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Dong Yu
Benjamin Petsch *Editors*

mRNA Vaccines

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mRNA Vaccines

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Preface

What a difference the recent two years have made for mRNA technology, particularly in the field of vaccines! mRNA is the intermediate transcribed from coding DNA and translated into proteins by the host cell. In principle, the mRNA technology is rather straightforward. An mRNA molecule encoding a vaccine antigen is generated by in vitro transcription, formulated with a synthetic delivery vehicle, such as lipid nanoparticles (LNPs), and delivered to the target cells of the host. The antigen is translated from the delivered RNA by the host cell and elicits innate and antigen-specific adaptive immune responses to protect against the targeted pathogen. To date, two major forms of mRNA vaccines have currently been developed: conventional mRNA mimicking endogenous RNAs (The chapter "mRNA-Based Vaccines and Mode of Action") and self-amplifying mRNA, derived from a viral genome and capable of intracellular RNA amplification driving abundant protein expression (The chapter "Self-amplifying mRNA-Based Vaccine Technology and Its Mode of Action"). The first report of using in vitro transcribed mRNA to express a reporter gene in animals was published in the 1990s, and the mRNA vaccine field has been advancing rapidly since then. Over the last 20 years, there has been increasing interest in the application of mRNA-based technology for the development of vaccines against infectious diseases and other disease targets. In parallel, tremendous progress has been made to make the mRNA technology amenable to these applications. A large body of proof-of-concept data has been accumulated in preclinical animal models, followed by multiple clinical trials that have generated promising data over the past several years. Companies such as CureVac, Novartis/GSK, Moderna, and BioNTech paved the way in both technology innovation and clinical development of mRNA-based vaccines. However, it was not until 2019, when the COVID-19 pandemic occurred, that the mRNA vaccine technology entered a new era. In collaboration with leading vaccine researchers, BioNTech/Pfizer and Moderna successfully developed two highly effective SARS-CoV-2 vaccines, namely Comirnaty and SpikeVax, in record time. These vaccines have saved countless lives from severe COVID-19 illness and death and now have been fully approved across multiple age cohorts by FDA and EMA. With the success of these two vaccines, the use of the mRNA platform for a rapid

response to emerging infectious diseases and outbreaks and for scale up of manufacturing has been truly appreciated, and the utility of this platform to produce highly effective vaccines has been fully materialized.

This book series reviews both mRNA platforms, the conventional mRNA-based and the virally derived self-amplifying mRNA. The advancements in RNA biology, chemistry, stability, and delivery that have enabled the development of fully synthetic mRNA vaccines are discussed. Applications of the RNA technology are covered, focusing on infectious disease vaccines, but also other applications are reviewed , such as immunotherapies and molecular therapies. Potent and long-lasting immune responses observed in animal and early human studies, importantly, together with the most recent success of two SARS-CoV-2 mRNA vaccines, support the potential of mRNA-based vaccination as a major alternative to conventional vaccine approaches. Consequently, the clinical development, regulatory issues, and remaining challenges unique to the mRNA vaccination approach are reviewed.

In the chapter of "mRNA-Based Vaccines and Mode of Action", Gergen et al. provide insight into the function and optimization of key elements of a mRNA vaccine molecule (e.g., CAP-structure, 5' and 3' UTRs, 3' end of the mRNA, and codon usage). The use of modified nucleotides, which is the foundation of the two licensed SARS-CoV-2 mRNA vaccines, is being discussed. The chapter offers an in-depth review on the effect of innate recognition of mRNA molecules on their immunogenicity (humoral and cellular), potency, and reactogenicity. The chapter concludes with a summary on the recent advancements and opportunities to further improve the existing technology. Nonetheless, with the launch of SARS-CoV-2 vaccines, the field has progressed at an unprecedented speed and the amount of data generated in humans using the licensed mRNA vaccines could not be fully captured in this chapter. These new progresses will be the subject of subsequent reviews.

