Molecular Therapy
Review

mRNA as a Transformative Technology for Vaccine Development to Control Infectious Diseases

Giulietta Maruggi,[1] Cuiling Zhang,[2] Junwei Li,[2] Jeffrey B. Ulmer,[1] and Dong Yu[1]

[1]GSK, 14200 Shady Grove Road, Rockville, MD 20850, USA; [2]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao 266109, China

In the last two decades, there has been growing interest in mRNA-based technology for the development of prophylactic vaccines against infectious diseases. Technological advancements in RNA biology, chemistry, stability, and delivery systems have accelerated the development of fully synthetic mRNA vaccines. Potent, long-lasting, and safe immune responses observed in animal models, as well as encouraging data from early human clinical trials, make mRNA-based vaccination an attractive alternative to conventional vaccine approaches. Thanks to these data, together with the potential for generic, low-cost manufacturing processes and the completely synthetic nature, the prospects for mRNA vaccines are very promising. In addition, mRNA vaccines have the potential to streamline vaccine discovery and development, and facilitate a rapid response to emerging infectious diseases. In this review, we overview the unique attributes of mRNA vaccine approaches, review the data of mRNA vaccines against infectious diseases, discuss the current challenges, and highlight perspectives about the future of this promising technology.

Vaccination is one of the most effective public health interventions to prevent and control infectious diseases. Since the first known clinical trial conducted with cowpox in 1796,[1] vaccines have resulted in the eradication of many infectious diseases, and today ~30 diseases worldwide can be prevented by vaccination.[2,3] Over the last two centuries, vaccinology has evolved from Pasteur's principles of pathogen "isolation, inactivation, and injection" to a rational vaccine design based on genetic engineering, immunology, structural biology, and systems biology.[4,5]

Despite the advancement in conventional vaccine approaches, challenges remain, and new vaccine technologies are necessary. The list of unmet medical needs includes vaccines against pathogens causing chronic infections that can evade adaptive immune responses or require cellular immune responses, and against emerging diseases, such as Zika, Ebola, Nipah, and pandemic influenza.[6] Epidemic outbreaks caused by virus infections are emerging or reemerging almost every year and in all cases are characterized by unpredictability, high morbidity, exponential spread, and substantial social impact. A "vaccine on demand" approach that enables rapid development, large-scale production, and distribution of the vaccine would be desirable. Such an approach may not be compatible with conventional vaccine technology platforms that often require complex and lengthy research and development processes.

Nucleic acid-based vaccines, including viral vectors, plasmid DNA (pDNA), and mRNA, will be suitable for rapid response applications because of their ability to induce broadly protective immune responses and their potential of being produced by rapid and flexible manufacturing processes. Because the manufacturing processes of nucleic acid-based vaccines are independent of encoded antigens, different vaccines based on the same nucleic acid platform can utilize the same production and purification methods, as well as manufacturing facilities, requiring adaptations only in validation methods, therefore cutting both costs and time for vaccine production.[7] Upon vaccination, nucleic acid-based vaccines mimic a viral infection to express vaccine antigens *in situ*, resulting in induction of both humoral and cytotoxic T cell responses.[8] This advantage is critical for the elimination of intracellular pathogens or infections in which potent humoral and cellular immune responses are required to achieve protective efficacy. In addition, nucleic acid vaccines have intrinsic adjuvant properties because of their recognition by specific pattern recognition receptors (PRRs) and elicitation of innate immune responses, which are critical for maturation of dendritic cells (DCs) to enhance the induction of subsequent adaptive immune responses.[9,10] pDNA and viral vectors have been evaluated as vaccine platforms in human clinical trials and demonstrated to be safe and immunogenic. However, pDNA delivery into the nucleus of target cells is rather inefficient, and viral vectors can induce interfering vector-specific immune responses against viral structural proteins especially upon boosting. A growing interest in and research activities into mRNA-based vaccines have started to emerge in the past decade.[11]

mRNA-based vaccines have several advantages over pDNA and viral vector-based vaccines. mRNA vaccines do not generate infectious particles or integrate in the genome of the host cells. They can be delivered for antigen expression *in situ* without the need to cross the nuclear membrane barrier for protein expression, and can express complex antigens without packaging constraints.[12] mRNA vaccines can be produced rapidly, possibly within days of obtaining gene sequence information, using completely synthetic manufacturing

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Correspondence: Giulietta Maruggi, GSK, 14200 Shady Grove Road, Rockville, MD 20850, USA.
E-mail: giulietta.x.maruggi@gsk.com
Correspondence: Dong Yu, GSK, 14200 Shady Grove Road, Rockville, MD 20850, USA.
E-mail: dong.x.yu@gsk.com

processes.[13] The platform is versatile and amenable to multiple targets, and thereby ideal for rapid responses to newly emerging pathogens.[14–16]

The seminal work from Wolff et al.[17] in 1990 provided the first successful example of *in vitro*-transcribed mRNA-expressing reporter proteins in the muscle after injection, which was followed by the studies of Martinon et al.,[18] Conry et al.,[19] and Hoerr et al.[20] demonstrating that vaccinations with viral or cancer antigen-encoding mRNA elicited antigen-specific immune responses. Despite the early promising results, it was only in the last decade that technological advancements in RNA biology, chemistry, and delivery systems have enabled the efficient and stable manufacture of mRNA products.

The recent Ebola and Zika outbreaks have demonstrated how quickly emerging infectious diseases can spread, and underline the crucial need for having a rapid response vaccine on demand platform technology in place.[6] mRNA vaccines have all of the attributes of a vaccine on demand, even though clinical validation remains to be confirmed.[16]

In this manuscript, we review the current state of mRNA vaccine approaches against infectious diseases, summarize the latest preclinical and clinical proof-of-concept data, and provide perspectives on the future of this promising new technology.

mRNA Vaccines: Types, Biology, and Latest Advancement

The concept for the development of an mRNA vaccine is rather straightforward. Once the antigen of choice from the pathogen target is identified, the gene is sequenced, synthesized, and cloned into the DNA template plasmid. mRNA is transcribed *in vitro*, and the vaccine is delivered into the subject. The mRNA vaccine utilizes the host cell machinery for *in vivo* translation of mRNA into the corresponding antigen, thereby mimicking a viral infection to elicit potent humoral and cellular immune responses. The final cellular location of the antigen is determined by the signal peptide and transmembrane domain. This can be intrinsic to the natural protein sequence or engineered to direct the protein to the desired cellular compartment.[21] Therefore, the antigen can be expressed as intracellular, secreted, or membrane-bound protein. Importantly, given its fully synthetic nature, virtually any sequence could be designed *in silico*, synthesized, delivered as an mRNA vaccine, and tested rapidly *in vivo* in animal models. For example, tagging antigen sequences with targeting sequences to major histocompatibility complex (MHC) class II compartments, with MHC class I trafficking signals, or with immunodominant helper CD4 T cell epitopes could amplify antigen presentation efficiency and enhance cellular immune responses. Arrays of antigen sequences can also be designed and rapidly tested to generate vaccines with efficient leader sequences, optimal codon usage, enhanced neutralization capacity, or reduced undesired cross-reactivity, as recently shown by Zika mRNA vaccines developed by Richner et al.[15]

Due to the ability of the host's innate system to sense and respond to RNA sequences of viral origin (reviewed in Chen et al.[9] and Vabret et al.[22]), mRNA vaccines induce robust innate responses, including production of chemokines and cytokines such as interleukin-12 (IL-12) and tumor necrosis factor (TNF) at the injection site.[23–25] These are factors crucial to successful induction of effective adaptive responses against the encoded antigen.[26] Currently, two forms of mRNA vaccines have been developed: conventional mRNA encoding the antigen of interest flanked by 5′ and 3′ UTRs, and self-amplifying mRNA derived from the genome of positive-stranded RNA viruses. Self-amplifying mRNA encodes not only the antigen but also the viral replication machinery required for intracellular RNA amplification leading to high levels of antigen expression (Figure 1). Unique attributes of each mRNA technology, as well as the roadblocks that need to be overcome for advancement, are summarized in Table 1.

