831467). The vaccine was administered in 5 doses intradermally at weeks 1, 3 and 7 to prime the adaptive antigen-specific immunity, and at weeks 15 and 23 to retain or boost immunity. The recommended dose determined in phase I was found to be the highest tested dose of 1280 µg. This vaccine was found to be well tolerated and safe as the adverse events were only of grade 1 and 2.

It was also deemed immunogenic since 79% of evaluable vaccinated patients developed an antigen-specific T cell immune response and it was directed against multiple antigens in 58% of patients. Another vaccine designated as CV9104 was developed and its mRNA sequence encoded for 2 additional antigens PAP and MUC1. It was tested in a randomized placebo-controlled phase I/IIa clinical trial (NCT01817738) to assess whether immunotherapy with this vaccine in addition to standard of care can provide longer overall survival (OS) than placebo plus standard of care in patients with metastatic castration-resistant prostate cancer. The vaccine failed to show any significant improvement in OS or radiographic progression-free survival. Accordingly, research is ongoing for the optimization of this vaccine with focus on improved formulations, modes of administration, and combination with checkpoint blocking antibodies [27–29]. Another study adopted the RNActive® vaccine CV9201 encoding 5 non-small cell lung cancer (NSCLC)-associated tumor antigens (MAGE-C1, MAGE-C2, NY-ESO-1, BIRC5, 5T4) in a phase I/IIa clinical trial (NCT01915524) comprising 46 patients with stage IIIB/IV NSCLC with a response or stable disease after first-line chemotherapy or chemoradiation. As no dose-limiting toxicities were recorded, the highest dose of 1600 µg was selected for phase IIa where patients received 5 doses within 15 weeks. The vaccine was well tolerated by patients, and it induced T and B cell responses against all included antigens. One important finding is that the vaccine induced a significant increase of pre-germinal center (GC) B cells which was correlated with an increase in CD4$^+$ effector T cells. GC B are implicated in producing long-lived antibody secreting plasma cells and memory B cells, which can provide protection against reinfection. This new finding may be used later as a biomarker in cancer immunotherapy. A phase Ib study is currently ongoing, and it involves the use of the vaccine CV9202 which includes mRNA that encodes for the 5 mentioned TAAs and an additional antigen of MUC1. It will assess the safety and tolerability of CV9202 vaccination combined with local radiation designed to enhance immune responses and it will include patients with stage IV NSCLC and a response or stable disease after first-line chemotherapy or therapy with an EGFR tyrosine kinase inhibitor [30,31]. The last RNActive® clinical trial (NCT02241135), to our knowledge at least, is a phase I uncontrolled study that enrolled 101 participants to receive the vaccine CV7201 encoding rabies virus glycoprotein as previously mentioned. The initial results of this study published in 2017 showed that the vaccine can be considered safe as only 10 participants reported grade 3 adverse events, with 1 serious adverse reaction that occurred and was later resolved without any consequences. Regarding the vaccine's immunogenicity, it was found that only needle-free injections (80, 160, 200 or 400 µg) applied by intradermal or intramuscular injection devices were able to elicit virus neutralizing antibody titers of ≥ 0.5 IU/ml, which is considered an adequate response by the World Health Organization (WHO). Moreover, a booster dose of 80 µg using needle-free injection and administered after 1 year was able to further increase antibody titers (Table 3) [32]. The inability of the vaccine to promote immunogenicity by typical needle injection is quite alarming and indicates the vital need for further improvements in the vaccine's formulation
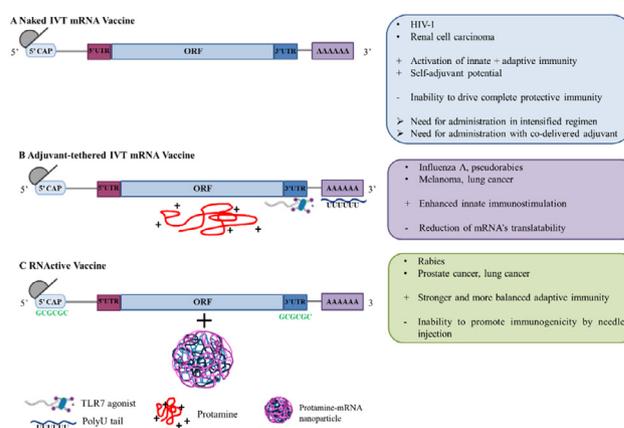
Fig. 2 – Schematic figure representing the three types of IVT mRNA vaccines along with their applications, main advantages, missing requirements, and needed improvements: (A) naked IVT mRNA vaccine; (B) adjuvant-tethered IVT mRNA vaccine whereby the adjuvant could be a TLR7 agonist, a polyU tail, or a CPP such as protamine; and (C) RNActive vaccine which is composed of a naked IVT-mRNA with GC enrichment at the 5′ and 3′ ends along with a protamine-mRNA nanoparticulate complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to provide easier transfection of immune cells and subsequent immunization (Fig. 2).

3. Current state of mRNA vaccines

Despite the advances made since the first mRNA studies, many challenges remained. While previous work that aimed to increase the mRNA's immunostimulatory potential via adjuvant addition was indeed a breakthrough in mRNA vaccine technology, one major disadvantage that remained was the accompanied decreased translational potential. Another challenge that still limits this technology is the ineffective transfection of DCs which requires sometimes atypical needle-free injections to obtain the desired immune response. Other limitations still facing mRNA vaccines included the poor stability of mRNA, its sensitivity and degradability by nucleases (5′ exonucleases, 3′ exonucleases, and endonucleases), its low half-life of only 7 h, as well as its high negative charge density and high molecular weight (MW) which hinder its entry into cells by passive diffusion across the plasma membrane [33–35]. Accordingly, novel non-viral delivery systems were proposed as a safer solution to overcome the previously mentioned challenges and provide more potent and versatile vaccines.

3.1. Liposome-Entrapped IVT mRNA vaccines

Liposomes are small vesicles at the nanoscale made up of one or more lipid bilayers mimicking the cell's membrane. They can consist of neutrally and/or positively charged

Table 3 – RNActive vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Preclinical/ Clinical Setting	Immune Response	Ref.
RNActive	Cancer	Ovalbumin	Mice	- Induction of balanced adaptive immune responses - Induction of antigen-specific memory T cells	[23]
RNActive	Cancer	Ovalbumin	Mice	- Promotion of immunostimulation at injection site and lymphoid organs - Induction of adaptive immunity	[24]
RNActive CV7201	Rabies	RABV-G	Mice Pigs	- Induction of high and stable antibody titers lasting for 1 year - Activation of CD4+/CD8+ T cells - Protection against lethal viral challenge	[25]
RNActive CV9103, CV9104	Advanced prostate cancer	PSA, PSCA, PSMA, STEAP1 ± PAP, MUC1	Phase I/II clinical trials (NCT00831467/ NCT01817738)	- Development of antigen-specific T cell immunity directed against multiple antigens	[27–29]
RNActive CV9201, CV9202	Non-small cell lung cancer	MAGE-C1, MAGE-C2, NY-ESO-1, BIRC5, 5T4 ± MUC1	Phase I/II clinical trials (NCT01915524)	- Induction of T and B cell responses against all included antigens - Increase of pre-germinal center B cells	[30,31]
RNActive CV7201	Rabies	RABV-G	Phase I clinical trial (NCT02241135)	- Induction of virus-neutralizing antibody titers ≥ 0.5 IU/ml	[32]

lipids and they entrap mRNA molecules within their internal aqueous core. They were the first delivery systems used for the entrapment and delivery of mRNA vaccines in 1993 [9]. Martinon et al. enveloped their nucleoprotein mRNA within simple neutrally charged liposomes made up of cholesterol, dipalmitoylphosphatidylcholine (DPPC), and phosphatidylserine [9]. In 2017, neutral lipids were still used in the synthesis of liposomes for mRNA entrapment. Persano et al. synthesized liposomes of 1,2-dioleoyl-sn–glycero-3-ethylphosphocholine / 1,2-dioleoyl-sn–glycero-3-phosphatidyl-ethanolamine / 1,2-distearoyl-sn–glycero-3-phosphoethanolamine-N-amino (polyethylene glycol)−2000 (EDOPC/DOPE/DSPE-PEG) for the encapsulation of TAA mRNA complexed with a cationic poly-(β-amino ester) polymer. The resulting formulation consisted of a polymeric polyplex core of polymer-mRNA loaded into a phospholipid bilayer shell. This nanoparticulate formulation not only protected the mRNA from RNase degradation but also promoted its internalization within DCs by macropinocytosis and enhanced antigen presentation. Interestingly, this lipopolyplex showed improved DC uptake and enhanced adjuvant effect by stimulating IFN-β and IL-12 expression via TLR7/8 signaling as compared to protamine-mRNA complexes, indicating the importance of the neutrally charged liposomal vesicles in mediating both effects [36].

On the other hand, cationic liposomes based on the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) are more often used as they can entrap or adsorb the mRNA with higher efficiency as a result of the electrostatic interaction between the positively charged amine groups in DOTAP and the negatively charged phosphate groups in the mRNA. They can also enhance the efficiency of intracellular

mRNA delivery by promoting endocytosis or fusion of the positively charged liposome with the negatively charged cytoplasmic membrane.

Several studies aimed to ameliorate the targeted delivery of mRNA vaccines to DCs, increase the antigen expression of mRNA within the cells, and enhance the induction of the innate and adaptive immune systems. As DCs typically overexpress the mannose receptor CD206, it is possible to achieve active targeting of DCs by modifying the mRNA-loaded liposomes with mannose molecules. Mannose-cholesterol conjugates, with PEG$_{1000}$ as linker molecules, incorporated in a DOTAP/DOPE liposomal mixture were found to significantly enhance DCs transfection efficiency *in vitro* from 12% to 52% as compared to a commercial transfection reagent. The spherical liposomes of 132.93 nm size and +37.93 mV surface charge protected the mRNA from degradation and enhanced its cellular expression, indicating that this mannose-modified liposome might be a good candidate for DC-targeting mRNA nanovaccine for *in vivo* applications [37]. Another successful approach for enhancing DCs transfection efficiency was through modifying mRNA-loaded DOTAP liposomes with a cholesterol-modified cationic peptide DP7-C. This peptide, developed by Zhang et al., has a double function: it can enhance antigen loading into cells via the clathrin- and caveolin-dependent pathways, and it can act as an adjuvant and stimulate DC maturation by activating the TLR2 signaling pathway. Indeed, *in vitro* and *in vivo* studies found that TAA mRNA-loaded DOTAP/DP7-C liposomes promoted DC transfection, maturation, and proinflammatory cytokine secretion, as well as CD8$^+$ T cell response, and exhibited anti-tumor effects [38]. DP7-C-modified liposomes may represent an alternative

method for further improving the intracellular delivery efficiency of mRNA vaccines and generating a stronger immune response. Another technique to boost the induction of the innate and adaptive immunities is by promoting the activation of invariant natural killer T cells (iNKT). These unconventional immune cells not only contribute to the induction of both immunities but also exert anti-tumor effects by positively modulating tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment. DOTAP/cholesterol liposomes were co-loaded with nucleoside-modified (5-methylcytosine, 5mC and N1-methylpseudoUTP, m1Ψ) mRNA encoding the TAA ovalbumin and α-galactosylceramide (α-GC), a glycolipid antigen acting as an iNKT ligand. *In vivo*, the intravenously administered vaccine induced a 7-fold increase in tumor-infiltrating antigen-specific cytotoxic T cells, a strong iNKT and NK cell activation, and a suppression of MDSCs. All of these anti-tumor effects combined translated to a significant reduction in tumor growth with complete tumor rejection in 40% of lymphoma mice models (Table 4) [39].

Taken together, these studies demonstrate the advantages of using liposomes for the intracellular delivery of mRNA vaccines and the feasibility of inducing surface modifications for enhancing the mRNA-loaded liposomes' transfection efficiency and immunostimulation potential.

3.2. Lipid nanoparticle-entrapped IVT mRNA vaccines

The first non-viral vectors used for the delivery of mRNA vaccines were liposomes and while they did show great potential in shielding mRNA from nuclease degradation and facilitating its uptake by APCs, they remain simple spherical vesicular formulations made up mainly of phospholipids and consisting of mostly an interior aqueous core. Lipid nanoparticles (LNPs) are solid particles at room and body temperature that can be engineered to take a variety of forms and are especially geared towards encapsulating nucleic acids. They consist of solid lipids or a mixture of solid and liquid lipids. While work on LNPs started in the 1980's, it took several decades for scientists to refine their formulation, ensure reliable and reproductive manufacturing techniques, and guarantee their delivery to different organs without accumulating at high rates in the liver. Currently, they represent the most popular non-viral gene delivery systems and several studies have adopted them for the delivery of mRNA vaccines since 2017. Unlike liposomes, LNPs are characterized by homogeneous morphologies of solid spheres that lack an aqueous core. They rarely have a contiguous lipid bilayer to qualify them as lipid vesicles; they most often assume a micelle-like structure, entrapping the nucleic acids within a low or minimally aqueous internal core, rendering them suitable for stable and efficient encapsulation of genetic payloads.

LNPs were adopted in several studies for the delivery of mRNA vaccines for prophylactic and therapeutic purposes. In most cases, mRNA molecules encoding the immunogens of choice are entrapped in LNPs by mixing an aqueous phase containing the mRNA with an ethanol phase containing the lipids using a microfluidic mixing device. The organic phase generally consists of 4 different types of lipids at the molar ratio of 50:10:38.5:1.5: (1) an ionizable cationic lipid which is positively charged at low pH enabling mRNA complexation and neutral at physiological pH reducing therefore potential cytotoxic effects, (2) a helper phospholipid to promote cell binding, (3) cholesterol to contribute to the NPs structure and fill the gaps between the lipids, and (4) a PEGylated lipid to help stabilize the NPs and reduce opsonization by serum proteins and reticuloendothelial clearance. LNP formulation, mainly the choice of the ionizable lipid, significantly affects the delivery and expression efficiency of the antigen of choice as well as the LNP potency and tolerability. In fact, LNPs formulated with an ionizable cationic amino lipid containing an ethanolamine linker and an acid-sensitive hydrophobic tail were found to induce the highest immunogen expression and the strongest humoral and cellular responses, and activate APCs via both the intradermal and intramuscular routes, as compared to lipids with hydrazine or hydroxylamine liner moieties [40]. Furthermore, ionizable lipids with a biodegradable property and a pK_a between 6.6 and 6.8 are ideal for intramuscular administration and for yielding high antigen expression and immunogenicity with improved tolerability of the lipid metabolites. Indeed, clearance of LNPs is preferred to extended residence time, which often results in undesirable inflammation at the site of injection even after clearance of the protein antigen [41].