The chapter of "Self-amplifying mRNA-Based Vaccine Technology and Its Mode of Action" describes how non-virally delivered self-amplifying mRNA vaccines have the potential to be as highly versatile, potent, scalable, and less expensive compared to their conventional mRNA counterparts, with the addition of dose-sparing potential. By amplifying the antigen-encoding mRNA in the host cell, the self-amplifying mRNA mimics a viral infection, resulting in sustained levels of the target antigens combined with self-adjuvanting innate immune responses, ultimately leading to potent and long-lasting antigen-specific humoral and cellular immune responses. Maruggi et al. highlight the progress made in using non-virally delivered self-amplifying mRNA-based vaccines against infectious diseases in animal models. It also provides an overview of unique attributes of this vaccine approach, summarizes the growing body of work defining its mechanism of action, discusses the current challenges and latest advances, and presents perspectives about the future of this promising technology. The recent development of self-amplifying mRNA-based SARS-CoV-2 vaccine candidates not only highlights the promises but also identifies current limitations of this technology, as the vaccines not only elicited the desired immunogenicity profile but also faced challenges concerning manufacturing. More work is needed to fully understand the mechanism

of action of this platform and to mitigate the technical challenge associated with manufacturing self-amplifying mRNA vaccines with high quality and potency.

In the chapter of "Formulation and Delivery Technologies for mRNA Vaccines", Zeng et. al. review the progress and challenges in the formulation and delivery technologies for mRNA vaccines with a perspective for future development. Although lipid nanoparticles have been proven an effective and safe delivery vehicle by the success of two SARS-CoV-2 mRNA vaccines, there are other alternative delivery methods under development. The alternative delivery formats being tested or in development include encapsulation by polymers, peptides, or free mRNA in solution. These formulation and delivery strategies are designed to facilitate enhanced antigen expression, presentation, and immune stimulation by an mRNA vaccine. Vaccine efficacy could be further enhanced by an optimized route of administration or co-delivery of multiple mRNAs.

In the chapter of "Messenger RNA-Based Vaccines Against Infectious Diseases", Alameh and coworkers review RNA for immunization for infectious disease applications. The mRNA-based platform could address key gaps that some of the traditional vaccine platforms may have, including lack of potency and/or durability of vaccine protection, time-consuming and expensive manufacturing, and, in some cases, safety issues. These attributes, which are critical for mRNA to be a platform of choice for the development of new vaccines for human use, are supported by a growing body of evidence, particularly the success of the two mRNA-based SARS-CoV-2 vaccines. This chapter reviews the recent publications on infectious disease mRNA vaccines and highlights the remaining challenges to overcome before this transformative novel vaccine platform can be applied broadly to diverse infectious disease targets.

The licensure and the observed safety profile of the two mRNA-based SARS-CoV-2 vaccines also open treatment options beyond COVID-19 and the prophylactic vaccines space. There is an enormous potential for applying mRNA to therapeutic approaches, including therapeutic vaccines against infectious diseases or cancer, and protein replacement therapy in which mRNA is used to substitute a missing or non-functional version of a human protein. Huang et al. provide in their chapter "Advances in Development of mRNA-Based Therapeutics" an overview of the exciting use of mRNA in therapeutic cancer vaccines by encoding either common tumor-associated antigens for "off the shelf use" or neoantigens derived from individual tumor biopsies. The latter approach customizes the therapy to best match the individual need of a patient (i.e., personalized medicine). The review also focuses on mRNA application in protein replacement therapy for liver (e.g., Fabry disease, hemophilia B, and methylmalonyl-CoA mutase deficiency) or lung disease (Cystic Fibrosis) and touches on the potential application as an interventional therapy for myocardial infarction. The most visionary application of mRNA is gene editing, with the potential to permanently cure an existing genetic defect obviating the need for life-long therapeutic treatment. It will be exciting to observe these therapeutic approaches progress in development.

The pace of clinical development for mRNA as a novel vaccine platform has been extraordinary. The first clinical testing of mRNA-based prophylactic vaccines was published in 2017, and by the end of 2020, the SARS-CoV-2 mRNA vaccine already achieved emergency use licensure for human use. August et al. describe in their chapter "Clinical Development of mRNA Vaccines: Challenges and Opportunities" the history of mRNA-based vaccines and provide a detailed overview of the first cautious and subsequently successful steps leading to the first two licensed mRNA vaccines employing modified nucleotides to mitigate excess of innate immune responses ("Kariko paradigm"). Leveraging the knowledge from the licensed SARS-CoV-2 mRNA vaccines, August et al. provide their perspective on important questions such as whether LNP used to formulate mRNA vaccines are to be considered an adjuvant. The authors also identify open questions that need further investigation or lead to important next development steps for mRNA vaccines, such as combination vaccines targeting multiple pathogens to simplify vaccination schedules. Another important perspective discussed in this chapter is the concept of platform safety, supported by the extensive safety database from the current SARS-CoV-2 mRNA vaccines. The acceptance of the platform safety concept would allow the acceleration of clinical development of new vaccines derived from the same platform. This exciting opportunity will allow an increased pace of development of prophylactic vaccines against other infectious diseases that have a high and urgent medical need.