Cell-free Production and Purification of mRNA Vaccines

Both conventional mRNA and self-amplifying mRNA vaccine approaches share essential elements of an eukaryotic mRNA: a cap structure [m7Gp3N (N, any nucleotide)], a 5′ UTR, an open reading frame (ORF), a 3′ UTR, and a tail of 40–120 adenosine residues [poly(A) tail].[27] Both types of mRNAs are produced in a cell-free system, using an enzymatic transcription reaction from a linearized pDNA template.[12,28] Manufacturing of mRNA vaccines against different disease targets requires the replacement of the sequence encoding the target antigen, without affecting the overall physicochemical characteristics of the RNA molecule (Figure 2). The first step in RNA production is the construction of a pDNA that contains a promoter sequence with high binding affinity to a DNA-dependent RNA polymerase (e.g., T7, SP6, or T3) and the sequence encoding the specific mRNA vaccine. The pDNA is linearized with a restriction enzyme and used as a template for an *in vitro* transcription (IVT) reaction using a DNA-dependent RNA polymerase. The enzyme moves along the template, elongating the RNA transcript until it runs off the end of the template. The template DNA is then degraded by incubation with DNase, and a cap [m7Gp3N] is enzymatically added to the 5′ end of the mRNA.[29] Alternatively, a synthetic cap analog can be added during the IVT reaction in a single-step procedure.[30,31] The presence of a 5′ cap structure is crucial for efficient translation *in vivo* and protects mRNA from intracellular nuclease digestion.[32,33] Once synthesis is complete, mRNA is purified to remove reaction components, including enzymes, residual template DNA, truncated transcripts, or aberrant double-stranded transcripts. A highly purified RNA drug substance is critical for the potency of an mRNA vaccine, because contaminants can activate non-specific or undesirable innate sensors.[34–37] Karikó et al.,[36,38] and more recently Foster et al.,[39] have demonstrated that purification of mRNA from double-stranded RNA (dsRNA) contaminants can enhance *in vivo* translation and reduce innate immune activation, and is critical for the success of RNA-based gene therapy and CAR T cell therapy applications. After purification, mRNA is exchanged into a final storage buffer or formulated with the delivery system for use.

These approaches are appropriate to produce almost any mRNA sequence, with low batch-to-batch variability, potentially saving time and reducing costs compared with other vaccine platforms.

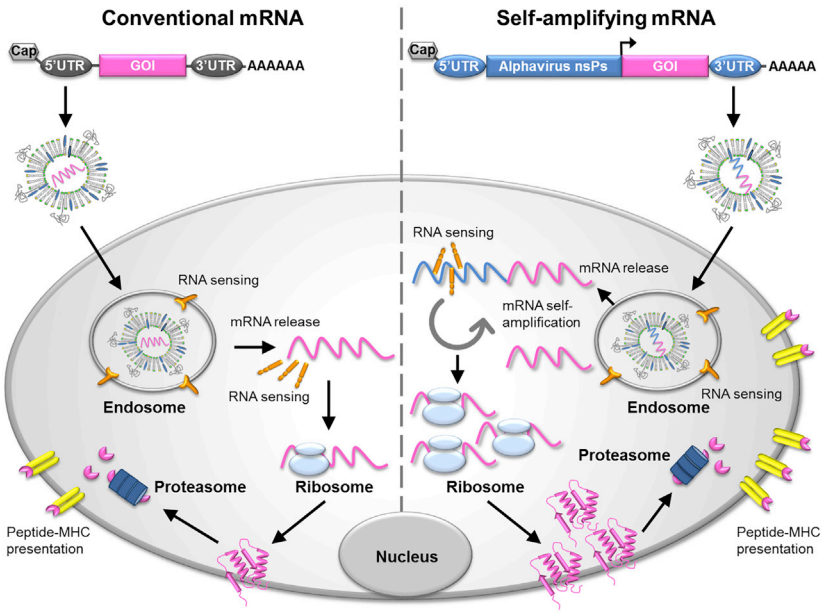

Figure 1. Schematic Representation of mRNA Vaccines and Mechanism of Antigen Expression

Conventional mRNA carries the coding sequence of the antigen of interest (GOI) flanked by 5′ and 3′ UTRs, a terminal 5′ cap structure, and a 3′ poly(A) tail. Once delivered into the cell and released from the endosome into the cytoplasm, the mRNA is translated immediately. The self-amplifying mRNA is often derived from the genome of positive-sense single-stranded RNA viruses, such as alphaviruses. It encodes both the antigen of interest and viral nonstructural proteins (nsPs) required for intracellular RNA amplification and high levels of antigen expression. The self-amplifying mRNA can direct its self-amplification to generate RNA intermediates and many copies of antigen-encoding subgenomic mRNA, producing high levels of the encoded antigen. Both conventional mRNA and self-amplifying mRNA vaccines require a delivery system for cell uptake, usually by endocytosis, which is followed by unloading of mRNA cargo from the endosome into the cytosol, where translation and protein processing for MHC presentation occur. Once delivered in the cell, the mRNA is almost immediately sensed by pattern recognition receptors (PRRs) in the endosome and in the cytoplasm. PRRs such as Toll-like receptors TLR3, TLR7, and TLR8 are localized in the endosome, and cytosolic sensors such as RIG-I, MDA5, PKR, and OAS also recognize double-stranded and single-stranded RNAs in the cytoplasm. GOI, gene of interest; MHC, major histocompatibility complex; nsPs, nonstructural proteins.

The mRNA drug substance and formulated drug product undergo testing and in-process analytics to assess identity, appearance, content, integrity, residual DNA, endotoxin contamination, and sterility.[40] A potency test is used to verify the ability of the mRNA to be translated into a desired protein product after delivery into target cells. Depending on the specific mRNA construct, the above-described procedure may be slightly modified to accommodate modified nucleosides, capping strategies, or purification protocols.

All components needed for mRNA production are available at the Good Manufacturing Practice (GMP) grade, and industrial-scale production facilities designed to produce up to 30 million doses of RNA-based products per year are being established.[41,42]

Conventional mRNA Vaccines

A conventional mRNA vaccine carries only the coding sequence of the antigen of interest flanked by regulatory regions (Figure 1). The major advantages of the conventional mRNA vaccine approach are the simplicity and relatively small size of the RNA molecule. In the simplest form, the stability and activity of the conventional mRNA *in vivo* is limited, because of propensity of cells to limit duration of expression. However, optimization of RNA structural elements and formulation can increase antigen expression and durability.[43] The cap (often a cap 1 structure[15,44–46]), UTRs, and the poly(A) tail are crucial for stability of the mRNA molecule, accessibility to ribosomes, and interaction with the translation machinery,[47–50] therefore representing targets for optimization (reviewed in Ross,[43] Lundstrom,[51] and Sahin et al.[52]). Additionally, codon usage can have a beneficial impact on protein translation, and replacing rare codons with frequently used synonymous codons is a common practice to enhance protein expression from DNA, RNA, and viral vector vaccines. However, this approach has been recently reviewed, suggesting that codon optimization may not necessarily increase protein production for mRNA therapeutics.[53,54]

Unlike proteins, mRNA vaccines need to be expressed *in situ* to induce an antigen-specific immune response; therefore, understanding the magnitude and durability of antigen expression after mRNA injection is important for vaccine optimization. Results by Vogel et al.[55] showed that naked, unmodified mRNA induced protein expression *in vivo* at 12–24 h after intramuscular (i.m.) injection and lasted for at least 6 days. Kinetics and magnitude of translation can be influenced by mRNA formulation, route of administration, nucleoside modifications, and sequence optimization.[45,56,57]