The roles of LNPs can be broken down to 3 steps: (1) binding to, condensing, and encapsulating the mRNA molecules; (2) shielding them from ribonucleases present in the body and the environment; (3) helping them internalize APCs, cross the lipid membrane, and reach the cytoplasm where protein translation can take place to promote antigen presentation and immune response. This last step is crucial for antigen presentation and subsequent activation of the immune system. Typically, the mRNA-entrapped LNP enters APCs such as DCs via endocytosis and is trapped as such within an endosome vesicle. Unlike exogenous antigens, the LNP-mRNA needs to escape from the endosome to release the mRNA into the cytosol and promote translation via ribosomes. As the pH in the endosome decreases below the pKa of the ionizable lipids of the LNPs, these lipids gain a positive charge and interact as such with anionic lipids in the endosomal membrane. The pair of cationic and anionic lipids take on a cylindrical shape, also known as the porous hexagonal phase, which disrupts the endosome and facilitates the release of the mRNA into the cytoplasm (Fig. 3). After translation of the mRNA vaccine, the obtained endogenous antigen is then processed in the proteasome and the generated peptide epitopes enter the endoplasmic reticulum, are loaded onto major histocompatibility complex (MHC) class I molecules, and are presented on the surface of DCs to stimulate CD8+T cells and drive a cellular immune response. Antigen fragments are also loaded onto MHC class II molecules and presented on the plasma membrane of DCs to stimulate CD4+T cells and activate B lymphocytes to produce antigen-specific antibodies and generate a humoral immunity [42].

3.2.1. LNP-mRNA for cancer immunotherapy
For cancer immunotherapy, it was found that LNPs consisting of the ionizable lipid cKK-E12, DOPE, cholesterol, and C14-

Table 4 – Liposome-entrapped IVT mRNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical /Clinical Setting	Immune Response	Ref.
IVT mRNA	Influenza	Nucleo-protein	Neutral liposomes (cholesterol/ DPPC/ phosphatidylserine)	Mice	- Induction of virus-specific CTL that can target and lyze cells infected with the nucleoprotein or the WT influenza virus	[9]
IVT mRNA + cationic poly-(β-amino ester) polymer	Lung metastatic melanoma	Oval-bumin	Neutral liposomes (EDOPC/ DOPE/DSPE-PEG)	Mice	- Protection from the RNase degradation - Increased internalization within DCs by macropinocytosis - Enhanced adjuvant effect by stimulating IFN-β and IL-12 via TLR7/8 signaling	[36]
IVT mRNA	Melanoma	TRP2	DC-targeting cationic liposomes (DOTAP/ DOPE with mannose-cholesterol conjugate)	Mice	- Enhanced DC transfection efficiency - Protection of mRNA from degradation - Enhanced cellular expression of mRNA	[37]
IVT mRNA	Cancer	LL2	DC-targeting cationic liposomes (DOTAP/ DP7-C)	Mice	- Enhanced transfection and maturation of DCs - Increased proinflammatory cytokine secretion and CD8+ T cell response - Exhibited anti-tumor effects	[38]
5mC/m1Ψ IVT mRNA	Lymphoma	Oval-bumin, α-galactosyl ceramide	iNKT-targeting cationic liposomes (DOTAP/ cholesterol)	Mice	- Increased tumor-infiltrating antigen-specific cytotoxic T cells - Strong iNKT and NK cell activation - Suppression of myeloid-derived suppressor cells - Reduction in tumor growth with complete tumor rejection in 40% of animals	[39]

PEG$_{2000}$, at the molar percentages of 15:26:40.5:2.5 along with 16 mol% of the additive sodium lauryl sulfate (SLS) were optimal for the delivery of TAA-encoding mRNA in terms of mRNA entrapment efficiency (84.07%), transfection of different immune cells (DCs, macrophages, neutrophils, and B cells), and induction of antigen-specific CD8$^+$ T cells (4.2%). When tested in an aggressive melanoma murine model, this vaccine formulation with mRNA encoding the self-TAAs TRP2 and gp100 was able to overcome the self-tolerance and to significantly extend the overall mice survival. The authors also found that adding lipopolysaccharide, a TLR4 agonist, at 1 mol% further enhanced the vaccine's potency as it extended survival [43]. Addition of other adjuvants, such as palmitic acid-modified TLR7/8 agonist (C16-R848), has also proved beneficial for the enhancement of transfection efficiency and antigen presentation on DCs, as well as for increasing the adaptive T cell immunity and anti-tumor activity [44].

Moreover, another study found that TRP2-encoding mRNA can elicit not only a cytotoxic T cell response but also a humoral immune response in a melanoma mouse model, by entrapment in LNPs with a calcium phosphate core and a lipid shell including mannose-conjugated PEGylated DSPE for targeted transfection of DCs. One advantage of this LNP formulation is that the calcium phosphate core allows acid-mediated dissolution in the endo-lysosomal compartment, a phenomenon known as the "proton sponge" mechanism. In fact, once a weakly basic molecule such as calcium phosphate is present in the acidic lysosomal compartment (pH 4.8), the ATP-mediated pH-dependent proton pumps open followed by passive influx of chloride ions and water molecules which results in a high osmotic pressure and hence immediate swelling and rupture of the endosomal membrane [45]. Accordingly, these LNPs promote endosomal escape and rapid release of the mRNA after cellular internalization of the
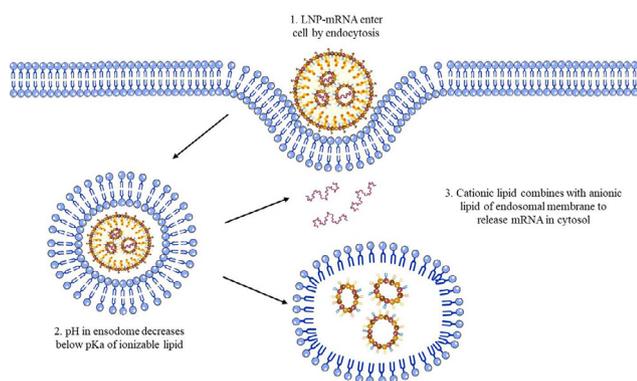
Fig. 3 – The mechanism of endosomal escape of LNP-mRNA.

NPs, which translates into higher transfection efficiency [37]. LNPs formed by water-in-oil-in-water double emulsions have also demonstrated strong potential for the entrapment and delivery of TAA-encoding mRNA. While they are not the most adopted NP formulation, these LNPs have already proven to be highly efficient vectors for the delivery of plasmids and small interfering RNA (siRNA) and they are now also considered as clinically translatable vehicles for the delivery of mRNA vaccines [46].

According to our knowledge, there is currently one published clinical study regarding LNP-entrapped mRNA vaccine for cancer immunotherapy. A small phase I/II clinical trial (NCT03480152) was carried out on 4 patients with metastatic gastrointestinal cancer to assess the immunogenicity and safety of a treatment with an LNP-delivered personalized mRNA vaccine encoding neoantigens expressed by the autologous cancer. This vaccine, named mRNA-4650, consisted of an mRNA backbone encoding 20 different neoantigens, expressed by the autologous cancer and recognized by the patient's tumor-infiltrating lymphocytes that were functionally tested for their immunogenicity. In addition to these defined antigens, the vaccine backbone contained any mutation in TP53, KRAS, or PIK3CA driver genes identified by exome sequencing of the autologous tumor and up to 15 human leukocyte antigens (HLA) class I candidate neoantigens that were predicted to bind to a patient's MHC alleles. mRNA-4650 was entrapped in LNPs made up of ionizable lipid:1,2-distearoyl-sn–glycero-3-phosphocholine (DSPC):cholesterol:PEG-lipid. The ionizable lipid used was heptadecan-9-yl 8-((2-hydroxyethyl)(8-(nonyloxy)−8-oxooctyl)amino)octanoate, an amino lipid that contributed to an enhanced mRNA delivery, an improved endosomal escape, and a safe repeated mRNA administration, as proven in both rodent and primate models. Results of this clinical trial showed that the vaccine was safe as only grade 1 and 2 toxicities were reported and immunogenic as it elicited CD8$^+$, and mainly CD4$^+$, mutation-specific T cell responses against predicted neoepitopes. While this study aimed to develop a personalized mRNA vaccine, it failed to show any advantage in the use of identified cancer neoantigens as they did not seem to contribute to the clinical effectiveness of the vaccine. This underlines the need for larger clinical trials, and the possibility of combining this vaccine with other

treatments such as checkpoint inhibitors or adoptive T cell therapy to achieve a clinical response (Table 5) [47,48].

3.2.2. LNP-mRNA for prevention of viral diseases

mRNA vaccines delivered by LNPs were studied on various viral diseases for the investigation of their prophylactic activities. The LNPs consisted in most cases of the same four-lipid formulation adopted in cancer immunotherapy, with some studies adding adjuvants for enhanced immune responses or tethering ligands for improved transfection of APCs. An LNP formulation of (3-(dimethylamino)propyl(12Z,15Z)−3-((9Z,12Z)-octadeca-9,12–dien-1-yl)henicosa-12,15-dienoate (DMAP-BLP)):DSPC:cholesterol:PEG-lipid was adopted for the delivery of nucleoside-modified mRNA against Zika virus (structural premembrane (prM) and envelope (E) genes) and against H10N8 and H7N9 influenza A viruses (H10 and H7 genes). The nucleoside uridine-5′-triphosphate (UTP) was substituted with the naturally occurring base modification m1Ψ as a mean to enhance mRNA translational efficiency while avoiding excessive and indiscriminate innate activation and reducing inflammatory side effects. Typically, naked and unmodified mRNA is recognized by PRRs of the innate immune system, which trigger the release of type I IFN and activate IFN-inducible genes that inhibit translation [49]. Indeed, the nucleoside modification along with the LNP delivery of mRNA vaccines promoted high neutralizing antibody titers ($\sim$ 1/100 000) against the Zika virus in mice and nonhuman primates after two and single doses, respectively; driven HA inhibition and potent neutralizing antibodies against H7N9 in mice and ferrets after a single dose; and promoted high seroconversion rates against H10N8 in a phase I human trial (NCT03076385), demonstrating robust prophylactic immunity [50–52]. The same LNP formulation was adopted for the delivery of mRNA encoding influenza H10, with the addition of 0.17% (M:M) glucopyranosyl lipid adjuvant (GLA), a TLR4 agonist. Non-human primates receiving 2 intradermal immunizations of the vaccine developed transient IFN-polarized innate immunity which resulted in priming of CD4$^+$ T cell responses in the vaccine-draining lymph nodes. Importantly, the 2-dose vaccine regimen also generated circulating H10-specific ICOS+ PD-1+ CXCR3+ T follicular helper cells, and this was accompanied by robust GC formation in vaccine-draining lymph nodes. These GC were responsible for the observed continuous increase in antibody avidity, seeding of H10-specific long-lived antibody-secreting plasma cells to the bone marrow, and production of circulating memory B cells in the blood. Collectively, these events resulted in excellent protective HA inhibition antibody titers sustained over 25 weeks. Of note, the GLA adjuvant did not show any significant increase in antibody titers, indicating that the LNP-mRNA formulation itself was sufficiently immunogenic [53,54]. Moreover, it was found that optimal translation and transfection efficiencies for influenza A-mRNA vaccines can be achieved by cloning human β-globin for both 5′ and 3′ UTRs instead of human α-globin and by conjugating mannose to the LNPs, respectively (Table 6) [55].

Nucleoside-modified mRNA vaccines entrapped in LNPs were also tested against Powassan and Dengue, two flaviviruses that are distantly and closely related to Zika

Table 5 – LNP-entrapped IVT mRNA vaccines for cancer immunotherapy.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref.
IVT mRNA	Melanoma	TRP2, gp100	LNP (cKK-E12: DOPE: cholesterol: C14-PEG2000: SLS, 15:26:40.5:2.5:16)	Mice	- Transfection of various immune cells - Induction of antigen-specific CD8$^+$ T cells - Overcoming of tumor self-tolerance and extension of mice survival	[43]
IVT mRNA	Lymphoma Prostate cancer	Oval-bumin	LNP (G0-C14/ C16-R848/ ceramide-PEG)	Mice	- Enhancement of transfection efficiency and antigen presentation on DCs - Increased adaptive T cell immunity and anti-tumor activity	[44]
IVT mRNA	Melanoma	TRP2	DC-targeting LNP (Calcium phosphate core and a lipid shell of mannose-conjugated PEG-DSPE)	Mice	- High transfection efficiency mediated by endosomal escape - Induction of cytotoxic T cell response and humoral response	[37]
IVT mRNA	Lymphoma	Oval-bumin	LNP (Double emulsions of PEG5000-PLGA/PLGA/ BHEM-cholesterol)	Mice	- Stimulation of DCs' maturation - Activation and proliferation of antigen-specific T cells - Slowed tumor growth	[46]
IVT mRNA (mRNA-4650)	Metastatic gastro-intestinal cancer	20 TAAs, 15 HLA-I neoantigens	LNP (ionizable lipid:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5)	Phase I/II clinical trial (NCT03480152)	- Induction of CD4+ and CD8+ mutation-specific T cell responses against predicted neoepitopes	[47,48]

Table 6 – Sequences of optimal UTR configurations [55].