Finally, in the chapter on "Regulatory Considerations on the Development of mRNA Vaccines", Naik and Peden provide their perspective on the regulatory path to licensure of mRNA vaccines. This topic is of particular importance since the understanding of regulatory approval pathways is often not the focus of academic or technical reviews. The authors offer interesting insights into the regulatory approach used to assess the safety and efficacy of mRNA-based medicinal products. These comprise not only clinical development considerations aimed at demonstrating safety and efficacy, but also key aspects regarding the quality of a biopharmaceutical product such as guidance on chemistry, manufacturing, and control (CMC); consistency of manufacturing; release parameters for the final product; and critical quality attributes. The chapter also highlights future developments in manufacturing and potential safety-related information that might be leveraged from existing similar mRNA vaccine products to new ones.

The mRNA technology is still in its infancy. Indeed, clinical proof of concept and utility for rapid responses have just recently been firmly established by the development and approval of two successful SARS-CoV-2 vaccines. Promising clinical data have also been generated for other infectious disease targets such as cytomegalovirus (CMV) and respiratory syncytial virus (RSV). Late-stage clinical data will tell if the current RNA technology, particularly conventional mRNA and lipid nanoparticle delivery, will be equally successful for new disease targets. Some new targets could be more challenging than SARS-CoV-2, requiring greater persistence and levels of T cell immunity, which have not been optimized for the current SARS-COV-2 mRNA vaccines and may be critical for a successful vaccine against another disease target. In addition, while to date conventional mRNA has

become a mainstream technology, the potential for self-amplifying mRNA continues being evaluated in clinical studies. Each chapter of this book series highlights various opportunities and challenges of mRNA platform technology. Further enhancement of delivery efficiency; improvements to reactogenicity, tolerability, and stability; and targeted delivery may represent additional opportunities to advance the platform. Improvement of T cell responses, particularly $CD8^+$ T cell immunity, and durability of vaccine-elicited protective responses are also areas for future investment. With the accelerated improvement of the platform, it is anticipated that the application of mRNA technology in other therapeutic areas, such as protein replacement, immuno-oncology, gene editing, or infectious disease therapeutics, could advance to fruition in the next few years.

In closing, we hope that this book series provides a unique value to readers. mRNA-based vaccine technology is progressing rapidly, and this book is intended to be an end-to-end review series, covering topics from basic RNA biology, science, and data supporting the platform and applications, to clinical development and regulatory approval. It offers a comprehensive overview of this transformative technology, its application, and future potential, providing established RNA researchers and developers with updates on the field.

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Contents

mRNA-Based Vaccines and Mode of Action

Janina Gergen and Benjamin Petsch

Contents

Abstract In the past 20 years, the mRNA vaccine technology has evolved from the first proof of concept to the first licensed vaccine against emerging pandemics such as SARS-CoV-2. Two mRNA vaccines targeting SARS-CoV-2 have received emergency use authorization by US FDA, conditional marketing authorization by EMA, as well as multiple additional national regulatory authorities. The simple composition of an mRNA encoding the antigen formulated in a lipid nanoparticle enables a fast adaptation to new emerging pathogens. This can speed up vaccine development in pandemics from antigen and sequence selection to clinical trial to only a few months. mRNA vaccines are well tolerated and efficacious in animal models for multiple pathogens and will further contribute to the development of

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vaccines for other unaddressed diseases. Here, we give an overview of the mRNA vaccine design and factors for further optimization of this new promising technology and discuss current knowledge on the mode of action of mRNA vaccines interacting with the innate and adaptive immune system.