Nucleoside base modification has been used to produce so-called modified mRNA. In this review, the term modified mRNA or unmodified mRNA refers to conventional mRNA with or without chemically modified nucleotides, respectively. Nucleoside base modification, often coupled with chromatographic purification to remove dsRNA contaminants, is a fast-emerging approach to improve mRNA potency by reducing activation of PRRs. Several RNA structures, such as dsRNA produced by annealing of complementary RNAs or short RNA stem loops, as byproducts of IVT, or by viral RNA replication intermediates, are immunostimulatory.[9,58–60] Although activation of innate immunity is required for vaccine application, its improper or

Table 1. Advantages and Disadvantages of Conventional or Self-Amplify mRNA Vaccines

Vaccines	Advantages	Disadvantages
Conventional mRNA Self-amplifying mRNA	synthetic production; egg and cell free	concerns with instability
	rapid and scalable production compared with other vaccine platforms (e.g., subunit proteins, viral vectors)	limited immunogenicity data in humans
	noninfectious, non-integrating, and naturally degraded	potential toxic effect of free extracellular mRNA[164,165]
	expression *in situ* to produce antigens with structure unaltered by *in vitro* manufacturing process	inflammation due to enhanced type I IFN activation
	expression *in situ* to stimulate innate immune response, enhancing broad T and B cell immune responses	efficient delivery required to deliver and launch self-amplifying mRNA
	–	efficient delivery required to deliver and/or provide adjuvanting effect for conventional mRNA
	–	unproven toxicity profiles of delivery system components
Conventional mRNA	shorter RNA length compared with self-amplifying mRNA	potential toxicity from modified nucleotides
	applicable to nucleoside base modification	shorter duration of expression
	direct antigen expression from mRNA	higher effective RNA doses
	no risk of anti-vector immunity	–
Self-amplifying mRNA	enhanced and prolonged antigen expression	potential elevated inflammation due to self-amplification
	lower effective RNA doses, potentially resulting in better safety	longer RNA length, may lead to more challenging production of high-quality RNA compared with conventional mRNA
	intrinsic adjuvant effect	interaction between nsPs and host factors yet to be addressed
	potential apoptosis of vaccine-carrying cells due to vaccine self-amplification, leading to enhanced cross-presentation	–
	option for single-vector delivery of multiple or complex antigens	–

IFN, interferon; nsPs, nonstructural proteins.

excessive activation can also interfere with antigen expression and adaptive immunity.[61,62] For example, Karikó et al.[57] and others[44,63–69] have shown that pseudouridine-containing mRNAs reduce activation of Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), protein kinase R (PKR), and 2′-5′-oligoadenylate synthetase (OAS), while increasing translational activity, resistance to RNase L-mediated degradation, and *in vivo* stability. The highest levels of protein production and immunogenicity are observed when nucleoside-modified mRNA is also purified by high-performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC), where aberrant double-stranded transcripts are removed.[38,46] This enhanced protective immune response has been demonstrated in mice, ferrets, and non-human primates (NHPs) against influenza[70,71] and Zika viruses,[14,15] among other targets.

In addition to modified nucleosides, sequence optimization is also used to ensure robust protein expression and immunogenicity. Thess et al.[45] have shown that an mRNA sequence enriched in G:C content and carrying optimized UTRs is superior to a nucleoside-modified counterpart *in vitro* and *in vivo*. Sequence-optimized mRNA delivered with lipid nanoparticles (LNPs) also elicited robust functional antibody titers against rabies and influenza antigens in NHPs.[72] Conversely, Pardi et al.[46] reported that 1-methylpseudouridine-modified mRNA delivered by LNPs produced 20 times more protein than its sequence-optimized but nucleoside-unmodified counterpart after intradermal (i.d.) injection in mice. Codon-optimized, FPLC-purified, unmodified mRNA encoding an influenza antigen induced weaker $CD4^+$ T cell, T follicular helper (Tfh), and germinal center (GC) responses than the 1-methylpseudouridine-modified counterpart. The discrepancies among these findings are not yet clear and might be due to the difference in routes of administration, sequence optimization algorithms, modified nucleosides, and experimental conditions. Nonetheless, these data show that both approaches are superior to unmodified mRNA. Both sequence-optimized RNA and nucleoside-modified RNA delivered by LNPs induce a robust activation of innate immunity, rapid infiltration of neutrophils, monocytes, and DCs to the injection site and the draining lymph nodes (dLNs) in mice and NHPs, as well as upregulation of key co-stimulatory receptors (CD80 and CD86) and type I interferon (IFN)-inducible genes, including Mx1 and CXCL10.[23,72]

In addition to mRNA modification, increasing adjuvant properties in formulation might also be advantageous for vaccination, as shown by the RNActive technology. This vaccine contains a naked, unmodified, sequence-optimized mRNA, whose potency depends on its carrier that consists of a non-coding RNA complexed with protamine, a cationic protein activating TLR7.[25,73–79] Loomis et al.[80] also explored the effects of adjuvants and activation of local innate responses on mRNA vaccination by coupling naked 1-methylpseudouridine-modified mRNA to small-molecule TLR2 and TLR7 agonists. By using

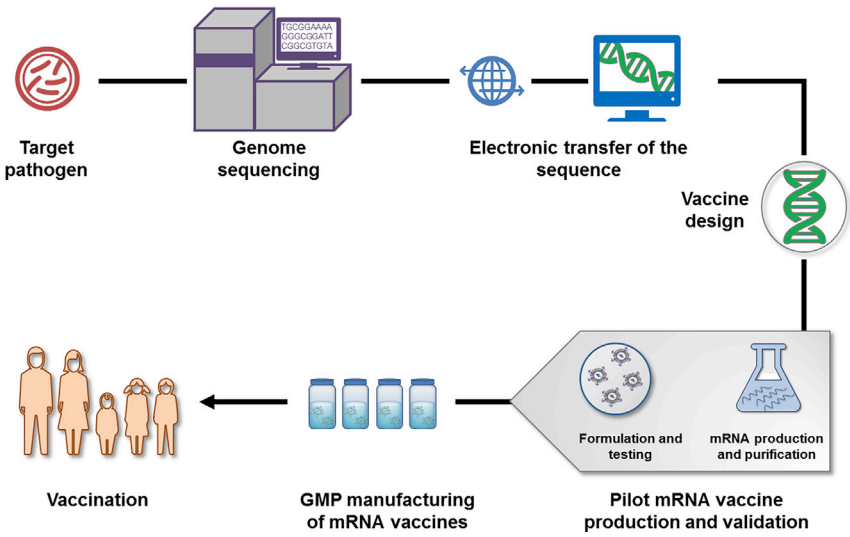

Figure 2. Schematic Illustration of mRNA Vaccine Production

Once a pathogen is identified or an outbreak declared, the genome of the pathogen and antigen(s) are determined, if not already available, by the combined sequencing, bioinformatics, and computational approach. Candidate vaccine antigen sequences are deposited electronically and available globally for *in silico* design of mRNA vaccines, followed by construction of plasmid DNA template by molecular cloning or synthesis. Pilot vaccine batches are generated in a cell-free system by *in vitro* transcription and capping of the mRNA, purification, and formulation with the delivery system. In-process analytic and potency tests are performed to assess the quality of pilot mRNA vaccine batches. If needed, pilot mRNA vaccine batches can be further tested in the immunogenicity and/or disease animal model. The final mRNA vaccine is scaled up and manufactured through a generic process with minimal modifications, rapidly tested, and dispatched for use. GMP, Good Manufacturing Practice.

ovalbumin as the model vaccine antigen, they observed increased innate, cellular, and humoral immune responses in mice compared with naked mRNA.

In summary, the most advanced conventional mRNA vaccines under evaluation in preclinical and clinical trials consist of unmodified but sequence-optimized mRNA, nucleoside-modified mRNA, or mRNA adjuvanted with protamine-complexed non-coding RNA.