5′UTR (β-globin-2)	AGAGCGGCCGCTTTTTCAGCAAGATTAAG CCCAGGGCAGAGCCATCTATTGCTTACATTT GCTTCTGACACAACTGTGTTCACTAGCAAC CTCAAACAGACACC
3′UTR (2β-globin)	AGCTCGCTTTCTTGCTGTCCAATTTCTAT TAAAGGTTCCTTTGTTCCCTAAGTCCAACT ACTAAACTGGGGGATATTATGAAGGGCCTTG AGCATCTGGATTCTGCCTAATAAAAAACA TTTATTTTATTGCAGCTCGCTTTCTTGCTG TCCAATTTCTATTAAAAGGTTCCTTTGTTCC CTAAGTCCAACTACTAAACTGGGGGATATT ATGAAGGGCCTTGAGCATCTGGATTCTG CCTAATAAAAAACATTTTTTTTCATTGC

virus, respectively. The LNP-mRNA vaccine developed against Zika virus mentioned earlier was also deemed to have a protective role against Powassan infections as one or two doses were able to elicit high titers of neutralizing antibody and sterilizing immunity against lethal challenge with different Powassan virus strains. Interestingly, the vaccine also induced cross-neutralizing antibodies against multiple other tick-borne flaviviruses and protected mice against the distantly related Langat virus, highlighting the ability of this vaccine to promote cross-protective immunity [56]. However, the generation of cross-reactive antibodies by mRNA vaccines is not always favored. In fact, Zika vaccine-induced antibodies can cross-react with Dengue virus and augment its infectivity by a phenomenon termed antibody-dependent enhancement (ADE) of infection. Accordingly, researchers developed a modified prM-E mRNA Zika vaccine encoding mutations destroying the conserved fusion-loop epitope in the E protein. This variant protected against Zika virus and diminished production of cross-reactive antibodies in cells and mice [50]. As the direct role of AED in the pathogenesis of severe Dengue disease remains controversial, development of a vaccine that can promote protective immunity solely against Dengue virus was needed. A modified LNP-mRNA vaccine encoding 3 non-structural immunodominant T cell antigens (NS3, NS4B, NS5) was developed and tested on transgenic mice expressing different human HLA alleles. The vaccine was able to induce a strong CD8$^+$ T cell response that was independent of IFN-induced innate immunity and conferred protection against Dengue infection. Of note, further experiments in HLA class I transgenic mice are required to determine whether

immunization with this vaccine can induce significant protection against other Dengue virus serotypes in the context of different HLA class I molecules [57].

LNP-entrapped modified mRNA vaccines encoding viral glycoproteins were examined in preclinical studies against various viruses including Ebola [58], HIV-1 [59], Nipah virus [60], among others. These studies have demonstrated the ability of the vaccines to induce high glycoprotein-specific neutralizing antibody titers as well as antibody-dependent cellular cytotoxicity and protect as such the immunized animals from lethal viral challenges. Moreover, the study on LNP-mRNA vaccine encoding Ebola virus' glycoprotein E found that swapping the authentic signal peptide of the viral glycoprotein with that of the human kappa immunoglobulin (Igκ) contributed to improved translocation through the intracellular secretory network for subsequent display on the cell surface, which resulted in a more potent vaccine. This suggests the possibility of administering one dose of an mRNA vaccine which incorporates Igκ signal peptide instead of the usual two-dose regimen [58]. Furthermore, it was found that LNP-mRNA vaccines encoding viral glycoproteins can be used in the prevention of infections caused by a closely related virus, underlining their versatility. This was seen with the LNP-mRNA vaccine encoding the soluble Hendra virus glycoprotein which protected 70% of animals from lethal Nipah virus challenge as both Hendra and Nipah are closely related paramyxoviruses. However, this vaccine only induced suboptimal primed immune responses after single immunization as Nipah virus-specific antibodies were not detected in the plasma of immunized hamsters. This limitation was partially attributed to the suboptimal immunogenicity of the antigen itself. A booster dose in this case may be beneficial for inducing significantly relevant antibody titers [60].

LNP-entrapped nucleoside-modified mRNA vaccines encoding viral glycoproteins have been compared side-by-side in preclinical studies to live attenuated vaccines, inactivated vaccines, viral vector DNA vaccines, and subunit protein vaccines, whereby they demonstrated enhanced immune responses and improved prevention from lethal viral challenges [58,61-67]. Indeed, comparison of an LNP-mRNA vaccine encoding the full-length cytomegalovirus (CMV) glycoprotein gB and the adjuvanted subunit protein vaccine gB/MF59, found that LNP-mRNA promoted enhanced durability of vaccine-elicited antibody responses and increased the breadth of IgG binding responses against gB peptides. Regarding gB-specific T cell immunity, the response detected was of low magnitude similarly to what was observed with gB/MF59, indicating that this novel mRNA technology provides viable improvements on the partial efficacy of gB/MF59 vaccination [61]. A similar CMV vaccine encoding gB glycoprotein as well as the gH/gL/UL128/UL130/UL131A pentameric complex (PC) and the immunodominant CMV T cell pp65 antigen was developed for the prevention of maternal acquisition of CMV in an attempt to reduce the incidence of congenital disease. Immunized mice having received a first dose of pp65 mRNA vaccine followed by a booster dose with the three-antigen mRNA (gB + PC + pp65) vaccine demonstrated multi-antigenic T cell responses whereby cells mainly

secreted IFN-γ and TNF-α, and to a lesser extent IL-2 (Th1). Furthermore, this vaccine allowed the expression of all antigens on the surface of transfected fibroblast cells and promoted the formation of conformation-dependent neutralizing monoclonal antibodies, indicating that mRNA technology is ideal for the rapid development of vaccines for complex multimeric antigens [62]. LNP-mRNA vaccines developed against herpes simplex virus type 2 (HSV-2) and varicella-zoster virus (VZV) were more immunogenic than trivalent subunit protein vaccine and live attenuated virus vaccine, respectively. A versatile HSV-2 vaccine was developed based on nucleoside-modified mRNA entrapped in LNPs and encoding 3 glycoproteins: the entry molecule glycoprotein D (gD2) and two immune evasion molecules, glycoprotein C (gC2) that binds complement C3b and glycoprotein E (gE2) that blocks IgG Fc (fragment crystallizable region) activities. Phenotypically, it protected immunized animals from genital lesions in a similar fashion to the protein vaccine however; it provided enhanced protection from recurrent virus shedding following lethal HSV-2 challenge as compared to the protein vaccine. On the immunological level, the mRNA vaccine enhanced humoral and cellular responses including memory responses. It was also able to provide protection against neonatal herpes as it induced high titers of IgG binding and neutralizing antibodies in both mothers and newborns, and protected as such the first and second litter newborns against disseminated infection. However, these effects were comparable using both mRNA and protein vaccines. But since the mRNA vaccine provided better protection against genital herpes, it was deemed as the preferred candidate for future studies. Importantly, the vaccine also generated cross-reactive antibodies that were able to neutralize HSV-1 and accordingly, it prevented death and genital disease in 100% of mice infected with HSV-1 or HSV-2, and prevented HSV-1 and HSV-2 DNA from reaching the dorsal root ganglia, inhibiting therefore viral latency [63–65]. LNP-mRNA vaccine encoding the VZV glycoprotein E (gE) was also found to generate comparable protective effects as the adjuvanted protein subunit vaccine however; its favored immunogenicity was evident when compared to the traditional live attenuated VZV vaccine. The mRNA vaccine included a Y569A mutation which modulated subcellular trafficking and ensured cell surface and Golgi antigen expression, a combination necessary for optimal stimulation of both humoral and cellular immune responses. Indeed, the vaccine elicited a robust immune response, after a single 100–200 µg dose or two 50 µg doses at 28-d interval, represented by peaks of gE-specific antibody binding titers (at d70) and CD4$^+$ T cell responses. Importantly, this study found that antibody decay kinetics, followed for 6 months after the second dose, were independent of the immunization dose or modality as antibody titers across all 3 groups decayed at roughly the same rate. This suggests that the magnitude of peak immunogenicity was the driving force in determining immune response longevity [66]. Another vaccine was developed using the same technology for protection against the respiratory syncytial virus (RSV). Several mRNA vaccines were developed for the expression of different forms of the RSV fusion (F) glycoprotein, including secreted, membrane associated, prefusion-stabilized, and non-stabilized native

structures. The mRNA vaccines were entrapped in LNPs consisting of DLin-MC3-DMA (MC3):DSPC:cholesterol:PEG$_{2000}$-dimyristoylglycerol in a molar ratio of 58:30:10:2. Vaccine candidates expressing either prefusion stabilized or native forms of F protein elicited robust neutralizing antibody responses in rodents however; the titers were similar to those obtained with a comparable dose of an adjuvanted prefusion stabilized F protein vaccine. The advantage of the mRNA vaccine was however manifested in the induction of robust cellular responses for both CD4$^+$ and CD8$^+$ T-cells. This finding implicates mRNA technology as the preferred choice of vaccines requiring a cellular immune response for efficacy [67]. This LNP-mRNA vaccine encoding the prefusion stabilized F protein was later tested in a phase I placebo-controlled dose-escalation clinical trial. All doses tested in young adults (25, 100, 200 µg) and older adults (25, 100, 200, 300 µg) were well tolerated indicating the safety of the vaccine. Following single intramuscular immunization, the vaccine led to robust humoral immune responses as indicated by the increase in RSV neutralizing antibody titers, serum antibody titers to the prefusion F protein, and competing antibody titers to the pre- and post-fusion F protein epitopes. Similarly, cellular immune responses were observed to RSV-F peptides, and they were driven by CD4$^+$ T cells as no CD8$^+$ T cell responses were detected in either age group [68]. Another phase I clinical trial (NCT03713086) was conducted for assessment of the safety and immunogenicity of an RNActive® rabies vaccine CV7202. We had previously reported the *in vivo* study conducted on the CV7201 vaccine. However, as immune responses to this vaccine were dependent on the route of administration, notably requiring intradermal or intramuscular administration with specialized needle-free injection devices, the mRNA vaccine CV7201 was then entrapped in an LNP formulation to improve transfection efficiency and designated as CV7202. Dose-escalation studies found that 1–2 µg of the vaccine are well tolerated while the higher dose of 5 µg induced unacceptably high reactogenicity. Upon intramuscular administration of the vaccine, low and dose-dependent rabies virus glycoprotein-specific neutralizing titers (VNT) were detectable from d15, and starting d43, all participants that received two 1 or 2 µg doses, separated by 28-d interval, had high antibody titers ($\geq$ 0.5 IU/mL). When compared to a licensed inactivated rabies vaccine (Rabipur), CV7202 elicited similar GMT at d57 and induced comparable adequate responses regarding both neutralizing and IgG antibodies (Table 7) [69].

3.2.3. *LNP-mRNA for prevention of COVID-19*

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been identified as the infectious agent responsible for the coronavirus disease-19 (COVID-19) pandemic that has affected more than 36 million individuals and taken the lives of over one million people since December 2019. Due to the urgent need for an effective vaccine that can be produced rapidly to respond to the needs of affected countries worldwide, there has been a surge in preclinical and clinical studies conducted to develop an effective vaccine against SARS-CoV-2. While several vaccine candidates are being developed, the leading vaccine candidates are based on LNP-entrapped mRNA encoding the virus' full length

spike protein (S) or its subunits such as the S1 and S2 subunits, and the receptor binding domain (RBD), usually translated in its native trimeric conformation. SARS-CoV-2 enters cells by binding via S glycoprotein to its receptor, angiotensin-converting enzyme 2 (ACE2), typically expressed on the surface of lung alveolar epithelial cells, enterocytes of the small intestine, arterial and venous endothelial cells and arterial smooth muscle cells in almost all organs of the body (oral and nasal mucosa, nasopharynx, lung, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney, and brain) [70].

Preclinical studies have aimed to develop various SARS-CoV-2 LNP-mRNA vaccine candidates and tested them *in vitro* and *in vivo*. Nucleoside-modified mRNA encoding the RBD or S1 subunit proteins were entrapped in GenVoy-ILM, a commercial ionizable lipid mix that is devoid of PEG and enables rapid and easy production of mRNA-loaded LNPs. Both LNP-mRNA vaccines induced robust antigen expression within cells and as secreted form for a minimum of 160 h, indicating long-term and broad expression of mRNA-encoding proteins, particularly RBD, in ACE2-expressing cells. They were also localized within cells' lysosomes, implicating that they may be resistant to lysosomal degradation. Moreover, prime and boost intradermal immunizations with 30 µg LNP-mRNA in mice induced significant T follicular helper cells and GC B responses in draining lymph nodes and plasma cell response in splenocytes, particularly for the RBD-encoding mRNA. Cellular responses were also recorded indicating that the vaccine elicits Th1 CD4$^+$ and CD8$^+$ T cell responses. Humoral responses were recorded as well indicating that RBD mRNA-LNP vaccine administered at different immunogen doses (10–30 µg) and variant routes induces strong RBD-specific IgG antibodies and potent neutralizing antibodies against pseudotyped and live SARS-CoV-2 infection, with long-lasting high titers for 70 d post-second immunization. Moreover, it was found that RBD mRNA-LNP-induced antibodies can potently block binding between SARS-CoV-2 RBD and its ACE2 receptor, in a dose-dependent manner, as well as cross-react with SARS-CoV RBD and cross-neutralize SARS-CoV infection, providing protection against both coronaviruses. Interestingly, both vaccines showed thermostability for 3 d at 4°C and 25°C [71]. Another thermostable LNP-mRNA vaccine encoding RBD but lacking the 5′-cap, termed ARCoV, was found to maintain stability at 4°C and 25°C for up to 1 week. It was also found to have high *in vitro* transfection efficiency which resulted in high translation and expression of recombinant RBD in culture supernatants. Indeed, the RBD protein retained high affinity for recombinant human ACE2 (rhACE2), inhibited entry of a vesicular stomatitis virus (VSV)-based pseudovirus expressing the SARS-CoV-2 S protein, and it was recognized by several monoclonal antibodies against SARS-CoV-2 RBD and convalescent sera from COVID-19 patients. Upon intramuscular injection of two 2–10 µg doses of the vaccine, it was detected at the site of injection and in the upper abdomen with a peak at 12 h up until 48 h. While the liver was the most abundant RBD-expressing tissue, the translated immunogen was also found within muscle cells co-localized with CD11b+ monocytes as well as CD163+ macrophages and CD103+ dendritic cells, showing the potential of the vaccine to transfect APCs and induce