1 Introduction

Vaccines provide the only durable protection against primary infections by pathogens. Since the smallpox vaccine development in 1798, countless lives and billions in health care costs have been saved (Plotkin 2014; Ozawa 2017). The World Health Organization (WHO) estimates that 2–3 million human lives are saved every year due to vaccination programs. Morbidity or crippling is prevented in numerous additional cases. Protective vaccines reduced annual poliomyelitis cases from 350,000 in 1988 to 33 in 2018. As of today, 26 infectious diseases can be prevented by vaccination, and four viruses have been eradicated from global circulation. Smallpox was eradicated in 1980 (World Health Organization 1980), wild type polio virus 2 and 3 in 2015 and 2019, respectively (https://www.who.int/news-room/feature-stories/detail/two-out-of-three-wild-poliovirus-strains-eradicated), and the animal pathogen rinderpest virus in 2011 (Mariner 2012). No other medical intervention is able to eradicate a disease.

Although a very successful medical intervention, existing vaccine technologies have their limitations, and progress in vaccine development is slowing down. Hence, new technologies are used to develop vaccines against pathogens such as SARS-CoV-2, which threaten our way of living.

In this review article, we summarize prophylactic vaccines with focus on mRNA-based vaccine technologies and their mode of action.

1.1 Established Vaccination Approaches

The main principle of vaccination is the induction of durable immunity against a pathogen by introducing either a part of the pathogen or the inactive or attenuated version of the pathogen into a vaccinee. The subsequent activation of the immune system, the induction of an adaptive immune response, and the establishment of a memory response against the pathogen allow the immune system to respond faster and more efficiently against this pathogen during subsequent infection to prevent disease manifestation. There are different classes of vaccines established. The first vaccines were attenuated versions of the pathogen that mimicked natural infection without causing disease in humans. These so-called live attenuated vaccines are able to replicate and express a variety of antigens. The resulting immune response is strong, broad, and long-lasting, sometimes due to low level of replication in the vaccinee. However, the attenuated virus might mutate and regain its pathogenicity, which

occurred with the polio vaccine resulting in vaccine-associated paralytic poliomyelitis (Burns et al. 2014). Additionally, attenuated vaccines are reduced in their pathogenicity, but often cannot be safely administered to specific immunocompromised target populations, e.g., pregnant women, immunocompromised or human immunodeficiency virus (HIV) infected individuals (Hesseling 2009). Although the safety of these vaccines is excellent in most cases, the class has some limitations.

These vaccine-associated risks can be limited by using inactivated bacteria or viruses, e.g., the rabies, influenza, or the Hepatitis A virus vaccines (Plotkin 2014; Innis 1994). While inactivated pathogens are considered safer than live attenuated pathogens, they are also less immunogenic. This can partially be overcome by the use of adjuvants. The proper inactivation is key to safety, since incomplete viral inactivation might lead to vaccine-induced infections. Therefore, proof of inactivation is a critical release parameter for inactivated vaccines. The last severe cases associated with incomplete inactivation happened in 1955, when insufficient formalin-inactivation of the newly (inactivated) polio vaccine produced by Cutter pharmaceutical company caused 250 cases of atypical paralytic polio (Juskewitch et al. 2010). Today's safety regulations and quality controls reflect lessons learned from those events and are designed to prevent reoccurrence. Nevertheless, quality inactivation control assessment can be demanding and intense. For the polio vaccine, WHO recommends a three week cell culture period with the vaccine virus (Chumakov et al. 2002); for rabies vaccine, it is even more intense, and it includes cell culture cultivation of the inactivated virus stained directly for virus replication or injected intracerebrally in mice (Bourhy 2007).

For live attenuated or inactivated whole organisms, replicating pathogens have to be produced in large quantities, often requiring individualized growth conditions for each vaccine, e.g., embryonated chicken eggs or cell culture for influenza virus. Reproducible vaccine production quality is challenging, and some vaccines suffer from high rate of batch failures. Moreover, the vaccine is more vulnerable to mutations that can decrease its efficiency. This is a problem especially for influenza vaccines. For egg-based influenza vaccines, the virus regularly adapts to the chicken cells by accumulating mutations within the receptor binding site which negatively influences vaccine efficiency, as observed for the vaccines of the last seasons (Zost 2017; Skowronski 2014).