Self-Amplifying mRNA Vaccines

Self-amplifying mRNA vaccines are commonly based on the engineered RNA genome of positive-sense single-stranded RNA viruses, such as alphaviruses, flaviviruses, and picornaviruses.[81,82] In all cases, the mRNA mimics the replicative features of positive-sense single-stranded RNA viruses with the goal of increasing the duration and magnitude of the expression, as well as subsequent immunogenicity of the encoded antigen. The best-studied self-amplified mRNA molecules are derived from alphavirus genomes, such as those of the Sindbis virus (SINV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis viruses (VEEVs) (reviewed in Ljungberg and Liljeström[83] and Atkins et al.[84]). Negative-sense single-stranded RNA viruses, such as rhabdoviruses and measles viruses, can also be utilized for the development of RNA-based vaccines (reviewed in Mühlebach[85] and Humphreys and Sebastian[86]). However, the negative-sense RNA genomes need to be rescued by reverse genetics where cell culture-based systems are required, and therefore are not the focus of this review.

Alphavirus RNA genomes, typically around 10–11 kb, encode four non-structural proteins, nsP1–4, which are translated directly from the genomic RNA and together form the RNA-dependent RNA polymerase (RDRP) complex.[87,88] RDRP interacts with host factors to form replication factories on the cytoplasmic side of modified membrane structures to synthesize the negative-sense RNA intermediates.[89–91] This results in the generation of new viral genomes (up to 2×10^5 copies/cell)[92] and, depending on temperature, of an even larger amount of capped subgenomic mRNA encoding structural proteins.[93,94]

Self-amplifying mRNA replicons are created by replacing viral structural genes with the antigen gene of interest, which, upon delivery in the cytoplasm of target cells, are capable of RNA amplification to express high levels of the antigen of interest. Because these replicons lack endogenous viral structural genes, they are unlikely to produce infectious virions or virus-like vesicles at the injection site in subjects after vaccination, hence greatly reducing safety concerns associated with a live attenuated virus vaccine. The full-length self-amplifying mRNA can be readily produced by IVT from a pDNA template using the process previously described and delivered as either viral replicon particles (VRPs) when structural genes are provided in *trans*, or as synthetically formulated RNA. Alternatively, self-amplifying RNA can also be produced directly in target cells, for example, by delivering a pDNA expressing the alphavirus-derived RDRP complex and the antigen of interest into such target cells, which has been reviewed elsewhere.[82] The efficacy of alphavirus VRPs as vaccine in preclinical models and humans has been previously described and has been largely attributed to the production of high levels of correctly processed heterologous proteins and to their ability to deliver antigen to a variety of cell types, including antigen-presenting cells.[95–103] Activation of DCs upon VRPs infection results in a wave of cytokine secretion and subsequently a robust adjuvanting effect, which markedly amplifies the magnitude of vaccine-elicited adaptive immune responses.[104]

In the 1990s, Zhou et al.[105] were the first to report that the self-amplifying SFV-derived IVT RNA could be used to elicit an immune response against a heterologous antigen. In 2001, the same group reported that as little as 10 μg of naked SFV-derived IVT RNA expressing respiratory syncytial virus (RSV) fusion protein (F), influenza virus hemagglutinin (HA), or Louping ill virus pre-membrane

and envelope (prME) were sufficient to induce significant antibody responses and partial protection against the lethal challenge of RSV, influenza, or Louping ill virus in vaccinated mice, respectively.[106] An important step for self-amplifying mRNA vaccine development was the use of complexing agents to protect and formulate IVT RNA. Geall et al.[107] reported that encapsulation of a 9-kb self-amplifying mRNA with LNP considerably improved the durability and magnitude of *in vivo* antigen production compared with naked RNA, and as little as 0.1 μg of LNP-formulated self-amplifying mRNA elicited potent T and B cell responses in mice. Similar results were later reported using a cationic nanoemulsion (CNE) to formulate RNA.[108]

Self-amplifying mRNA vaccines enable high levels of antigen production from low doses of vaccine for an extended duration, likely because of their self-amplification in cells. By using a secreted alkaline phosphatase (SEAP) as reporter gene, Brito et al.[108] showed that antigen expression from self-amplifying mRNA peaked at 3 days and remained measurable at more than 14 days postadministration in NHPs. In a recent study, Vogel et al.[55] compared antigen expression kinetics, immune responses, and protective efficacy after injection of self-amplifying mRNA or unmodified conventional mRNA in mice. They showed that the self-amplifying mRNA vaccine expressed much higher levels of the protein for a longer period of time and induced more potent immune responses than the conventional mRNA vaccine. Similar superior kinetics and potency have been previously described for CNE-formulated self-amplifying mRNA.[108] Leyman et al.[109] recently compared luciferase expression of self-amplifying mRNA, nucleoside-modified conventional mRNA, and unmodified conventional mRNA after i.d. electroporation in pigs. Both conventional mRNAs reached their peak expression 1 day after electroporation, followed by a 2.5- to 5-fold decrease at day 6. Self-amplifying mRNA-driven expression in the skin of pigs was similar to that of conventional mRNA at day 1, then slightly increased at day 6, and persisted until day 12. The high levels of antigen expressed from the self-amplifying mRNA vaccine may play an important role in its potency, as reported for other nucleic acid vaccines. Using VRP-delivered alphavirus self-amplifying mRNA, Kamrud et al.[110] demonstrated that increased antigen expression from VRP-delivered self-amplifying mRNA vaccines correlated with increased protection in immunized mice after challenge.

When used in an influenza challenge model, both naked self-amplifying mRNA and conventional mRNA protected against infection with the homologous virus.[55] However, 64-fold less self-amplifying mRNA (1.25 μg of self-amplifying mRNA versus 80 μg of mRNA) was required to generate a similar level of protection. Considering that self-amplifying mRNA is much larger than mRNA (9.3 versus 2.2 kb), this suggested that 270-fold fewer self-amplifying mRNA molecules are required to achieve the similar vaccine efficacy. The dose-sparing quality of self-amplifying mRNA vaccines may facilitate scale-up and manufacturing large numbers of vaccine doses. On the other hand, the production process and stability of the longer self-amplifying mRNA molecule could be more challenging than conventional mRNA products and require process improvement.

Another factor, in addition to the magnitude and duration of antigen expression, which may also contribute to the increased immune response elicited by self-amplifying mRNA, is the dsRNA amplification intermediates that trigger host sensing machinery, activate innate immunity, and confer an adjuvant effect. Indeed, co-administration of LNP-formulated self-amplifying mRNA, regardless of the encoded antigen (GFP, matrix protein 1, or nucleoprotein), could act as an adjuvant for the subunit monovalent inactivated influenza vaccine to elicit H1-specific effector $CD8^+$ T cells, increase the magnitude of H1-specific $CD4^+$ T cells, and shift their polarization toward a Th1 phenotype.[111]

However, the role of innate immunity, such as type I IFN induction, in self-amplifying mRNA vaccination is complex. Delivery of self-amplifying mRNA by LNPs has strong local pro-inflammatory effects with activation of innate immune, anti-viral, and inflammatory signaling pathways.[24] Early and robust induction of type I IFN and IFN-stimulated responses was detected at the site of injection in mice within a few hours. However, although balanced IFN activation is beneficial for generating potent immune responses, elevated type I IFN early responses impair self-amplifying mRNA expression and potency.[112,113] Consistently, antigen expression and immunogenicity were both enhanced in the absence of type I IFN signaling, as previously shown with conventional mRNA.[24,114,115] The negative impact from excessive IFN activation could also derive at the level of T cells. Although type I IFN can determine the differentiation of antigen-primed $CD8^+$ T cells into cytolytic effectors, they may also promote T cell exhaustion.[62] Whether type I IFNs inhibit or stimulate the $CD8^+$ T cell response to mRNA vaccines might depend on the timing and intensity of type I IFNs induced.[116]

The usage of modified nucleosides to silence self-amplifying mRNA recognition by innate immune sensors is not an option, because it could impair RDRP-mediated mRNA amplification in target cells, and the effect of modified nucleoside would also be lost after the first round of amplification. Beissert et al.[117] have demonstrated that antigen expression from self-amplifying mRNA can be significantly improved *in vitro* and *in vivo* by co-transferring mRNAs encoding IFN and PKR inhibitory proteins, such as viral protein B18R from vaccinia virus. These data are consistent with previous results showing an increase in translation from an alphavirus self-amplifying mRNA co-expressing a dominant-negative PKR[118] or co-delivered with a recombinant vaccinia B18R protein.[119] Other potential strategies to enhance the potency of self-amplifying mRNA vaccines include sequence modification to generate IFN-insensitive RNA, novel formulations to limit IFN induction upon vaccine delivery, and inclusion of small-molecule modulators to target various components of the IFN signaling cascade.[61] Finally, potential interactions between the encoded nsPs and host factors, as well as their immunogenicity, merit additional investigation. So far, no evidence has been generated in preclinical models that immune responses against the

nsPs interfere with the immune response induced by subsequent booster doses,[120] but this needs to be confirmed with synthetic self-amplifying mRNA vaccines.