Table 7 – LNP-entrapped IVT mRNA vaccines for prevention of viral diseases.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref.
m1Ψ IVT mRNA	Zika	prM, E	DMAP-BLP:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice Non-human primates	- Induction of high neutralizing antibody titers (∼ 1/100 000)	[50,51]
m1Ψ IVT mRNA	Influenza A H7N9, H10N8	H7, H10	DMAP-BLP:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice Ferrets Phase I clinical trial (NCT03076385)	- Promoted HA inhibition and potent neutralizing antibodies - Promoted high seroconversion rates	[52]
m1Ψ IVT mRNA	Influenza A H10N8	H10	DMAP-BLP:DSPC:cholesterol: PEG-lipid:GLA, 50:10:38.5:1.33:0.17	Non-human primates	- Induction of IFN-polarized innate immunity - Activation of H10-specific T follicular helper cells and robust GC formation in vaccine-draining lymph nodes - Production of sustained HA inhibition antibody titers	[53,54]
m1Ψ IVT mRNA	Powassan	prM, E of Zika virus	DMAP-BLP:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Production of high neutralizing antibody titers and sterilizing immunity - Induction of cross-neutralizing antibodies against multiple tick-borne flaviviruses - Protection of mice against Langat virus	[56]
m1Ψ IVT mRNA	Dengue	NS3, NS4B, NS5	ionizable lipid:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Induction of a strong CD8+ T cell response independently of IFN-induced innate immunity - Protection against Dengue infection	[57]
m1Ψ IVT mRNA	Ebola	gE	DMAP-BLP:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Guinea pigs	- Improved vaccine potency mediated by human Igκ signal peptide - Induction of high glycoprotein-specific neutralizing antibody titers - Protection of immunized animals from lethal viral challenge	[58]
m1Ψ IVT mRNA	HIV-1	Env 1086C	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Rabbits Rhesus macaques	- Induction of high glycoprotein-specific neutralizing antibody titers and antibody-dependent cellular cytotoxicity	[59]
m1Ψ IVT mRNA	Nipah	Soluble Hendra virus glycoporotein	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Syrian hamsters	- Induction of suboptimal primed immune response - Protection of 70% of immunized animals from lethal viral challenge	[60]
m1Ψ IVT mRNA	Cytomegalovirus	gB	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5)	Rabbits	- Enhanced durability of antibody responses and increased breadth of gB-specific IgG binding antibodies compared to gB/MF59 - Induction of low magnitude gB-specific T cell immunity	[61]
m1Ψ IVT mRNA	Cytomegalovirus	gB, pentameric complex, pp65	ionizable lipid: DSPC:cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Activation of Th1-biased multi-antigenic T cell responses - Formation of conformation-dependent neutralizing monoclonal antibodies	[62]
m1Ψ IVT mRNA	HSV-2	gD2, gC2, gE2	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Enhanced humoral, cellular, and memory responses - Protection against neonatal herpes - Generation of cross-reactive antibodies that neutralized HSV-1 and inhibited viral latency	[63–65]
m1Ψ IVT mRNA	VZV	gE	DMAP-BLP:DSPC: cholesterol: PEG-lipid, 50:10:38.5:1.5	Rhesus macaques	- Induction of similar and favored immunogenicity compared to the adjuvanted protein subunit and the live attenuated vaccine, respectively - Activation of robust humoral and CD4+ T cell responses	[66]
m1Ψ IVT mRNA	RSV	F	MC3:DSPC: cholesterol: PEG2000-dimyristoylglycerol, 58:30:10:2	Mice Cotton rats	- Enhanced induction of cellular responses compared to an adjuvanted prefusion stabilized F protein vaccine	[67]
m1Ψ IVT mRNA	RSV	Prefusion stabilized F protein	MC3:DSPC: cholesterol: PEG2000-dimyristoylglycerol, 58:30:10:2	Phase I clinical trial	- Activation of robust humoral immune responses - Induction of F-specific CD4+-biased cellular immune responses	[68]
m1Ψ IVT mRNA CV7202	Rabies	RABV-G	ionizable lipid:DSPC: cholesterol:PEG-lipid, 50:10:38.5:1.5	Phase I clinical trial (NCT03713086)	- Induction of similar neutralizing and IgG antibody titers to a licensed inactivated rabies vaccine (Rabipur)	[69]

antigen presentation. ARCoV also induced RBD-specific IgG and neutralizing antibodies, Th1-biased cellular response, and protected 100% of mice against the challenge of a SARS-CoV-2 mouse-adapted strain by preventing SARS-CoV-2 replication in the lower respiratory tract and protecting mice from lung lesions. This study also found that placebo-LNPs stimulated massive infiltration of monocytes and macrophages, demonstrating again the role of LNPs not only as delivery vehicles but also as vaccine adjuvants. Owing to its promising results and its adequate safety profile, this vaccine is currently being evaluated in phase I clinical trials (NCT04283461) [72]. Of note, LNP-mRNA vaccines encoding RBD protein in its monomeric conformation failed to produce significant antibody titers even after a booster dose, indicating the need for expression of RBD in its native trimeric form [73]. Moreover, delivery of mRNA encoding RBD with Fc region of human IgG1 (hFc) fused both as mRNA and as a recombinant protein was shown to increase the half-life, immunogenicity, solubility, and delivery efficiency of the immunogen. As a result, intramuscular administration of an LNP-mRNA encoding RBD-hFc elicited specific anti-RBD humoral responses, a high level of neutralizing antibodies that blocked viral infection in a spike-pseudotyped VSV and a Th1-biased cellular response in BALB/c mice [40].

Despite ongoing efforts regarding SARS-CoV-2 mRNA-LNP vaccines, no peer-reviewed preclinical studies have been published to date. Accordingly, one study conducted in 2020 evaluated two nucleoside-modified SARS-CoV-2 mRNA-LNP vaccine formulations encoding RBD or the full-length S protein with deleted furin cleavage site (S∆furin), present at the S1-S2 boundary. Remarkably, after single immunization in mice, both vaccine formulations induced potent CD4$^+$ and CD8$^+$ T cells, particularly in the lungs, with more than half the cells producing IFN-γ (Th1), suggesting the minimal stimulation of a Th2 response which may induce vaccine-associated enhanced respiratory disease. This suggests that SARS-CoV-2 mRNA-LNP vaccines elicit T cells that preferentially home to the lungs, and that they are safer than other vaccines that elicit Th2-biased responses. Another safety concern with coronaviruses is the risk of inducing ADE of infection, as is the case with flaviviruses that we had previously discussed. This study found that none of the SARS-CoV-2 mRNA vaccine-elicited antibodies mediated ADE under *in vitro* conditions further proving the safety profile of these vaccines. Moreover, both vaccines elicited potent long-lived plasma cells and memory B cells responses, as well as rapid generation of neutralizing antibodies that persisted at high levels for 9 weeks after immunization, indicating the ability of the vaccines to generate durable protective humoral immunity, which is vital to achieve in the midst of a global pandemic [74]. To study the protective efficacy of SARS-CoV-2 mRNA-LNP vaccines in preclinical settings, hACE2 transgenic mice were immunized with a single dose of RBD-encoding mRNA-LNP and infected with the wild-type SARS-CoV-2 one month post-vaccination. Results of this study demonstrated that a single immunization can induce robust and durable neutralizing antibodies, lasting for a minimum of 6.5 months, and provide as such near-complete protection against wild SARS-CoV-2 challenge in the lungs of transgenic mice. The long-term protection provided by the vaccine is of

great importance since several studies reported that antibody-mediated immunity in convalescent humans from SARS-CoV-2 infection only persisted for 2–3 months, indicating the need for vaccines to provide long-lasting protection and memory immunity [75,76]. Another study investigated an LNP-mRNA vaccine RQ3013-VLP formulated from a cocktail of mRNAs encoding 3 structural proteins: S, M, and E which formed SARS-CoV-2 virus-like particles (VLPs) with an average diameter of 100 nm. To increase the expression capacity of the vaccine, all mRNAs were subjected to an in-depth sequence optimization procedure of 2 parameters (codons in the DNA template and modified nucleotides in the mRNA) and the final vaccine candidate had an optimal combination of codon and modified nucleotides that provided the most robust antigen expression. After prime and boost immunization, RQ3013-VLP promoted the production of S-specific antibodies and neutralizing antibodies in immunized mice with elevated titers that remained stable for 8 weeks. Moreover, the VLP vaccine induced a cellular response with a 0.67:0.29 CD4$^+$/CD8$^+$ T cell ratio along with robust VLP- and S-specific T cell responses. This pilot study implicates that when S is presented in secreted vesicles such as VLPs, it induces more robust humoral and cellular immune responses than when it is displayed on the cell membrane [73]. To further show the enhanced efficacy of LNP-formulated SARS-CoV-2 mRNA vaccines, 2 formulations encoding RBD and the full length S glycoprotein were compared to a recombinant SARS-CoV-2 RBD protein (rRBD) with an MF59-like adjuvant. Single immunization with both mRNA vaccines, but not with rRBD, elicited potent GC B and T follicular helper cell responses as well as long-lived plasma cells and memory B cells, suggesting that mRNA vaccines are strong candidates for promoting robust GC-derived immune responses (Table 8) [77].

While these LNP-mRNA vaccines have been tested in preclinical settings, there are to date 3 SARS-CoV-2 LNP-mRNA vaccines that are already being investigated in clinical trials: LNP-entrapped nucleoside-modified mRNA-1273 encoding the viral S protein stabilized in its prefusion form from Moderna (the United States of America), LNP-entrapped BNT162b1 encoding the RBD protein and BNT162b2 encoding the full-length S protein from Pfizer/BioNTech (Germany).

Moderna's mRNA-1273 encodes the S-2P antigen, consisting of the SARS-CoV-2 S glycoprotein with a transmembrane anchor and an intact S1–S2 cleavage site. The phase I dose-escalation study (NCT04283461) included 45 healthy adults (18–55 years) who received the vaccine intramuscularly in 2 shots, 28 d apart, in doses of 25, 100, and 250 µg. Their results showed that the 100 µg dose elicited high titers of S-2P and RBD specific binding antibodies (782,719 GMC at d57), the highest titers of neutralizing antibodies (343.8 geometric mean ID50 at d43) and Th1-biased CD4$^+$ T cell responses, along with a more favorable safety profile where only mild to moderate local and systemic adverse events were reported in all participants following the second dose [78]. Accordingly, a phase II randomized placebo-controlled study (NCT04405076) was then conducted in 600 healthy adults of 2 age cohorts (18–55 and >55 years) who received 50 or 100 µg of the vaccine or the placebo. Safety was again confirmed as only mild to moderate adverse events were reported with the 2 doses with the most common

Table 8 – LNP-entrapped IVT mRNA vaccines for prevention of COVID-19 in preclinical trials.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref
m1Ψ IVT mRNA	SARS-CoV-2	RBD in trimeric form, S1	GenVoy-ILM	Mice	- Induction of T follicular helper cells and GC B responses - Activation of Th1 CD4+ and CD8+ T cell responses - Production of RBD-specific IgG antibodies, potent neutralizing antibodies, cross-reactive antibodies that neutralize SARS-CoV	[71]
m1Ψ IVT mRNA ARCoV)	SARS-CoV-2	RBD in trimeric form	ionizable lipid:DSPC: cholesterol:PEG-lipid, 50:10:38.5:1.5	Mice/ Phase I (NCT04283461)	- RBD protein expression at injection site, liver, APCs - Production of RBD-specific IgG and neutralizing antibodies and Th1-biased cellular response - Protection of 100% of mice against the viral challenge	[72]
m1Ψ IVT mRNA (RQ3011-RBD, RQ3012-S, RQ3013-VLP)	SARS-CoV-2	RBD in monomeric form, S, S + M + E	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Failure of RQ3011-RBD to produce significant antibody titers even after a booster dose - Production by RQ3013-VLP of S-specific and neutralizing antibodies, and induction of a cellular response	[73]
IVT mRNA	SARS-CoV-2	RBD-hFc	ionizable lipid:DSPC: cholesterol:DMG-PEG, 50:10:38.5:1.5	Mice	- Production of specific anti-RBD and neutralizing antibodies that blocked viral infection - Activation of a Th1-biased cellular response	[40]
m1Ψ IVT mRNA	SARS-CoV-2	RBD, SΔfurin	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Activation of Th1-biased CD4+ and CD8+ T cell responses in lungs - Production of long-lived plasma cells, memory B cells, long-lasting neutralizing antibodies	[74]
m1Ψ IVT mRNA	SARS-CoV-2	RBD	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	hACE2 transgenic mice	- Production of robust and durable neutralizing antibodies - Near-complete protection against wild SARS-CoV-2 challenge in the lungs	[76]
m1Ψ IVT mRNA	SARS-CoV-2	RBD, SΔfurin	ionizable lipid: phosphatidylcholine: cholesterol: PEG-lipid, 50:10:38.5:1.5	Mice	- Induced more potent GC B, T follicular helper cells, long-lived plasma cells, and memory B cells than recombinant SARS-CoV-2 RBD protein with an MF59-like adjuvant	[77]

ones being pain at injection site, headache, and fatigue following each vaccination in both age cohorts. These adverse effects were transient lasting no more than 4 d The vaccine also triggered high titers of anti-SARS-CoV-2 S binding and neutralizing antibodies after the second vaccination with comparable titers across the 2 doses and both age cohorts. Antibody titers were also maintained at elevated levels until d57, implicating that mRNA-1273, given at 50 or 100 μg in a 2 dose-regimen is safe and immunogenic in healthy adults aged 18 and older [79]. Following these promising results, a phase III (NCT04470427) was conducted to assess the efficacy and safety of the vaccine in individuals at high risk for SARS-CoV-2 infection or its complication. A total of 30,420 participants, 18 years of age or older, received 2 intramuscular injections

of 100 µg mRNA-1273 or the placebo, at 28-d interval. The vaccine showed an impressive 94.1% efficacy at preventing symptomatic COVID-19 including severe disease. Aside from moderate transient reactogenicity and rare serious adverse events (0.6% in vaccine and placebo groups) after vaccination, no major safety concerns were reported [80]. Of note, young adult male patients reported myocarditis and pericarditis after the second dose however, the symptoms presented were mainly mild chest pain and fever and most patients recovered completely or partially [81].