Subunit vaccines, which contain only a protein of the respective pathogen, such as surface proteins (e.g., hepatitis B virus surface protein), or toxoids (e.g., Tetanus toxoid) are likely the safest. However, due to their high purity, they are less immune-stimulatory. They usually require an adjuvant, e.g., aluminum salts, which stimulate the immune system to support the induction of a protective immune response, but can induce adverse effects of their own (Petrovsky 2015). The first subunit vaccines were purified from cultured organisms, but with the rise of gene technology, recombinant proteins have become the standard. Manufacturing is more consistent and not as vulnerable to mutations as whole virus vaccines. However, for some pathogens, it is difficult to produce a stable, soluble antigen in the natural conformation needed to induce a protective immune response. Surface proteins, like viral envelope proteins, often have transmembrane domains and

assemble into multimers. For the expression of such recombinant proteins, the introduction of stabilizing mutations or protein-engineering is necessary to produce the antigen in its natural conformation. This is exemplified by the HIV envelope (ENV) protein that is a trimeric transmembrane glycoprotein described as very unstable even during natural infection (Burton and Hangartner 2016). For recombinant protein expression, the full-length protein (160kD) is truncated to create a soluble gp140 protein, an internal protease cleavage site needs to be altered, and disulfide bonds are introduced to stabilize the trimer. Still, even small mutations can have a big impact on stability and immunogenicity (Beddows 2006; Sanders and Moore 2017). Similar results were reported for the respiratory syncytial virus fusion protein that is meta-stable, but a much better immunogenic in the pre-fusion conformation (Rossey et al. 2018).

Overall, prospective vaccines need to be easily manufactured, safe, and immunogenic. New vaccine technologies, such as viral vectors, DNA, and mRNA vaccines, have been developed showing promising features (Rauch et al. 2018).

1.2 Novel Vaccination Approaches

Viral vectors are engineered viruses, e.g., adenoviruses, adeno-associated viruses, or vesicular stomatitis viruses that encode a heterologous antigen. They are replication-deficient and deliver the antigenic sequence information into the host cell, which produces the antigen and presents it to immune system. Viral vectors allow a strong and diverse immune reaction to an antigen. At the same time, pre-existing anti-vector immune responses to the natural virus, e.g., adenovirus 5, can drastically decrease vaccination efficiency (Lemckert 2005).

DNA vaccines also deliver the antigenic sequence into the cell and induce transient antigen expression. The introduction of DNA into the host cell is challenging, since it has to reach the cell nucleus, crossing two cellular membranes, in order to facilitate antigen expression. Furthermore, the delivery of foreign DNA into a host cell comes with a risk of integration into the host genome, which could lead to unwanted side effects, including oncogenesis, depending on the integration site (Lee et al. 2018).

Using messenger RNA (mRNA) as a vaccine is a fairly new approach although it has been known since the early 90s that mRNA can induce antigen expression upon immunization and the induction of antigen-specific cytotoxic T cells (Wolff 1990; Martinon 1993). In 2000, Hoerr et al. confirmed and extended the potential of mRNAs as vaccines. They showed that immunizations with mRNA can be at least as effective as DNA in inducing cellular and humoral immune responses, i.e., cytotoxic T cells and antigen-specific antibodies (Hoerr et al. 2000). mRNA vaccines allowed the expression of antigens by the host cells and the expression of transmembrane proteins and viral glycoproteins with a natural glycosylation profile. Compared to DNA vaccines, mRNA can be more easily delivered into the cell, since it only needs to reach the cytoplasm for translation. Consequently and due to

the absence of a reverse-transcriptase that could copy the mRNA into DNA, there is no risk of integration into the host DNA genome. Overall, use of mRNA is associated with a lower risk profile, as its production does not require cultivation of pathogens or any infectious materials at any step of the process. Production of an mRNA vaccine only requires the genetic sequence information, today often available via online databases. Moreover, only a limited number of antigens are expressed and for a short period of time. For a long time, RNA was perceived as a very unstable molecule for use as a genetic vector. However, handling in an RNAse-free environment and the formulation of the mRNA molecules allow the production of stable mRNA vaccines (Stitz 2017). In this chapter, we describe the underlying technology of mRNA vaccines in detail and discuss the immune responses that can be induced by mRNA vaccination.