Another attribute of the self-amplifying mRNA vaccine platform is its ability to encode multiple antigens in the same replicon. This could allow the development of a vaccine expressing both a target antigen and an immuno-modulatory biological molecule (e.g., biological adjuvant) for enhanced potency, vaccine encoding both B and T cell antigens, single combo vaccine simultaneously targeting multiple pathogens, or vaccine targeting multi-subunit complex antigen. For certain pathogens, neutralizing antibody responses are directed toward conformational epitopes resulting from protein complex formation, rather than the individual components. Using viral delivery of alphavirus replicon, Wen et al.[121] showed that a self-amplifying mRNA replicon can express five full-length subunits (gH, gL, UL128, UL130, and UL131A) of the human cytomegalovirus (HCMV) pentameric complex, form the properly folded pentameric complex, and elicit potent and broadly neutralizing antibodies in mice. Given no packaging constraints in a synthetic delivery system, the coding capacity of self-amplifying mRNA might be pushed even further. Proof-of-concept studies of multi-protein antigen delivery with self-amplifying mRNA formulated with LNPs were reported by Brito et al.[12] using the HCMV gH/gL protein complex and Magini et al.[111] using influenza virus nucleoprotein (NP) and M1 proteins. The self-amplifying mRNA vaccine encoding gH/gL induced antibodies and $CD4^+$ T cells at levels similar to those induced by an MF59-adjuvanted gH/gL protein complex. Immunization of mice with very low doses of self-amplifying mRNA vaccine (0.1–0.2 μg) encoding influenza M1 and/or NP elicited potent antigen-specific T cell responses and protection from viral challenge. Such an approach might be particularly useful for the generation of a cross-protective influenza single-vector vaccine able to induce both T cell- and B cell-mediated immunity. Indeed, a swine fever virus-derived self-amplifying mRNA vaccine encoding influenza HA and NP was shown to induce antigen-specific T and B cell responses in mice.[122,123]

In summary, the self-amplifying mRNA vaccine platform is a versatile technology that can be used to develop single- or multi-antigen vaccines, with the benefit of low effective dosage thanks to its high antigen expression and strong intrinsic adjuvant effect. Insights into the mechanisms controlling the interaction of self-amplifying mRNA replicons with host innate immunity and subsequent modulation of antigen-specific immune responses will enable the rational design of improved self-amplifying mRNA vaccines.

Synthetic Delivery Vehicles for mRNA Vaccines

mRNA vaccines need a delivery system to reach their full potential, because naked RNA not only is prone to nuclease degradation, it is also too large and negatively charged to passively cross the cell membrane.[124] It has been speculated that the cellular uptake rate of naked mRNA is less than 1 in 10,000 molecules.[52] As such, the mRNA delivery field has focused on the discovery and development of methods and materials that can transport RNA into cells, benefiting from extensive prior research into non-viral delivery of small interfering RNA (siRNA), recently reviewed elsewhere.[125,126] There are several major delivery vehicles currently under investigation, some encapsulating mRNA molecules into particles and others using positively charged polymers to bind to RNA through charge interactions. Several groups are also attempting to create particles that behave like viruses able to shield mRNA from endo-, 5′ exo-, and 3′ exo-nucleases, deliver mRNA efficiently into cells, and achieve a sufficient level of the encoded protein.[127–129] In all cases, the vehicle must pass through the target cell membrane, and after cell uptake, usually by endocytosis, it must escape the endosome and unload its mRNA cargo into the cytosol, where translation occurs. For some mRNAs, the delivery system may also influence the quantity and quality of local gene expression patterns, innate immune stimulation, and can provide a synergistic adjuvant effect.[24,130]

One of the most promising and commonly used systems for non-viral delivery of RNA is LNPs. LNPs are formulated using precise molar ratios of phospholipids that enhance fusogenicity and endosomal escape, cationic-ionizable amino lipids that condense with nucleic acid at low pH, poly(ethylene) glycol (PEG) lipids that provide steric stabilization of the formulation before use, and cholesterols that enable vesicle stability both *in vivo* and in vials.[131] Several amino lipids have been developed for siRNA delivery over the past two decades, and the first LNP-delivered therapeutic siRNA was approved by the US Food and Drug Administration (FDA) in 2018.[132] Leveraging the knowledge from siRNA delivery, many laboratories have used LNP delivery of self-amplifying mRNA and conventional mRNAs against various pathogens, such as rabies, Zika, and influenza viruses, and demonstrated rapid and robust immune responses in mice, ferrets, and NHPs.[13–15,70–72]

LNPs enter cells by exploiting membrane-derived endocytic pathways. Endosomal processing and release of the encapsulated genetic material into the cytosol is the rate-limiting step of delivery and a major area of research for formulation improvement. Gilleron et al.[133] have estimated that only less than 2% of LNP-delivered siRNAs taken up by cells are released into the cytosol, and endosomal escape occurs only during a brief phase of endolysosomal maturation. Using CRISPR-based genetic disruption of different stages of the lysosomal pathway, Patel et al.[135] demonstrated that, in addition to fusogenicity, late endosome-lysosome formation in the endocytic process is essential during LNP trafficking for mRNA release in the cytosol.[134] LNP-mediated mRNA delivery can be modulated through incorporation of bioactive lipids modulating cellular signaling or enriching in endo-lysosomal compartments. The link between endosomal escape and LNP potency has been further explored by Sabnis et al.,[136] who demonstrated that the improved delivery efficiency of their novel amino lipid, designed by a rational chemistry approach, was due to improved fusogenicity and endosomal escape. The use of high-throughput screening of nanocarriers *in vitro* toward the development of mRNA vaccine formulation with enhanced endosomal escape is hindered by the discrepancies observed between *in vivo* and *in vitro* data. Recent encouraging results using skeletal muscle

cells grown on an *in vitro* hydrogel may provide a better prediction of mRNA uptake, endosomal entry, release, and protein expression *in vivo* upon i.m. injection.[137]

Although not as clinically advanced as lipid-based systems for mRNA delivery, polymers have shown considerable potential in mRNA vaccine development. One example is the dendrimer-based nanoparticle system developed by Chahal et al.,[138,139] which comprises molecules of high amine density with organizing branching structures condensing around mRNA to form monodisperse spheres. After a single immunization, the dendrimer-encapsulated self-amplifying mRNA vaccine elicited $CD8^+$ T cell and antibody responses that fully protected against lethal challenge by Ebola virus, H1N1 influenza, and Zika viruses in mice, with 40 μg as the optimal RNA dosage. This dendrimer technology was used to generate a multiplexed vaccine consisting of six self-amplifying mRNA replicons simultaneously formulated in the same nanoparticle, which protected mice against lethal *Toxoplasma gondii* challenge. Although promising, this technology is still at the early stage and needs further optimization, given the high RNA amount required.

The capability to generate viable, potent single-dose vaccines against multiple antigens or even pathogens may have broad utility and reduce the number and frequency of vaccinations. Future research should also evaluate the lack or presence of potential interference in activation of target-specific immune responses among co-delivered mRNA vaccines encoding antigens from multiple pathogens.