Pfizer/BioNTech first developed the BNT162b1 vaccine which consists of an LNP-formulated, nucleoside-modified mRNA encoding the RBD of the S glycoprotein of SARS-CoV-2, trimerized by addition of a T4 fibritin foldon domain so as to increase its immunogenicity. In a phase I/II dose-escalation study (NCT04368728) involving 45 healthy adults, 3 doses of the vaccine were tested (10, 30, 100 µg) upon intramuscular administration of 2 shots with a 21-d interval. While the dose of 100 µg of mRNA-1273 was deemed safe and well tolerated, this dose of BNT162b1 triggered increased reactogenicity (one report of severe pain) without any significant enhanced immunogenicity after single immunization as compared to the 30 µg dose. The lower vaccine doses were found to have a safe profile as only mild to moderate events were reported and they were transient as well. Immunogenicity of the vaccine was confirmed as RBD-binding IgG concentrations and SARS-CoV-2 neutralizing titers were found to increase in a dose-dependent manner and after the second dose. Importantly, GMT of the neutralizing antibodies were up to 4.6-fold higher than that of a panel of COVID-19 convalescent human sera, implicating the enhanced protective immunity provided by the vaccine [82]. In another phase I/II clinical trial (NCT04380701), BNT162b1 applied in a 2-dose regimen at 1 and 50 µg showed potent humoral and cellular responses whereby RBD-specific binding antibodies and SARS-CoV-2 neutralizing antibodies were present in the participants' serum at much higher levels than those seen in serum from a cohort of individuals who had recovered from COVID-19. Moreover, RBD-specific CD4$^+$ Th1 cell responses were reported along IL-2 and IFN-γ producing CD8$^+$ T cells. These data suggest that BNT162b1 vaccine can provide protection against COVID-19 through multiple beneficial mechanisms [83].

In order to mimic the intact virus more closely, Pfizer/BioNTech developed another LNP-formulated SARS-CoV-2 mRNA vaccine, designated as BNT162b2, which encodes the full-length S glycoprotein, modified by two proline mutations to lock it in its prefusion conformation. A phase I dose-escalation study (NCT04368728, NCT04380701) was conducted on 195 healthy participants of 2 age cohorts (18–55 years and 65–85 years) who received BNT162b1, BNT162b2, or placebo in a 2-dose regimen with a 21-d interval when given doses of 10, 20, or 30 µg; while the 100 µg dose was given as a single shot most likely due to the previously reported severe reactogenicity. BNT162b2 was associated with a lower incidence and severity of systemic reactions than BNT162b1, particularly in older adults, with mostly short-term adverse events. Moreover, both vaccines elicited similar dose-dependent neutralizing GMT, which were similar to or higher than the GMT of a panel of SARS-CoV-2 convalescent serum samples. Studies conducted in the United States and

Germany also found that two 30-µg doses of BNT162b2 not only induce humoral responses but also S-specific CD8$^+$ and Th1-type CD4+ T-cell responses. Together, these findings supported progression of BNT162b2 into phase III [84,85]. Phase II/III trials (NCT04368728) for investigation of the vaccine's safety and efficacy are still on going. Results so far have showed that out of 21,720 participants, aged 16 years and older who received two 30 µg-doses of the vaccine, only 8 cases of COVID-19 were reported with only 1 severe case. Accordingly, the vaccine was deemed 95% effective with a well-tolerated safety profile as mainly short-term, mild-to-moderate pain at the injection site, fatigue, and headache were reported, along with a low incidence rate of serious adverse events, including myocarditis and pericarditis in young males similarly to what has been reported for the mRNA-1273 vaccine [81,86]. Importantly, a new study found that immunization with a single dose of BNT162b2 significantly reduces the viral load in SARS-CoV-2 positive patients by 2.8–4.5-fold for infections occurring 12–37 d after vaccination when compared to unvaccinated patients. These reduced viral loads suggest that BNT162b2 vaccine may promote lower infectiousness, further contributing to vaccine effect on virus spread [87]. Moreover, as several mutation variants of SARS-CoV-2 have emerged around the world, it was important to investigate whether vaccination with BNT162b2 can still confer protection against the new strains, some of which are more transmissible than the WT virus. Xie et al. engineered 3 SARS-CoV-2 viruses containing key spike mutations from the United Kingdom and South African variants: N501Y; 69/70-deletion + N501Y + D614G; and E484K + N501Y + D614G. They studied whether sera obtained from 20 patients vaccinated with 2 doses of BNT162b2 can neutralize the 3 mutant viruses. Indeed, GMTs of neutralizing antibodies against all mutant viruses were only 0.81–1.46-fold of the GMTs against the WT virus. The magnitude of the differences in neutralization GMTs against the mutant viruses is small compared to the greater than 4-fold differences in HA-inhibition titers that are used to indicate potential need for a strain change in influenza vaccines [88]. Other studies found that neutralizing antibodies elicited by both primary infection and BNT162b2 vaccination can still provide protection from several variants including COH.20 G/677H in Columbus Ohio and 20A.EU2 in Europe. However, vaccine-elicited antibodies neutralized the E484K-containing B.1.1.7 and B.1.351 spike variants with a few fold reduction in titers. This reduction is most likely attributable to the E484K mutation in the RBD. Accordingly, the partial resistance of the B.1.1.7 and B.1.351 variants could render some vaccinated individuals less well protected, suggesting the potential need to develop modified vaccines containing E484K [89,90]. Fortunately, with the ease and flexibility of manufacturing LNP-mRNA vaccines, we are experiencing for the first time during the COVID-19 pandemic a rather facilitated route for adapting vaccines to new viral strains (Table 9).

While all these results look encouraging, further investigations are required to study whether the vaccines are safe and effective in children less than 16 years of age. Moreover, it is vital to investigate whether certain side effects reported after immunization with mRNA-1273 and BNT162b2 are indeed related to the vaccines.

Table 9 – LNP-entrapped IVT mRNA vaccines for prevention of COVID-19 in clinical trials.

Vaccine Platform	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref
m1Ψ IVT mRNA (mRNA-1273)	S-2P	LNP (ionizable lipid:DSPC: cholesterol:PEG-lipid, 50:10:38.5:1.5)	Phase I/II/III (NCT04283461/ NCT04405076/ NCT04470427)	- Production of high S-2P and RBD-specific and neutralizing antibodies by 100 µg dose, and Th1-biased CD4+ T cell response without major safety concerns - Confirmation of safety and immunogenicity of 50–100 µg in a 2 dose-regimen in healthy adults aged 18 and older - 94.1% efficacy at preventing symptomatic COVID-19 including severe disease in individuals at high risk for SARS-CoV-2	[78–80]
m1Ψ IVT Mrna (BNT162b1)	RBD in trimeric form	LNP	Phase I/II (NCT04368728/ NCT04380701)	- Safety and immunogenicity of 10–30 µg doses that induced dose-dependent RBD-binding IgG concentrations and SARS-CoV-2 neutralizing titers - Induction by 1–50 µg doses of potent humoral and cellular responses (RBD-specific binding and SARS-CoV-2 neutralizing antibodies, RBD-specific CD4+ Th1 cell responses, IL-2 and IFN-γ producing CD8+ T cells)	[82,83]
m1Ψ IVT mRNA (BNT162b2)	S in prefusion conformatiion	LNP	Phase I/II/III (NCT04368728/ NCT04380701/ NCT04368728)	- Safety and immunogenicity of 30 µg dose which induced S-specific CD8+ and Th1-type CD4+ T-cell responses - 95% efficacy at preventing COVID-19 - Promotion of lower infectiousness in COVID-19 vaccinated patients - Protection against United Kingdom, South African, Columbus Ohio, and European variants, with partial protection against E484K-containing B.1.1.7 and B.1.351 spike variants	[84–90]

These include morbilliform rash, blue-toes, supraclavicular lymphadenopathy, lymphoma-mimicking lymphadenopathy, and deep vein thrombosis, among other reports [91–95].

3.3. Polymeric NP-Entrapped IVT mRNA vaccines

Intranasal delivery of mRNA vaccines is a flexible and convenient approach. However, the nasal epithelium remains a major biological barrier to deliver immunogens to the nasal associated lymphoid tissue (NALT), rich in APCs and T and B lymphocytes. While liposomes and LNP are more often used for the delivery of mRNA vaccines, lipids are incapable of opening the epithelial tight junctions to reach underlying APCs. Accordingly, a nanoparticulate system was designed for the delivery of HIV-1 mRNA vaccine encoding gp120 glycoprotein by electrostatically complexing the negatively charged mRNA with a cationic conjugate composed of β-cyclodextrin (β-CD) and polyethylenimine (PEI) with a MW of 2k kDa (CP2k) [96]. Cyclodextrins (CDs) are macrocyclic oligosaccharides that are composed of glucose subunits and are synthesized by the bacterial enzymatic digestion of starch. They are FDA-approved as solubilizing agents

to improve delivery of various drugs as they can form inclusion complexes with drugs by trapping the entirety of the molecule or part of it into their hydrophobic cavity [97]. PEI is a hydrophilic cationic polymer that can take linear or branched structures. Its strong positive charge enables it to condense negatively charged particles such as RNA and improve its delivery *in vivo*. Its backbone structure contains one nitrogen atom in every three atoms, allowing PEI-based NPs to escape from lysosome degradation by working as a "proton sponge" [98]. The CP2k polymeric delivery system of only 117.3 nm in size efficiently transfected dendritic cells, tight junction-forming epithelial cells, and bronchial epithelial cells *in vitro*, with minimal cytotoxic effects. After endocytosis into APCs, the mRNA/CP2k NPs successfully escaped the endosome by the "proton sponge" mechanism, mediated by the basic buffering property of PEI, which enhanced mRNA expression in the cytoplasm. In turn, this promoted antigen presentation on the surface of DCs via MHC I which induced activation of CD8+ T cells, and via MHC II on the other hand which activated CD4+ T cells and helped generate gp120-specific IgG1/2a and secreted IgA antibodies. Moreover, CP2k enhanced the mRNA's nasal residence time

by 1.5-fold, implicating an increased adhesion to the nasal mucosa which permits enhanced mRNA uptake by the NALT. Importantly, complexation of mRNA with the polymers led to a decrease in TLR3-induced type I IFN production when compared to naked mRNA, suggesting that this polyplex ensures a balance between antigen-specific immune response and innate immunity. Overall, combining the high mucosal affinity of CD and the good adjuvanticity of the cationic PEI polymer promoted enhanced immune responses by ensuring delivery of mRNA via the paracellular route by reversibly opening the epithelial tight junctions and the intracellular route by transfecting cells of the NALT [96]. Moreover, the chemical structure of PEI was found to strongly influence how well nanocomplexes of PEI/CD and mRNA migrate to the lymph nodes and elicit immune responses. Compared to CP600 and CP25k, CP2k was found to be the optimal nasal delivery vehicle for mRNA vaccines as it induced significantly higher transfection efficiency of APCs, stronger potential to migrate from superficial to deeper lymph nodes where it stimulated DC maturation, and enhanced capacity to stimulate humoral and cellular immune responses. One major concern was the known toxic effect of PEI partly due to its positive charge however; the complexed CP600 and CP2k were associated with lower systemic and local toxicities as compared to the unmodified CP25k, implicating CP2k/mRNA nanocomplexes as self-adjuvanted vaccine delivery vehicles that traffic to lymph nodes with high efficiency and safety [99]. Moreover, a recent study published the optimal formulation of CP2k for the delivery of mRNA vaccines. It was found that the ideal CP2k/mRNA ratio, expressed as N/P ratio, is 16 where N refers to the nitrogen atoms in PEI and P refers to the phosphate groups in the mRNA backbone. Compared to N/P ratios of 4, 8, and 24, at an N/P of 16, the NPs of CP2k had a small size of 234.7 nm, with a homogeneous spherical morphology, a high encapsulation efficiency of mRNA encoding the model antigen ovalbumin, and the highest transfection efficiency of DCs. Moreover, CP2k was found to transfect DCs with significantly higher and moderately lower efficiencies than the classic nucleic acid transfection reagents branched-PEI25k and lipo-2000, respectively. Also, DCs internalized CP2k/mRNA within only 1 h of incubation at much higher rate than branched-PEI25k. This further indicated that modification of PEI2k with CD renders it a more potent mRNA delivery vehicle. Regarding the mRNA sequence, it was demonstrated that substituting cap0 with a cap1 structure, lengthening the polyA tail from 30 to 47 adenosine residues, and incorporating 5′ and 3′ UTR sequences provided optimal mRNA expression and protein translational efficiencies. Once applied *in vivo*, the CP2k/mRNA complex induced Th2 and Th1 responses, indicated by IgG1a and IgG2 antibody titers respectively, upon intramuscular and intradermal administrations but not with the subcutaneous route. Of note, the intramuscular route promoted a Th2-skewed response while the intradermal route induced a Th1-biased response. Taken together, this CP2k-based mRNA vaccine platform, combined with an optimized mRNA structure and an appropriate administration route, holds great promise for application to specific antigens in the future [100].