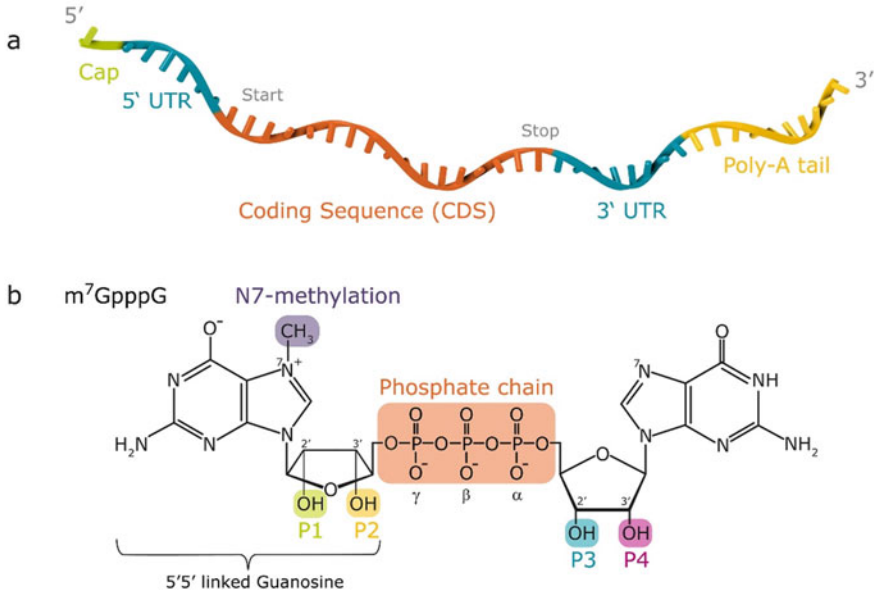

Fig. 1 Schematic structure of the mRNA and cap. **a** The general structure of an mRNA is based on a 5′ Cap, a 5′ UTR, an open reading frame (ORF) coding for the respective antigen, a 3′ UTR and a 3′ end containing a poly(A) stretch. **b** The 5′-cap structure is a N7-methylguanosine (methyl = purple) binding to the first nucleotide of the mRNA by 5′-5′ phosphodiester bond. Cap analogs can be modified at several position. P1 (green) and P2 (yellow) are used for methylation to generate the anti-reverse cap analog (ARCA). The phosphate chain (orange) can be prolonged or substituted with sulfur or other elements. At position P3 (blue), the next nucleoside will be attached by normal 5′-3′ bond, and Position P4 (pink) can be methylated to generate a cap1 structure

2 mRNA Technology

A classical cellular mRNA has the minimal structural requirement of a 5′ cap, the open reading frame (ORF) and a 3′ poly(A) tail to enable efficient translation of the encoded protein. Untranslated regions (UTR) with regulatory function before and after the ORF can improve mRNA properties. Synthetic mRNAs are modeled after cellular mRNAs. They contain the ORF of the antigen complemented by UTRs, a 5′ cap, and a 3′ poly(A) tail (Fig. 1a). Synthetic mRNA vaccine are produced in a similar way (Schlake et al. 2012). First, the mRNA sequence is cloned into a plasmid downstream of a bacteriophage promotor, e.g., T7 or Sp6. The plasmid is subsequently linearized and used as a template for in vitro transcription by an RNA polymerase. After purification, the produced mRNA is formulated with proteins and/or lipids, which facilitate uptake by host cells and protect the mRNA against RNAses (Geall 2012).

In the following paragraphs, different designs for these structural elements are presented, and their impact on mRNA stability and protein expression are reviewed.

2.1 5′ Cap Structure

Each eukaryotic mRNA starts with a 5′-cap structure. The most common natural cap is a N7-methylguanosine (m^7G) which is connected to the mRNA via a 5′-5′ phosphodiester bond, followed by a ribose 2′-*O*-methylation on the first nucleotide (Banerjee 1980) (Fig. 1b). The cap interacts with cellular cap binding proteins, e.g., eukaryotic initiation factor 4E(eIF4E), which regulate mRNA processing, nuclear export, translation initiation, and prevent mRNA decay by blocking access of RNA decapping proteins, e.g., decapping protein 1 and 2 (DCP1/2). The cap is also involved in the discrimination between self and non-self mRNAs by the innate immune system (Lässig and Hopfner 2017; Galloway and Cowling 2019). To achieve maximum efficiency, synthetic mRNAs need to be capped, usually in parallel to, or subsequently to in vitro transcription (IVT).