As an alternative to a single-vial formulation, such as LNP, a two-vial approach where the delivery formulation can be manufactured and stockpiled separate from the target mRNA has been developed by Brito et al.[108] In this context, an emulsion-based delivery vehicle was used to bind self-amplifying mRNA to the nanoparticle surface, prior to administration. The CNE-delivered self-amplifying mRNA was shown to elicit potent immune responses after two or three immunizations in mice, rats, rabbits, and NHPs with a variety of viral, bacterial, and recently, parasite antigens.[21,108,140–142] This two-vial approach allows for the delivery vehicle to be stockpiled and mixed with any target RNA, designed, and produced whenever an outbreak occurs.

There are additional aspects of delivery to consider for the efficacy of mRNA vaccines. The route of administration is critical for RNA uptake and expression. Vaccines are typically administered via i.d., i.m., or subcutaneous (s.c.) injections, in order to target tissues, such as the skin and skeletal muscle, which are densely populated by DCs. For LNP-formulated nucleoside-modified mRNA vaccine, the greatest magnitude and duration of antigen expression were observed after i.m. or i.d. injection in mice.[56] However, although both i.d. and i.m. administration of LNP-formulated nucleoside-modified mRNA influenza H10 vaccine induced protective titers in NHPs, the responses occurred more rapidly after i.d. vaccination.[143] DC targeting is another active area of research for mRNA delivery. In an effort to improve targeting of DCs via s.c. injection, Englezou et al.[144] explored and optimized cationic lipids and lipoplex formation to increase the uptake, release in the cytoplasm, and translation of a self-amplifying mRNA molecule in DCs. The lipoplex, optimized for increased *in vitro* translation in DCs, elicited pro-inflammatory cytokines, humoral responses, and T cellular responses against the self-amplifying mRNA-encoded influenza NP antigen after s.c. vaccination in mice and in an adoptive transfer model. Although extensive studies have been performed for *ex vivo* mRNA loading of DCs to generate cell-mediated immunity against cancer or for therapeutic vaccination (reviewed in Benteyn et al.[145] and Gornati et al.[146]), *in vivo* targeting to DCs of synthetic mRNA vaccines against infectious diseases is still in the early phase. Formulation design, optimization, and combination with adjuvants or surface molecules to target DCs after immunization might increase the potency of synthetic mRNA vaccines, given the central role of DCs in processing antigens and initiating antigen-specific adaptive immune responses.

Thermal stability of vaccines can pose a major logistical problem for stockpiling and distribution, particularly in countries that lack infrastructure to maintain the cold chain. Jones et al.[147] reported that a Kunjin virus-derived self-amplifying mRNA freeze-dried in trehalose was stable for 10 months when stored at 4°C–6°C, indicating the feasibility of using lyophilization to stabilize RNA during storage. In addition, a lyophilized protamine-complexed mRNA vaccine was shown to retain its full biological activity even after exposure to thermal stress conditions for several weeks and up to 36 months at 5°C–25°C.[73,75] Similarly, oscillating temperatures between +4°C and +56°C for 20 cycles, to simulate interruptions of the cold chain during transport, did not impact the protective efficacy of a lyophilized mRNA vaccine in a lethal challenge mouse model.[148]

The development of a formulation enabling potent single-dose mRNA vaccines that are thermostable, can be stockpiled, and can simultaneously target multiple antigens and pathogens will have broad utility for a range of diseases, reduce the number and frequency of vaccinations, and alleviate healthcare worker burden.

mRNA Vaccines in the Prevention of Infectious Diseases: From Preclinical Proof of Concept to Initial Clinical Data

Early clinical evaluation of mRNA vaccines mostly addressed cancer immunotherapy, often with no validated benchmarks to compare.[149] These studies have been pivotal to prove that large-scale GMP production of mRNA is feasible, and mRNA vaccines have a favorable safety profile. However, the use of mRNA as a therapeutic drug or in cancer vaccination is not the focus of this review and has been extensively reviewed elsewhere.[52,150–152]

In contrast with cancer, where the lack of conventional vaccines makes it difficult to weigh the effectiveness of RNA as a platform, infectious diseases offer a portfolio of traditional vaccines to benchmark. To this end, considerable preclinical research (Tables 2 and 3) and early clinical trials (Table 4) on infectious diseases have been conducted.

Table 2. Summary of Representative Preclinical Studies of Conventional mRNA Vaccines for Prevention of Infectious Diseases

Antigen (Pathogen)	mRNA and Delivery	Route	Elicited Responses	Models Tested	References
HA (influenza virus)	sequence-optimized protamine-RNA	i.d.	humoral, cellular, protection	mice, ferrets, pigs	73
GP (rabies virus)	sequence-optimized protamine	i.d.	humoral, cellular, protection	mice, pigs	76,148
HA (influenza virus)	sequence-optimized LNP	i.m.	humoral, cellular, innate	mice, NHPs	72
HA (influenza virus)	nucleoside-modified LNP	i.d., i.m.	humoral, cellular, innate, protection	mice, ferrets, NHPs	23,46,70,71,143
prM-E (Zika virus)	nucleoside-modified LNP	i.d., i.m.	humoral, protection	mice, NHPs	14,161,166
PC, gB, pp65 (HCMV)	nucleoside-modified LNP	i.m.	humoral, cellular	mice, NHPs	163
GP (Ebola virus)	nucleoside-modified LNP	i.m.	humoral, protection	guinea pigs	167
Env (HIV)	nucleoside-modified LNP	i.d.	humoral, cellular	mice, NHPs	46
NP (influenza virus)	unmodified liposome	s.c.	cellular	mice	18
Gag (HIV)	unmodified cationic nanomicelles	s.c.	humoral	mice	168

Env, envelope; gB, glycoprotein B; GP, glycoprotein; HA, hemagglutinin; HCMV, human cytomegalovirus; i.d., intradermal; i.m., intramuscular; i.v., intravenous; LNP, lipid nanoparticle; NHP, nonhuman primate; PC, pentameric complex; prM-E, pre-membrane and envelope; s.c., subcutaneous.

mRNA Vaccines Targeting Influenza Virus Infection

mRNA vaccines against influenza virus are among the most extensively studied because of the availability of tools to measure T and B cell responses, the ease of testing efficacy in small-animal models, and the benefits that an mRNA-based influenza vaccine could bring. Production of conventional, FDA-approved vaccines against new influenza pandemic viruses could take at least 6 months, leaving the population unprotected during this period.[153] By comparison, Hekele et al.[13] have demonstrated that once the genetic sequence of the target influenza HA antigen is known, a self-amplifying mRNA vaccine can be generated within a very short period of time. In 2013, during an outbreak of a deadly strain of H7N9 influenza in China, the HA gene was cloned in a self-amplifying mRNA vaccine pDNA template, and the self-amplifying mRNA vaccine was produced within 8 days after publication of the HA gene sequence.