Polymeric NPs consisting of various cationic CPP have also been adopted as delivery vehicles for mRNA vaccines. In fact, low MW cationic polymers, such as the previously mentioned PEI2k, have been suggested as an alternative to the widely used protamine adjuvant. While protamine binds with strong affinity to mRNA molecules with an ineffective endosome-to-cytosol translocation, low MW cationic polymers are anticipated to associate with mRNA molecules with a diminished interaction strength enabling as such efficient mRNA expression provided that an intrinsic or extrinsic endosome disrupting agent is added. Indeed, this was achieved with the CP2k conjugate. It was also demonstrated that NPs consisting of CPPs can achieve similar effects as they combine lower charge densities with excellent intrinsic membrane disruptive abilities. Peptides containing the RALA, GALA, or LAH4-L1 motifs were shown to associate with mRNA molecules and form robust nanocomplexes that display acidic pH-dependent membrane disruptive properties. Accordingly, they promoted high cellular uptake by DCs, endosomal escape, and expression of the mRNA inside the cells' cytosol. This promoted efficient mRNA translation and subsequent antigen presentation on the surface of DCs. In turn, the CPP/mRNA NPs triggered DC maturation and potent CD8+ cytolytic T cell responses which were more enhanced when the mRNA sequences were modified with m1Ψ and 5mC. Moreover, it was shown that GALA and LAH4-L1 promoted endocytosis/phagocytosis-mediated entry into DCs via sialic acid and clathrin pathways, respectively. Also, RALA-mediated mRNA vaccines outperformed a standard liposomal mRNA formulation composed of DOTAP/DOPE, in terms of potency and immunogenicity. Accordingly, CPP-based NPs represent highly promising delivery vehicles for mRNA vaccines based on their ease-of-production, safety, and high immunogenicity [101–103].

Biodegradable polymers have also been adopted for the safe delivery of mRNA vaccines. Polyglucin (PG), a glucose polymer nontoxic to humans, and spermidine (S), a polyamine naturally present in all living organisms, were adopted for the formation of a cationic polymer conjugate. Due to electrostatic interactions, PGS self-assembled with mRNA encoding SARS-CoV-2 RBD antigen at a charge ratio of 5:1 into NPs of 164 nm in diameter and an overall neutral charge. PGS protected the entrapped mRNA from nuclease degradation and allowed it to be lyophilized and stored at +4°C without loss of nucleic acid activity, an important feature for the storage and transportation of vaccines which is not found with LNP-formulated mRNA vaccines. Moreover, the neutral surface charge may indicate that the positively charged PGS conjugate had completely packed the negatively charged mRNA molecules. It may also reflect the safety of these neutral NPs as compared to charged delivery vehicles since the latter tend to be more cytotoxic than the former. Indeed, mRNA-RBD-PGS cytotoxic concentration 50 (CC_{50}) was 30.6-fold higher than that obtained with conventional mRNA-RBD-Lipofectamine 3000 liposomes, confirming the enhanced safety profile of the NPs. Moreover, mRNA-RBD-PGS induced a 100–1000 increase in RBD- and S-specific IgG antibody titers compared to naked mRNA-RBD, and promoted SARS-CoV-2 neutralizing antibodies indicating the potential of the NPs to enhance mRNA immunogenicity without causing cytotoxicity [104]. Another method to address the toxic effects of positively charged mRNA delivery vehicles

is by developing charge-altering releasable transporters (CART). These dynamic polymers, specifically oligo(carbonate-*b*-*α*-amino ester)s, form polycations that can associate noncovalently to polyanionic mRNA, protect it, and deliver it to target cells. CARTs were able to transfect professional APCs (DCs, monocytes, and B cells) as well as T lymphocytes upon intravenous and subcutaneous administration in mice, implicating that CART uptake occurred both in the circulation and in secondary lymphoid organs. Once present in the APCs' endosomes, the polycations lose their positive charge through a controlled self-immolative degradation mechanism and disintegrate into neutral nontoxic byproducts, inhibiting therefore chelative electrostatic binding to anionic molecules and promoting endosomal escape. In turn, the newly freed mRNA is released into the cytosol for translation and antigen presentation on the surface of APCs. Importantly, the mRNA-CART delivery system was found to have superior transfection efficiency and antigen expression, as well as long lasting cytotoxic antigen-specific CD8$^+$ T cell activity than the commercial transfection reagent Lipofectamine 2000. The therapeutic effect of ovalbumin mRNA-CART was also demonstrated in tumor-bearing mice as CARTs were able to suppress, treat, and fully cure mice with established large tumors. Overall, CARTs are novel NPs with tunable, nontoxic, and dynamic properties that can serve as delivery agents for mRNA vaccines and have clinical promise for the treatment of established tumors (Table 10) [105].

3.4.   Scaffolds for delivery of IVT mRNA vaccines

Scaffolds are three-dimensional (3D) tissue engineered constructs. They serve as a matrix on which cells can be seeded along with chemical signals and/or drugs to promote tissue repair, replacement, or regeneration. Scaffolds are also employed for the entrapment of NPs to promote enhanced sustained release of the entrapped drugs and replace the need for repeated injections. They have been recently investigated for the improved delivery of mRNA vaccines. 3D polymeric scaffold constituted of poly (2-hydroxyethyl methacrylate) (pHEMA) was used for the delivery of commercial liposome Stemfect (SF)-entrapped mRNA vaccine. Lyophilization of the scaffold created a porous structure with pore size of 40 μm which facilitated cell seeding and homogeneous cell distribution. The optimal technique for loading SF:mRNA NPs onto the scaffold was by adopting the incubation/lyophilization technique whereby the lyophilized scaffold is incubated with an SF:mRNA NPs solution containing 1–2 μg of the lipoplex, subjected to flash freezing and lyophilization, then rehydrated with another SF:mRNA NPs solution and subjected to 1–4 cycles of incubation/lyophilization. The addition of the cryoprotectant trehalose to the lipoplex solution was found to be necessary to prevent the NPs from aggregating and losing their stability. Upon subcutaneous implantation in mice, scaffolds loaded with SF:mRNA NPs were found to induce optimal local prolonged release of mRNA from the scaffold, highest local transgene expression, and highest transfection efficiency (15%) as compared to naked mRNA entrapped in scaffold or bolus injections of naked mRNA or SF:mRNA NPs. The liposomes also protected mRNA from degradation and

improved its *in vivo* stability as scaffolds containing SF:mRNA NPs had 2.5–6.7–fold higher retained mRNA than scaffolds containing naked mRNA [106]. Another 3D porous polymer scaffold was developed for the delivery of mRNA vaccine encoding the model ovalbumin antigen. Biodegradable succinyl chitosan (S-CS) and oxidized alginate (O-Alg) were cross-linked and lyophilized for the formation of a gel scaffold with improved porous structure (pore size 100–200 μm) and swelling property for enhanced SF:mRNA loading. SF NPs promoted slow release of mRNA from the gel as only 30% was released over a course of 2 weeks while 80% of naked mRNA was released from the gel within only 3 d, proving again the importance of entrapping mRNA within NPs for more sustained release and better stability. An important aspect of using biodegradable polymers is that their byproducts are naturally eliminated from the body within 2 months, minimizing any risk for cytotoxicity. A 5-fold increase in local antigen expression was obtained with the SF:mRNA loaded scaffold along with an early production of ovalbumin-specific IgG antibodies (1 week *vs* 2 weeks for protein vaccine) and a 3-fold increase in IFN-*γ* producing cytotoxic T cells as compared to protein vaccine or systemically delivered mRNA vaccine [107]. These studies suggest that polymeric scaffold-based mRNA vaccine delivery may be an alternative approach to traditional nucleic acid immunization methods (Table 11) (Fig. 4).

4.   The future of mRNA vaccines

4.1.   Self-Amplifying RNA (saRNA) vaccines

Self-amplifying RNA (saRNA), also termed replicons, are considered the new generation of mRNA vaccines. They are most often derived from auses such as the Venezuelan equine encephalitis virus (VEEV), Sindbis virus replicon virus (SINV), and Semliki Forest virus (SFV). They consist of an alphavirus genome whose genes encoding the viral structural proteins have been deleted, rendering the mRNA incapable of generating infectious viruses. These deleted genes are replaced by the target gene(s) encoding the vaccine antigen(s). Similarly to conventional non-replicating mRNA, saRNA contain 5′ and 3′ UTRs as well as a 5′ cap and a polyA tail. However, they are larger in size (9–12 kDa) as they contain a larger ORF which includes a subgenomic promoter and four genes encoding four non-structural proteins (nsP1–4) required for the formation of a functional RNA-dependent RNA polymerase (RDRP) complex. RDRP binds to the subgenomic promoter placed right before the antigen sequence and uses the mRNA vaccine as a template to synthesize a complementary negative sense RNA which then serves as a template for synthesis of positive sense genomic and subgenomic RNA strands.

There are several advantages of saRNA vaccines compared to non-replicating mRNA. (1) saRNA lead to the production of excessive subgenomic RNA encoding the vaccine antigen up to ten-fold higher than the produced viral genome. Subsequently, they produce higher and more sustained levels of antigen expression relative to conventional mRNA. Hence, saRNA vaccines require lower doses of RNA which is an

Table 10 – Polymeric NP-entrapped IVT mRNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical	Immune Response	Ref
IVT mRNA	HIV-1	gp120	β-CD/ PEI (CP2k) NPs	Mice	- Induction of enhanced immune responses via paracellular delivery of mRNA by reversibly opening the epithelial tight junctions and the intracellular route by transfecting cells of the NALT - Decreased TLR3-induced type I IFN production	[96]
IVT mRNA	N/A	Ovalbumin	β-CD/ PEI (CP600, CP2k, CP25k) NPs	Mice	- Induction of the highest transfection efficiency of APCs, stimulation of DC maturation, and activation of humoral and cellular immune responses by CP2k - Safety profile of CP600 and CP2k	[99]
IVT mRNA	N/A	Ovalbumin	β-CD/ PEI (CP2k) NPs	Mice	- CP2k/mRNA ratio of 16 ideal for optimal size of NPs, encapsulation of mRNA, transfection of DCs - Ideal mRNA sequence for optimal mRNA expression and protein translation include cap1 structure and polyA tail of 47 residues - Th-2 and Th-1 skewed responses upon intramuscular and intradermal routes, respectively	[100]
5mC/m1Ψ IVT mRNA	HIV-1	Gag Ovalbumin	CPP NPs (RALA, GALA, LAH4-L1)	*In vitro* Mice	- Promotion of high cellular uptake by DCs, endosomal escape, and antigen presentation - Activation of DC maturation and potent CD8+ cytolytic T cell responses	[101–103]
IVT mRNA	SARS-CoV-2	RBD	PG/SNPs	Mice	- Protection of mRNA from RNase degradation - Enhanced safety profile and production of RBD- and S-specific IgG antibodies compared to Lifofectamine-3000 and naked mRNA-RBD, respectively - Production of SARS-CoV-2 neutralizing antibodies	[104]
IVT mRNA	Cancer	Ovalbumin	CART NPs	Mice	- Efficient transfection of APCs and T lymphocytes - Enhanced transfection efficiency, antigen expression, long lasting cytotoxic antigen-specific CD8+ T cell activity than Lipofectamine 2000	[105]

Table 11 – Scaffolds for delivery of IVT mRNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref
IVT mRNA in SFliposomes	GFP	pHEMA scaffold	Mice	- Induction of local prolonged release of mRNA, high local transgene expression, and high transfection efficiency	[105]
IVT mRNA in SF liposomes	Ovalbumin	S-CS and O-Alg scaffold	Mice	- Slow release of mRNA from liposomes entrapped in scaffold - Increase local antigen expression, early production of ovalbumin-specific IgG, and increased IFN-γ producing cytotoxic T cells compared to protein vaccine or systemically delivered mRNA vaccine	[106]
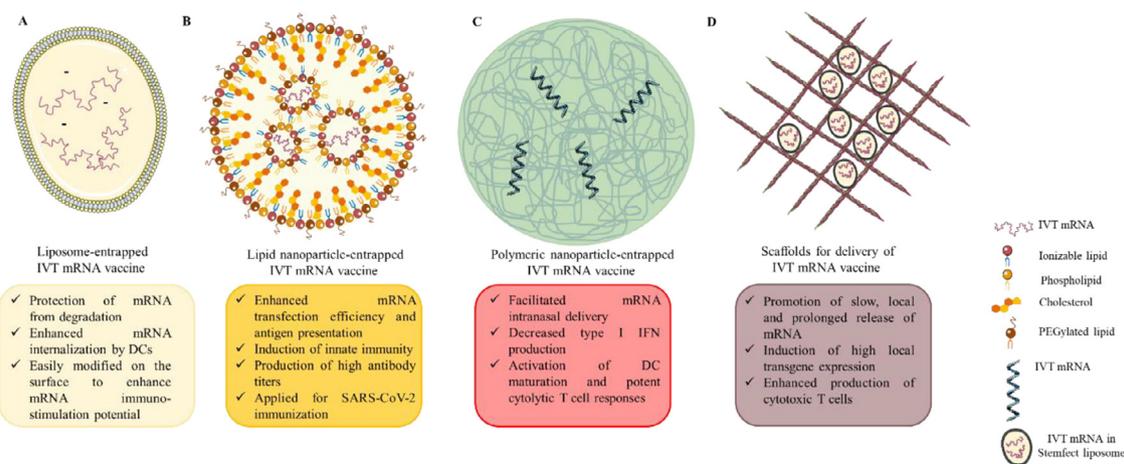
Fig. 4 – Schematic figure representing the four types of nanocarriers used for the delivery of IVT mRNA vaccines along with their main advantages compared to naked IVT mRNA vaccines: (A) liposome-entrapped IVT mRNA vaccine, (B) LNP-entrapped IVT mRNA vaccine, (C) polymeric NP-entrapped IVT mRNA vaccine, and (D) scaffolds for the delivery of IVT mRNA vaccines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

important factor to consider when there is a viral pandemic and large quantities of vaccine need to be distributed worldwide at high speed and low costs. Indeed, an saRNA encoding the HA gene from an influenza virus was able to induce similar protective efficacy as a conventional mRNA vaccine but at a 64-fold lower dose (1.25 µg *vs* 64 µg) [108]. (2) saRNA lead to more protein translation than conventional mRNA, generate double stranded RNA intermediates, and promote antiviral responses. All of these events cause cellular exhaustion, immune stimulation, and eventually cell apoptosis, mimicking as such a typical viral infection. As a result, saRNA generate enhanced antigen-specific humoral and cellular responses than conventional mRNA. In a similar fashion, saRNA simulate the sustained antigen presentation characteristics of traditional live attenuated vaccines and could offer as such more durable immunity than the conventional mRNA [109]. (3) Other than stimulating the adaptive immunity, saRNA also stimulate the innate immune system. In fact, pathogen-associated molecular patterns (PAMPs) present on the RNA and detected by PRRs expressed on APCs, mainly DCs, induce inflammatory responses and innate host defenses, increasing the vaccine's immunogenicity. In fact, saRNA vaccines were found to promote a type I IFN response which acted as an adjuvant effect, especially upon mutations affecting the nsP proteins. However, as seen with conventional IVT mRNA, an elevated type I IFN response decreases RNA's translation efficiency. In order to overcome this limitation, it was advised to induce sequence modifications to generate IFN-insensitive RNA or to optimize the delivery of saRNA vaccine such as entrapment in LNPs to prevent recognition of RNA by PRRs [110].