The first study demonstrating immunogenicity of an mRNA-encoding influenza NP in mice was published in 1993.[18] However, only in 2012 was the first complete protection from influenza virus after mRNA vaccination demonstrated.[73] Petsch et al.[73] showed that i.d. injection with unmodified conventional mRNA encoding various influenza virus antigens combined with a protamine-complexed RNA adjuvant was immunogenic in mice, ferrets, and domestic pigs, matching the immune responses conferred from a licensed inactivated virus vaccine. This pivotal work was followed by several publications (reviewed in Scorza and Pardi[154]) showing that mRNA-based influenza vaccines, either as conventional mRNA or self-amplifying mRNA formulated in LNP or CNE, induce broadly protective T and B cell immune responses. Chitosan, polyethylenimine (PEI), and dendrimer-based formulations of self-amplifying mRNA HA have also been shown to be effective.[55,123,138]

Lutz et al.[72] demonstrated for the first time that a single i.m. injection with 10 μg of LNP-formulated HA-encoding mRNA induced protective hemagglutination inhibition (HAI) titers in NHPs comparable with or even higher than vaccination with a full human dose of licensed vaccines based on inactivated virus. Responses could be boosted by a second dose and remained stable for over a year. Only limited reactogenicity at the injection site and minor changes in systemic cytokine and chemokine concentrations were reported. Lipid-complexed mRNA of influenza HA gene segments was also tested by intravenous immunization in mice and showed T cell activation following a single dose.[155]

Lindgren et al.[143] have characterized the quality of B cell immune responses to an LNP-formulated N1-methyl-psudouridine-modified conventional mRNA encoding HA of H10N8 pandemic influenza strain after both i.d. and i.m. vaccination of NHPs. They showed that circulating H10-specific memory B cells expanded after each immunization, along with a transient appearance of plasmablasts. They showed for the first time the formation of a GC in dLNs after mRNA vaccination, along with an increase in circulating H10-specific Tfh cells, previously shown to correlate with high-avidity antibody responses after seasonal influenza vaccination in humans and to be predictive of seroconversion.[156–158]

More recently, LNP-formulated mRNA has been used for the development of a "universal" influenza vaccine capable of inducing potent immune responses against viral epitopes conserved among virus strains. One current target for this approach is the immune subdominant HA stalk that is less tolerant to escape mutations.[159] Using a nucleoside-modified, FPLC-purified mRNA-LNP vaccine expressing influenza full-length HA, Pardi et al.[70] demonstrated that a single immunization raised durable antibodies against the stalk of HA in mice, ferrets, and rabbits, and was protective against both homologous and heterologous influenza challenges in mice.

The first human trial of an mRNA-based influenza vaccine, using an LNP-formulated, nucleoside-modified conventional mRNA encoding an H10N8 HA antigen, has been recently reported (ClinicalTrials.gov: NCT03076385).[71] Interim findings from 23

Table 3. Summary of Representative Preclinical Studies of Self-Amplifying mRNA Vaccines for Prevention of Infectious Diseases

Antigen (Pathogen)	Delivery	Route	Elicited Responses	Models Tested	References
HA (influenza virus)	CNE, LNP, MDNP	i.m.	humoral, cellular, protection	mice, ferrets	13,138,142
gp140 (HIV)	CNE	i.m.	humoral, cellular	mice, rabbits, NHPs	108,140
gB, pp65-IE1 (HCMV)	CNE	i.m.	humoral, cellular	NHPs	108
SLOdm, BP-2a (streptococci)	CNE	i.m.	humoral, protection	mice	21
PMIF (malaria)	CNE	i.m.	humoral, cellular, protection	mice	141
F (RSV)	CNE, LNP	i.m.	humoral, cellular, protection	mice, cotton rats	107,108
GP (rabies), gH/gL (HCMV)	LNP	i.m.	humoral	mice	12
NP, M1 (influenza virus)	LNP	i.m.	humoral, cellular, protection	mice	111
prM-E (Louping ill virus), F (RSV), HA (influenza virus)	naked	i.m.	humoral, cellular, partial protection	mice	106
HA, NP (influenza virus)	PEI, chitosan	s.c.	humoral, cellular	mice, rabbits	122,123
prM-E (Zika virus)	MDNP, NLC	i.m.	humoral	mice, guinea pigs	139,162
GP (Ebola virus)	MDNP	i.m.	humoral, cellular, protection	mice	138
Six antigens[a] (*Toxoplasma gondii*)	MDNP	i.m.	protection	mice	138

BP-2a, group B Streptococcus pilus 2a backbone protein; CNE, cationic nanoemulsion; F, fusion protein; gB, glycoprotein B; GP, glycoprotein; HA, hemagglutinin; HCMV, human cytomegalovirus; i.d., intradermal; i.m., intramuscular; i.v., intravenous; LNP, lipid nanoparticle; M1, matrix protein 1; MDNP, modified dendrimer nanoparticle; NHP, nonhuman primate; NLC, nanostructured lipid carrier; NP, nucleoprotein; PC, pentameric complex; PEI, polyethylenimine; PMIF, plasmodium macrophage migration inhibitory factor; prM-E, pre-membrane and envelope; Ref. References; RSV, respiratory syncytial virus; s.c., subcutaneous; SLOdm, double-mutated group A Streptococcus Streptolysin-O.
[a]AMA1, GRA6, ROP2A, ROP18, SAG1, and SAG2A from *Toxoplasma gondii*.

individuals vaccinated with 100 μg of the RNA vaccine i.m. showed that the vaccine was immunogenic in all subjects at 43 days after vaccination, with 100% and 87% of vaccinated subjects achieving HAI titers $\geq$40 and micro-neutralization titers $\geq$20, respectively. These results are encouraging, although antibody titers were lower compared with those reported in animal models. Most subjects reported an acceptable mild-to-moderate reactogenicity profile (injection-site pain, headache, fatigue, and chills) similar to adjuvanted vaccines.

mRNA Vaccines Targeting Rabies Virus Infection

Data from a first time in humans study of protamine-formulated, sequence-optimized conventional mRNA vaccine encoding rabies virus glycoprotein (RABV-G) have recently been published.[75] This vaccine had been previously shown to induce protective immunity against a lethal intracerebral virus challenge in mice and a potent neutralizing antibody response in pigs.[76] In the human study, subjects received 80–640 μg of mRNA vaccine three times by needle-syringe or needle-free devices, either i.d. or i.m. Within a 7-day window after vaccination, nearly all subjects reported mild-to-moderate injection-site reactions, and 78% reported solicited systemic adverse events, including fever, fatigue, and pain. The vaccine was generally safe, with a reasonable tolerability profile and with only one case of adverse events (Bell's palsy) in a subject vaccinated at a 640-μg mRNA dose. Unexpectedly, the vaccine failed to induce an adequate antibody response by needle injection, independent of dose or route of administration. Needle-free delivery provided superior results to needle-syringe injection, consistent with observations in animal models. However, immune responses declined after 1 year in subjects who were followed up long term. Subsequent preclinical work has demonstrated that the longevity of immune responses generated by this rabies mRNA vaccine can be improved by optimizing the delivery system.[72] LNP formulation of the above-described rabies mRNA showed induction in NHPs of virus neutralization titers above the World Health Organization (WHO) reference titer of 0.5 IU/mL, which could be boosted by a second dose and remained stable above this threshold for at least 5 months. A phase I clinical trial has been recently initiated to evaluate this LNP-formulated sequence-optimized mRNA vaccine (ClinicalTrials.gov: NCT03713086).[160]

mRNA Vaccines Targeting Zika Virus Infection

An LNP-formulated nucleoside-modified conventional mRNA vaccine targeting Zika virus has been recently moved into clinical evaluation (ClinicalTrials.gov: NCT03014089).

The preclinical assessment of this ZIKA mRNA vaccine had been described by Richner et al.[15,161] In parallel, Pardi et al.[14] independently described their own LNP-formulated, nucleoside-modified conventional mRNA against Zika infection. Both groups demonstrated impressive levels of neutralizing titers and protection against lethal challenge in mice after two 10-μg i.m. or one 30-μg i.d. vaccination, respectively, and in NHPs after a single 50-μg i.d. vaccination. Richner et al.[15,161] also tested an mRNA vaccine encoding a modified Zika prM-E antigen that carried mutations destroying the conserved fusion-loop epitope in domain II of the E protein, potentially further improving the safety of the vaccine. They demonstrated that this vaccine remained protective against Zika challenge while diminishing production of antibodies enhancing Dengue virus infection in cells or mice, which is a major concern for Dengue and Zika virus vaccines.

Table 4. mRNA Vaccines for Infectious Diseases in Clinical Trials

Sponsor	Indication	mRNA Vaccine: Delivery	Trial No. (ClinicalTrials.gov)	Stage	Status and References
CureVac	rabies	CV7201 (sequence-optimized mRNA: protamine-RNA)	NCT02241135	phase I	completed[75] PCD: February 2018
CureVac	rabies	CV7202 (sequence-optimized mRNA: LNPs)	NCT03713086	phase I	recruiting[160] PCD: December 2019
Moderna	influenza H10N8	mRNA-1440 (nucleoside-modified mRNA: LNPs)	NCT03076385	phase I	completed[71] PCD: October 2018
Moderna	influenza H7N9	mRNA-1851 (nucleoside-modified mRNA: LNPs)	NCT03345043	phase I	active PCD: September 2018
Moderna	hMPV/HPIV3	mRNA-1653 (nucleoside-modified mRNA: LNPs)	NCT03392389	phase I	active PCD: July 2019
Moderna	Zika	mRNA-1325 (nucleoside-modified mRNA: LNPs)	NCT03014089	phase I/II	active PCD: September 2018
Moderna	HCMV	mRNA-1647 and mRNA-1443 (nucleoside-modified mRNA: LNPs)	NCT03382405	phase I	recruiting PCD: February 2020
Moderna	chikungunya	mRNA-1388 (nucleoside-modified mRNA: LNPs)	NCT03325075	phase I	active PCD: March 2019

The table summarizes clinical trials that evaluate vaccination by direct injection of mRNA vaccines registered at ClinicalTrials.gov as of January 8, 2018. Clinical evaluation of vaccination with dendritic cells loaded with antigen-expressing mRNAs is reviewed elsewhere.[169] HCMV, human cytomegalovirus; hMPV, human metapneumovirus; HPIV3, human parainfluenza virus type 3; LNP, lipid nanoparticle; Moderna, Moderna Therapeutics; PCD, estimated primary completion date; Ref., References.

The same vaccine was evaluated in a mouse model of maternal vaccination and fetal infection, where two immunizations protected against maternal, placental, and fetal infection from Zika virus and completely rescued a defect in fetal viability. Finally, Erasmus et al.[162] have also tested a Zika self-amplifying mRNA vaccine formulated with nanostructured lipid carriers (hybrid formulation between oil-in-water nanoemulsion and LNPs), and showed that a single i.m. vaccination at doses as low as 0.01 μg resulted in durable immune responses and completely protected mice against a lethal virus challenge.

mRNA Vaccines Targeting Multivalent Antigens

Immunization against more than one antigen concurrently is an interesting concept, with the potential of generating immunity against multiple pathogens, different antigens of a same pathogen, or complex polyproteins. This is particularly important when it is necessary to target diverse antigens to stimulate different arms of immunity or to target antigen expressed in multiple life cycles of a pathogen, as in the case of *Toxoplasma gondii*. The work from Chahal et al.[138] demonstrated that six self-amplifying mRNA replicons can be co-formulated, express each of the encoded antigens, and induce protective immunity. A similar approach has been recently reported with a conventional mRNA vaccine. John et al.[163] used the HCMV pentameric complex as the model antigen to study multi-valency and interference of conventional mRNA vaccines. LNP encapsulation of all five nucleoside-modified mRNAs, each one encoding one subunit of the pentameric protein complex, was shown to allow complex formation *in vitro*, efficient delivery *in vivo*, and generation of robust immune responses in mice and NHPs. The inclusion of an additional mRNA encoding HCMV glycoprotein B (gB) did not affect the levels of antibodies against either the pentameric complex or gB, suggesting a lack of interference by combining the different antigens. The combination of the six mRNAs encoding the HCMV pentameric complex and gB is currently under evaluation in a phase I clinical trial (ClinicalTrials.gov: NCT03382405). Although preclinical data are promising, efficacy, safety, and manufacturability will need to be carefully evaluated to understand whether this multivalent conventional mRNA vaccine approach is feasible and effective, or if a single multivalent self-amplifying mRNA vector vaccine should be pursued.

mRNA Vaccines Targeting Bacterial and Parasitical Infections

In addition to viral targets, a self-amplifying mRNA vaccine has been shown to elicit immune responses against bacterial pathogens, such as group A and group B streptococci, and provided partial protection through passive and active immunization in the mouse challenge model.[21] After vaccination, protection was also transferred to newborns, suggesting that the self-amplifying mRNA vaccine may also be suitable for maternal immunization approaches.

Recently, the utility of self-amplifying mRNA against parasitic diseases has been evaluated by using malaria as target disease.[141] A self-amplifying mRNA vaccine encoding plasmodium macrophage migration inhibitory factor (PMIF), which suppresses memory T cells and allows the parasites to evade the immune system, protected against reinfection. Self-amplifying mRNA vaccination elicited both cellular and humoral immune responses against PMIF, and anti-PMIF immunoglobulin G (IgG) blocked the proinflammatory action of PMIF. It delayed blood-stage latency after sporozoite infection, augmented Tfh cell and GC response, and enhanced the differentiation of antigen-experienced memory $CD4^+$ T cells and liver-resident $CD8^+$ T cells. Moreover, mice that were cured from infection but received a second sporozoite challenge were fully protected against reinfection. Protection from reinfection was recapitulated by the adoptive transfer of $CD8^+$ or $CD4^+$ T cells. This work demonstrated the utility of self-amplifying mRNA vaccination to block an immune-evasion mechanism employed by the parasite.

Other Clinical Studies of mRNA Vaccines

Additional LNP-formulated nucleoside-modified conventional mRNA vaccines currently under clinical evaluation include vaccines targeting influenza H7N9 (ClinicalTrials.gov: NCT03345043),

chikungunya (ClinicalTrials.gov: NCT03325075), and human metapneumovirus and human parainfluenza virus type 3 (ClinicalTrials.gov: NCT03392389) viruses, but details of these studies are currently unavailable.

Conclusions and Perspectives

mRNA-based vaccines are a promising novel platform with the potential to be highly versatile, potent, streamlined, scalable, inexpensive, and cold-chain free. Importantly, mRNA-based vaccines may fill the gap between emerging pandemic infectious diseases and a rapid, abundant supply of effective vaccines. The mRNA vaccine technology holds great potential over conventional vaccines. This versatile RNA vaccine platform offers benefits in the speed and cost of discovery and development, the probability of success for many targets, and rapid production of an effective vaccine against novel threats (Figure 2). However, it is still too early to fully understand its safety and effectiveness in humans. Recently published results from two clinical trials of conventional mRNA vaccines against infectious diseases showed an overall good tolerability profile and promising immunogenicity, but with more modest responses than expected based on results from animals.[71,75] Further insights into the mechanism of action are needed to understand the impact of innate immune responses generated both by the mRNA and the delivery system, and to determine how learning from animal species will translate to humans.

Several questions remain unanswered, including the relative utility of nucleoside-modified versus unmodified mRNAs, self-amplifying mRNAs versus conventional mRNAs, the most efficient and safest delivery system, and the best route of administration. In addition, the safety and tolerability profile of these emerging technologies needs to be fully elucidated, including the relative roles of the RNA and delivery systems in stimulating proinflammatory innate immune responses, and how the doses of these components contribute. A further understanding of the sequence of events leading to antigen expression, innate activation, and adaptive responses could guide the development of a portfolio of mRNA molecules and delivery systems with differential attributes. This will create a toolbox to tackle different applications, such as prophylactic versus therapeutic vaccines, infectious disease versus host disease (e.g., cancer) targets, or delivery of vaccine antigen versus therapeutic molecules (e.g., molecular antibodies).

The next 5 years will be very important for the field of mRNA vaccines, with results from several human clinical trials providing a clearer understanding of the true prospects of the technology and insights into the strengths and weaknesses of the various mRNA technologies and delivery systems under development.

AUTHOR CONTRIBUTIONS

G.M. and D.Y. planned the manuscript. G.M. wrote and coordinated the draft. C.Z. wrote a part of the draft. J.B.U., J.L., and D.Y. reviewed the manuscript.

CONFLICTS OF INTEREST

G.M., J.B.U., and D.Y. are employees of the GSK group of companies and report ownership of GSK shares and/or restricted GSK shares.

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