Self-amplifying RNA vaccines have been tested for cancerous and infectious diseases. VEEV-based saRNA encoding IL-2 and entrapped in LNPs were tested on melanoma and colon carcinoma mice models. The LNPs composed of DOTAP:DSPC:cholesterol:PEG$_{2000}$ at a molar ratio of 40:10:48:2 promoted immunogenic cancer death (ICD), the saRNA itself stimulated TLR3-mediated type I IFN response, and the saRNA-encoded IL-2 primed systemic and memory antitumor immunity. Together, a single injection with the vaccine eradicated large tumors and enabled regression of distal uninjected tumors [111]. Moreover, introduction of mutations into the VEEV replicon backbone of an saRNA encoding IL-2 was shown to enhance intra-tumoral antigen expression by 5.5-fold and increase CD8+ T cell response which significantly halted melanoma tumor growth [112]. saRNA encoding tumor-specific neoantigens are also being tested in as prime boost vaccines for several metastatic solid cancers. Naked saRNA vaccines [113,114], as well as those entrapped in dendritic NPs [115,116], cationic nanoemulsions [117–120], and LNPs [121–123] have also shown promising results in promoting protective immunity against several viruses such as Ebola, Zika, HIV-1, RSV, and influenza viruses. An saRNA vaccine with a VEE-SINV backbone encoding the Rabies glycoprotein G and entrapped in cationic nanoemulsions is currently being tested in a phase I clinical trial (NCT04062669) for safety, reactogenicity and immunogenicity [124]. Moreover, a novel design of saRNA was introduced in 2020 by Beissert et al. It consists of a bipartite vector system whereby the vaccine antigen is encoded by an alphaviral saRNA from which the RDRP has been deleted. Accordingly, the replicase activity is provided in *trans* by a second RNA molecule which was either an saRNA or an optimized non-replicating mRNA (nrRNA). This system, termed trans-amplifying RNA (taRNA), was successful in inducing neutralizing antibodies and a protective immune response against live influenza virus challenge at doses as low as 50 ng. These results together with a favorable safety profile, a simple production process, and a universal applicability, implicate the potential of taRNA
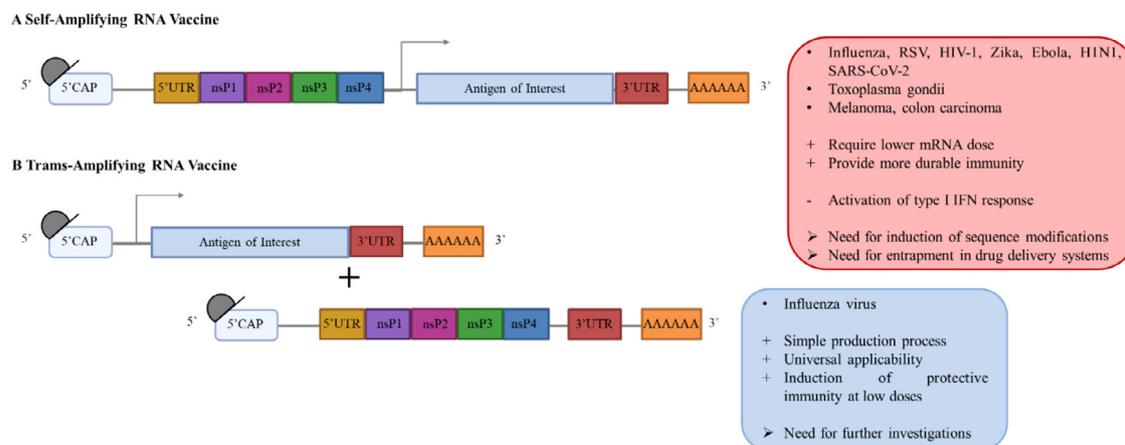
Fig. 5 – Schematic figure representing the structure of (A) a self-amplifying mRNA derived from an alphavirus genome and that of (B) a trans-amplifying mRNA which consists of a bipartite vector system whereby the vaccine antigen is encoded by an alphaviral self-amplifying RNA from which the RDRP has been deleted and a second RNA molecule encoding the RDRP which originates from either a self-amplifying RNA or an optimized non-replicating mRNA.

vaccines to be further investigated in various applications (Fig. 5) [125].

Taken together, these studies demonstrate the extraordinary properties of saRNA vaccines to induce immune responses. Accordingly, we believe that saRNA represent the next generation of mRNA vaccines, and accordingly research on saRNA will continue to grow and it will most likely be the focus of future mRNA vaccine studies.

Indeed, saRNA vaccines for prevention of the ongoing global pandemic of COVID-19 are already being tested in preclinical and clinical settings and are showing promising results. LNP-nCoVsaRNA is an saRNA vaccine candidate that was rapidly developed by the Imperial College London within only 14 d of first genetic sequencing. It is an engineered VEEV-based purified saRNA that encodes the pre-fusion stabilized SARS-CoV-2 S glycoprotein and is encapsulated in LNPs formulated as in previous studies (ionizable cationic lipid/phosphatidylcholine/cholesterol/PEG-lipid). *In vivo* studies conducted in mice showed that 0.01–10 µg of LNP-nCoVsaRNA, administered intramuscularly in a two-dose regimen, can generate highly-specific Th1-biased neutralizing antibodies as well as cellular responses in mice, at higher quantities compared to patients that had recovered from COVID-19. The vaccine is currently being tested in a phase I/II clinical trial (ISRCTN17072692) performed with healthy volunteers between 18 and 75 years of age for dose-escalation and safety studies [126]. repRNA-CoV2S is another VEEV-derived replicon RNA vaccine candidate which encodes the SARS-CoV-2 S glycoprotein. It is entrapped however within lipid inorganic NPs (LION). Upon single intramuscular injection of 50 µg of vaccine in mice and nonhuman primates, the LION/repRNA-CoV2S vaccine promoted the production of Th1-biased S-specific IgG antibodies that lasted for 70 d and efficiently neutralized SARS-CoV-2. A boost immunization was required however for induction of a potent T cell response in young mice and in nonhuman primates, and also

for induction of both humoral and cellular immunities in aged mice, representative of the older human population mainly affected by COVID-19. On the other hand, a 250 µg prime-only dose was sufficient for induction of neutralizing antibodies that can provide protection of nonhuman primates from infection and disease. This vaccine is currently in the pre-recruiting step of a phase I clinical trial (NCT04844268) [127]. Another VEEV-derived SARS-CoV-2 saRNA vaccine currently in phase I clinical trial (NCT04480957) is STARR™ saRNA with LUNAR® LNP. It consists of an saRNA encoding the full length pre-fusion stabilized S protein of SARS-CoV-2 and entrapped within LNPs of Arcturus Therapeutics proprietary ionizable lipid:DSPC:cholesterol:PEG_{2000}-dimyristoylglycerol at a molar ratio of 50:13:35.5:1.5. Preliminary data reported that a single immunization of mice with a dose of 2 or 10 µg was able to induce strong Th1-biased antibody responses with neutralizing antibody titers that lasted up to 60 d A strong CD8+ T cell response was also recorded. Together, this established immunity protected human ACE2 transgenic mice from mortality and infection following WT SARS-CoV-2 challenge. Importantly, equal doses of STARR RNA and conventional mRNA both encoding the same full-length S protein and entrapped in the same LUNAR® LNP formulation were compared head-to-head. At 2 and 10 µg doses, LUNAR®-COV19 vaccine was shown to induce higher and more prolonged expression of the S protein as compared to the conventional mRNA. Similarly, LUNAR®-COV19 vaccine upregulated the gene expression of several innate, B and T cell response genes in the blood and draining lymph nodes, and these properties were associated with greater humoral and cellular responses than those elicited by the conventional mRNA. Importantly, while the highest dose of conventional mRNA produced similar titers of S-specific antibodies as the lowest dose of STARR RNA, it was not sufficient to neutralize SARS-CoV-2 WT virus, further highlighting the advantages of saRNA over conventional mRNA (Table 12) [128].

Table 12 – Self-amplifying RNA vaccines for infectious and cancerous diseases.

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref.
saRNA	Influenza	HA	N/A	Mice	- Induction of similar protective efficacy as a conventional mRNA vaccine but at a 64-fold lower dose	[108]
saRNA	Influenza	Nucleoprotein	LNP (DLinDMA:DSPC: cholesterol:PEG-DMG 2000, 40:10:2:48)	Mice	- Antigen expression in muscle cells transferred to APCs is responsible for induction of MHC class-I restricted CD8+ T cells	[109]
saRNA	RSV	F	LNP (DLinDMA:DSPC: cholesterol:PEG-DMG 2000, 40:10:2:48)	Mice	- Enhanced immunogenicity by activation of type I IFN response	[110]
saRNA	Melanoma Colon carcinoma	IL-2	LNP (DOTAP:DSPC: cholesterol:PEG2000, 40:10:48:2)	Mice	- Induction of immunogenic cancer death, stimulation of TLR3-mediated type I IFN response, priming systemic and memory antitumor immunity - Eradication of large tumors and regression of distal uninjected tumors	[111]
saRNA with mutated VEEV backbone	Melanoma	IL-2	LNP (DOTAP:DSPC: cholesterol:DSPE-PEG2000, 40:10:48:2)	Mice	- Enhanced intra-tumoral antigen expression by 5.5-fold - Increased CD8+ T cell response - Inhibition of tumor growth	[112]
saRNA	HIV-1C	gag, env, polRT	N/A	Mice/ Phase I (NCT03639714)	- Activation of CD4+ and CD8+ T cells - Production of HIV-1C specific antibodies	[113]
saRNA	Zika	prM, E	N/A	Mice/ Phase II (NCT03953235)	- Moderate humoral and cellular immune responses due to type I IFNs	[114]
saRNA	Ebola, H1N1, Toxoplasma gondii	EBOV-GP, HA, cytoplasmic ovalbumin	Dendritic NPs	Mice	- Activation of CD8+ T-cell and antibody responses that fully protected against lethal exposures to the deadly pathogens	[115]
saRNA	Zika	E	Dendritic NPs	Mice	- Production of E-specific IgG antibodies - Activation of CD8+ T cells	[116]
saRNA	HIV-1	TV1 Env gp140	Cationic nanoemulsion	Rhesus macaques	- Induction of potent cellular responses greater in magnitude than those induced by saRNA packaged in viral replicon particle (VRP) or by a recombinant HIV envelope protein formulated with MF59 adjuvant - Production of anti-envelope binding and neutralizing antibodies that exceeded those induced by VRP vaccine	[117]
saRNA	Influenza	HA	Cationic nanoemulsion	Mice Ferrets	- Induction of potent neutralizing antibody and cellular immune responses - Protection of immunized animals from lethal viral challenge and containment of viral replication in upper respiratory tract	[118]
saRNA + GM-CSF	Influenza	Nucleoprotein	Cationic nanoemulsion	Mice	- Enhanced recruitment of APCs - Improved magnitude of nucleoprotein-specific CD8+ T-cell response - Improved protection against lethal challenge	[119]

Table 12 (continued)

Vaccine Platform	Disease Target	Antigen	Delivery Platform	Preclinical/ Clinical Setting	Immune Response	Ref.
saRNA	Zika	C, prM, E	Cationic nanoemulsion	Mice Non-human primates	- Production of potent neutralizing antibodies - Protection of animals from Zika lethal challenge	[120]
saRNA	HIV	gp120	LNP (DOTAP:DSPC: cholesterol:DSPE-PEG, 40:10:48:2)	Mice	- Effective delivery in the muscle and expression of mRNA for 30 d - Production of high titers of gp120-specific antibodies and antigen-specific GC B cells	[121]
saRNA	Influenza	Nucleoprotein	LNP (DLinDMA:DSPC: cholesterol:PEG-DMG 2000, 40:10:2:48)	Mice	- Activation of robust polyfunctional CD4+ T helper 1 cells, nucleoprotein-specific cytotoxic CD8+ T cells - Reduced lung viral titers and pathology, and increased survival	[122]
saRNA	Zika	NS3	LNP (Proprietary lipids)	Mice	- Failure to stimulate antibody production - Production of polyfunctional CD8+ T cells that prevented death in lethally infected adult mice and fetal growth restriction in infected pregnant mice	[123]
saRNA	Rabies	Glycoprotein G	Cationic nanoemulsion	Rat/ Phase I (NCT04062669)	- RNA detectable at injection site and in lymph nodes up to 2 months post-injection - Well tolerated by animals upon intramuscular administration	[124]
taRNA	Influenza	HA	N/A	Mice	- Induction of neutralizing antibodies and a protective immune response at low doses of 50 ng	[125]
saRNA	SARS-CoV-2	S	LNP (ionizable lipid: phosphatidylcholine: cholesterol:PEG-lipid, 50:10:38.5:1.5)	Mice/ Phase I/II (ISRCTN17072692)	- Induction of highly specific Th1-biased neutralizing antibodies and cellular responses	[126]
saRNA	SARS-CoV-2	S	LION	Mice, Non-human primates/ Phase I (NCT04844268)	Low dose of 50 μg: - Production of Th1-biased S-specific IgG antibodies that lasted for 70 d and efficiently neutralized SARS-CoV-2 - Induction of potent T cell response in young mice and nonhuman primates, and induction of both humoral and cellular immunities in aged mice after booster dose High dose of 250 μg: - Induction of neutralizing antibodies that protected non-human primates from infection and disease	[127]
saRNA	SARS-CoV-2	S	LNP (ionizable lipid: DSPC:cholesterol: PEG2000-DMG, 50:13:35.5:1.5)	hACE2 transgenic mice/ Phase I (NCT04480957)	- Activation of strong Th1-biased antibody responses with neutralizing antibody titers lasting 60 d - Induction of a strong CD8+ T cell response - Protected of mice from mortality and infection	[128]

Table 13 – The major advantages and disadvantages of the various types of mRNA vaccines.

Type		Advantages	Disadvantages
History of mRNA Vaccines	Naked IVT mRNA	- Self-adjuvant potential - Activation of innate and adaptive immunity - Inability to integrate the genome - Inexpensive, simple, reproducible, and fast synthesis procedure - Universal applicability	- Low transfection efficiency - Intensified and/or boost administrations required - Insufficient for providing complete protective immunity - High type I IFN signaling - Reduced translatability - Optimizations required to reduce immunogenicity (*i.e.* nucleoside modification and purification from double-stranded RNA)
	Adjuvant-tethered IVT mRNA	- Enhanced innate immunostimulation - Facilitated transfection of antigen-presenting cells - Low IFN-driven immunogenicity - Ease of production	- High type I IFN signaling - Reduced translatability - Intricate production to achieve optimal mass ratios of antigen/adjuvant
	RNActive mRNA	- Preserved translatibility - High antigen expression - Enhanced stimulation of innate immunity by TLR7 - Facilitated transfection of antigen-presenting cells - Stronger and more balanced adaptive immunity - Favorable safety profile with low-grade adverse events - Stable at room temperature - Ease of lyophilization for transportation and distribution	- Inefficient for driving immunogenicity by needle injection
Current status of mRNA Vaccines	Liposome-entrapped IVT mRNA	- Protection of mRNA from degradation - Enhanced internalization by dendritic cells - Easily functionalized with ligands to achieve targeted delivery - Good biocompatibility - Ease of fabrication - Good scalability - Low batch-to-batch variability	- Unrestricted protein binding - Colloidal instability - Risk of mRNA leakage - Risk of neutralization of cationic liposomes by anionic serum proteins leading to cytotoxicity and reduced efficacy - Difficulty with lyophilization for transportation and distribution
	LNP-entrapped IVT mRNA	- High encapsulation efficiency - Enhanced mRNA transfection efficiency and antigen presentation - Endosomal escape capacity - Protection of mRNA from degradation - Activation of cellular and humoral immunity - Favorable safety profile with low-grade toxicity - Reliable and reproductive production	- Risk of low delivery efficiency with cationic lipids - Insufficient information regarding the immunogenicity of lipids used - High frequency and moderate severity of local injection site reactions and systemic adverse events - Short-term and low stability - Difficulty with lyophilization for transportation and distribution
	Polymeric NP-entrapped IVT mRNA	- Enhanced immunogenicity - High stability - Facilitated uptake by dendritic cells - Facilitated intranasal delivery - Activation of dendritic cells maturation and cytolytic T cells - Enhanced safety - Biodegradability - Ease of production	- Polydispersity - Challenges with metabolism of large molecular weight polymers - Limited animal studies
	Scaffolds for delivery of IVT mRNA	- Slow, local, prolonged release of mRNA - High local transgene expression - Enhanced production of cytotoxic T cells - Biodegradability - Versatile production techniques for different scaffold shapes and porosities	- Invasive surgical implantation required
Future of mRNA Vaccines	Self-amplifying mRNA	- High level of RNA amplification and transgene expression - Humoral and cellular responses elicited against expressed antigen - Safe due to lack of viral genes for structural protein assembly - No risk of genome integration - Ease of large-scale synthesis for various antigens	- Prime and/or boost administrations may be required - Delivery via nanocarriers may be required - Insufficient information regarding the immunogenicity of RDRP complex - Insufficient information regarding the safety of prolonged RNA amplification and expression
	Trans-amplifying mRNA	- High translation efficiency - Enhanced intracellular delivery - Activation of protective immunity at low RNA doses - No interference with cellular translation - Favorable safety profile - Simple, fast, and cost-efficient production - Universal applicability - Ability to optimize each of the two components independently	- Limited preclinical and clinical data - Insufficient information regarding the safety of sustained trans-replicase activity

4.2. Advantages, challenges, and opportunities for future mRNA vaccine research

As the world is hit every few years with new viral pandemics, the critical need for novel technologies that can provide rapid and adaptable production of safe and effective vaccines is increasing. Generating such vaccines is therefore an urgent unmet clinical need. mRNA-based vaccines are currently the lead vaccine candidates for the ongoing COVID-19 pandemic and for several infectious and cancerous diseases owing to their additional beneficial characteristics over other vaccine platforms. First, mRNA vaccines have a favorable safety profile and higher efficiency when compared to viral vector and DNA vaccines as they are readily functional as soon as they enter the cell's cytoplasm since they do not need to reach the nucleus to get translated into the gene of interest. Also, as they cannot integrate into the host's genome, they are unable to introduce mutations, replicate, induce or transmit an infection. Synthesizing mRNA with proper purification and incorporation of modified nucleosides can further enhance mRNA vaccines' safety by reducing its inflammatory capacity. Second, mRNA vaccines can be produced at low cost, in a reproducible, cell-free, and rather simple manner, and at a fast rate rendering them the ideal candidates for providing life-saving vaccines amid a pandemic. Also, the translated antigen will have natural glycosylation and conformational properties, increasing as such its immunogenicity. Third, the various forms of conventional and self-replicating mRNA vaccines – naked, adjuvanted, or entrapped in delivery vehicles – have shown great potential in generating both neutralizing and binding antibodies, inducing CD4$^+$ and CD8$^+$ cellular responses, promoting long-lived and memory immunity, as well as activating the innate immune system which provides superior protection against infectious diseases. Single dose of mRNA vaccines was also sufficient in some cases for providing protective immunity, implicating again the importance of such vaccines in a pandemic setting where mass vaccination is a critical need. Fourth, mRNA can be synthesized to express any protein of interest and can be easily modified to alter its immunostimulatory potential. Accordingly, mRNA vaccines can be applied for the prevention of viral, bacterial, and parasitic diseases, as well as for the prevention and treatment of cancer and, in theory, any other infectious disease, rendering them versatile tools with infinite applications. Fifth, saRNA and taRNA vaccines are highly immunopotent, promote amplification of the antigen within the host's cells, and accordingly the accumulated immunomodulatory proteins may enhance genetic immunization strategies, rendering them ideal in pandemic settings and chronic infectious illnesses [129–131].

Despite all the efforts that have been made so far in mRNA vaccine research, there are still several challenges to overcome. First, RNA is an unstable molecule due to the presence of a hydroxyl group at the 2′ position on the ribose sugar. This renders it sensitive to environmental and biological nucleases which can cleave its phosphodiester bond by intramolecular transesterification. Freezing, freeze-drying, or lyophilizing RNA formulations was found to be a viable approach to enhance RNA's stability. Adding the cryoprotectant trehalose to freeze-dried RNA vaccines maintained its stability for a period of 10 months when stored at 4 °C [132]. However, these lyophilization cannot be applied to mRNA entrapped in lipid formulations such as liposomes and LNPs as their structure gets altered by the freezing/thawing cycles during the lyophilization process. Currently, Pfizer's COVID-19 mRNA vaccines are only stable in liquid form when stored at −80°C which is a limitation to certain developing countries that do not have the luxury of purchasing such heavy-duty freezers. Addition of 5% (w/v) of sucrose or trehalose to the LNP formulation is a viable approach to overcome the LNPs' colloidal instability and conserve the mRNA delivery efficiency. This technique has however proved effective for 3 months only and necessitates the storage of the LNP-entrapped mRNA in liquid nitrogen [133]. Another approach to protect mRNA from enzymatic degradation is by complexing it with protamine which is still associated with the risk of decreasing the nucleic acid's translatability [18,19]. Second, mRNA can stimulate the innate immunity through distinct PRRs expressed on the surface of APCs. While this adjuvant effect might be advantageous for protein vaccines, indiscriminate immune activation is associated with the risk of inhibiting mRNA translation, thus reducing antigen expression and immunogenicity of an mRNA vaccine. While several attempts have been made to overcome this limitation, there is a lot of progress left to be made to provide vaccines with a high potency. Third, the safety of mRNA vaccines in clinical settings remains an uncertain issue. Preclinical as well as short-term clinical studies have implied that mRNA are generally safe for use as vaccines since they were mainly associated with transient, mild to moderate adverse events. However, several reports have implicated the possible association of these vaccines with rare thrombotic events that were even fatal in some cases. Moreover, little is known regarding the safety of sustained RNA expression and/or amplification, as well as the fate of an mRNA vaccine in a host with a retroviral infection. Accordingly, there is critical need for conducting long-term clinical studies to further evaluate the effect of mRNA vaccines on children, young and older adults, as well as pregnant and lactating women, and people with allergic reactions and comorbidities (Table 13) [129–131].

5. Conclusion

Over the past two decades, researchers have extensively explored the potential of mRNA molecules as preventive and therapeutic vaccines for distinct infectious diseases, many of which have already undergone clinical trials, mainly for viral infections and cancer (Fig. 6). The development of mRNA vaccines has evolved tremendously, starting with the inexpensive and time-effective in vitro synthesis of naked mRNA using cell-free systems, along with intricate sequence optimizations such as introduction of nucleoside modifications, sequence engineering, and chromatographic purification of single-stranded mRNA. This easily reproducible production process further evolved with the addition of adjuvants, as co-delivered stimulatory factors or tethered to
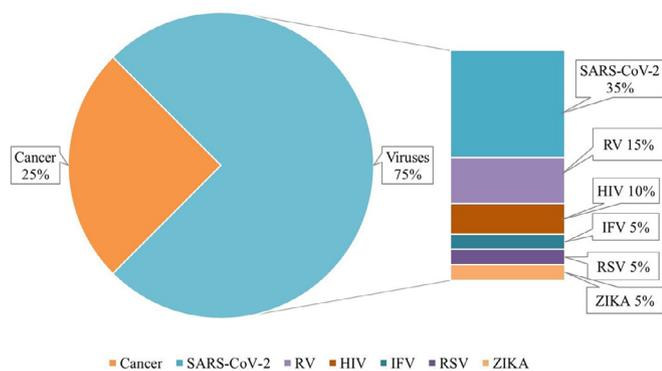
Fig. 6 – Bar of pie chart representing the clinical application percentage (%) of mRNA vaccines indicating that 75% of mRNA vaccines are being clinically tested for prevention of viral infections while 25% are being investigated for cancer therapy. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, RV: rabies virus, HIV: human immunodefiency virus, IFV: influenza virus, RSV: respiratory syncytial virus, ZIKV: zika virus.

the mRNA molecule, for the enhancement of the vaccine's immunogenic potential and uptake by antigen-presenting cells. As the major drawbacks limiting the clinical feasibility of mRNA vaccines remained their low stability and limited transfection efficiency, nanotechnology concepts were introduced. The entrapment of mRNA vaccines within novel nanocarriers, mainly liposomes, LNPs, polymeric NPs, and tissue engineered scaffolds, promoted the progression of mRNA vaccines from a conceptual proposition to a clinically-feasible versatile solution. The immense success of LNP-entrapped mRNA vaccines was clearly demonstrated during the COVID-19 pandemic which has incentivized more scientists and pharmaceutical industries to get involved in this promising field of research. The evolution of mRNA vaccines is steering towards saRNA and taRNA as they are proving to be highly immunopotent, are able to amplify the antigenic sequences within the transfected cells and can elicit both humoral and cell-mediated immune responses. As mRNA research is currently being heavily funded worldwide, we anticipate greater and faster improvements in this field. Future research should heavily focus on exploring the optimal ionizable lipids to be included in the structure of LNP-entrapped mRNA vaccines, determining the ideal surface moieties for enhanced selective targeting, as well as studying the vaccines' potency, adjuvanticity, and exact mechanism of action. These mentioned recommendations would eventually help fully exploit the potential of this novel therapeutic modality. Moreover, as studies continue to investigate new strategies to improve IVT mRNA synthesis, optimize their adjuvanticity and delivery formulations, and enhance their *in vivo* pharmacokinetics, it remains vital to pinpoint the best strategies to adopt. Accordingly, running parallel studies are encouraged to help compare, side-by-side, the most suitable vaccination approaches to adopt in clinical settings. Moreover, long-term safety studies are required to uncover any potential toxicities of mRNA vaccines, particularly the newly adopted saRNA and taRNA. The latter are mainly limited by the short

number of studies that have explored their application and hence, it is of great importance to verify their prophylactic functions in advanced preclinical studies as well as clinical trials. Recruitment of volunteers from different ethnicities, nationalities, and age groups is another vital criterion to consider since environmental and genetic differences can influence immunization responses. Finally, clinical studies should also consider distinct administration routes and regimens to help determine the optimal immunization strategy which would help guide the ideal storage procedure, transportation, distribution, and handling of mRNA vaccines.

In conclusion, mRNA technology offers great potential for synthesis of rapidly-, mass-produced, and environmentally-friendly vaccines with enhanced safety and efficacy. The versatility of mRNA vaccines permits their use in prophylactic as well as therapeutic applications for, theoretically, any infectious disease. Future research in this field as well as data from clinical trials will help uncover the long-term effects of mRNA vaccines and their implications. Finally, the future of mRNA vaccines seems bright and there is great hope for rapid advancement of this field towards clinical use.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Acknowledgements

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