There are three types of cap structures, cap0, where only m^7G is added to the mRNA (m^7GpppN), cap1, containing both m^7G and 2′-*O*-methylation of the first nucleotide ($m^7GpppNm$), and cap2, where m^7G is followed by two methylated nucleotides ($m^7GpppNmNm$). Cap1 and in theory cap2 are not only more efficiently incorporated into the mRNA and increase its translation, but they are also less likely to be detected by innate immune receptors. A detailed description of how mRNA vaccines interact with the innate immune response can be found in a latter part of this chapter (Sect. 3.2).

The first synthetic cap was mCap, a guanine dinucleotide m^7GpppG and cap0 structure (Pasquinelli et al. 1995). It is incorporated co-transcriptionally into the mRNA by the RNA polymerase itself, which uses the mCap to initiate the IVT. However, T7 and other bacteriophage RNA polymerases can initiate at both

guanines and therefore can incorporate mCap in forward ($_{m}^{7}$GpppG-mRNA) and reverse (Gppp$_{m}^{7}$G-mRNA) orientation with the approximated ratio of 30–50% reverse orientation (Pasquinelli et al. 1995). Reverse mCap is not recognized by the translation machinery, and no protein is expressed from these mRNA molecules. Hence, a significant portion of the mRNA will not be expressed.

There have been major efforts to improve capping of synthetic mRNAs (Table 1). Substitutions of the hydroxyl group at position C2 or C3 of m^{7}G by a simple hydrogen (m^7 2′dGpppG, m^{7}3′dGpppG) or the addition of a methyl group (m$_2^7$ $^{2'O}$GpppG, m$_2^7$ $^{3'O}$GpppG) prevent reverse incorporation of the cap and improve translation efficiency (Stepinski et al. 2001; Jemielity 2003). This cap was named "anti-reverse cap analog" (ARCA) (Stepinski et al. 2001). Later, mostly the m^7 $^{2'O}$GpppG is referred as ARCA. Additional modifications increase the translation efficiency further and are summarized in Table 1. They include the extensions of the phosphate chain, while a tetraphosphate (m^{7}GppppG) increases translation efficiency due to higher binding efficiency to eIF4E, a pentaphosphate (m^{7}GpppppG) shows decreased translation efficiency by preventing eIF4E release (Jemielity 2003). The insertion of bridging modifications, e.g., dichloromethylene insertions (m^{7}Gpp-CCl$_2$-ppG) or sulfur substitutions (m^7 $^{2'O}$ Gpp$_s$pG, named β-S-ARCA), prevents decapping (Rydzik 2017; Grudzien-Nogalska et al. 2007). The β-S-ARCA showed a nearly doubled half-life in vitro and improved stability in primary dendritic cells (DC) (Kuhn 2010). Furthermore, it induces increased T cell responses in vivo (intranodal application of unformulated mRNA) compared to the regular ARCA (Kuhn 2010). Other modifications affect the m^{7}G and can improve the mRNA stability as well. A benzyl at position P2 (Fig. 1b) enhances overall translation efficiency due to improved eIF4E binding, although it might be more sensitive to decapping by Dcp1/Dcp2 (Kocmik 2018). Finally, the locked nucleic acid with a modification of the first guanosine has a lower capping efficiency compared to ARCA, but still a higher translation efficiency due to improved binding to eIF4E (Kore et al. 2009).

More recently, TriLink has developed a synthetic cap1 (m^{7}GpppNmN), called CleanCap®, that is added co-transcriptionally (Vaidyanathan 2018; www.cleancapmrna.com). According to the company, it outcompetes ARCA (cap0) in capping efficiency (95 vs. 70% for ARCA) and translation efficiency. The CleanCap® is available as a natural m^{7}GpppNmN cap or with the ARCA modification (m^7 3′ dGpppNmN) and both with the different variants of the second and third nucleotide NN = GG, AU or AG.

Alternatively, caps can be added post-transcriptionally by the vaccinia virus capping complex (Schnierle et al. 1992; Venkatesan et al. 1980; Meis et al. 2006), e.g., the commonly used synthetic cap structure, called ScriptCap. It is post-transcriptionally incorporated by subsequent incubation with a capping enzyme adding m^{7}G, and a methyltransferase adding the 2′-*O*-methylation (Schnierle et al. 1992). Although the capping efficiency is nearly 100%, the addition of one or two enzymatic reactions and a purification step adds time and costs to the manufacturing process (Meis et al. 2006). Methyltransferases can also be used to add 2′-*O*-methylations to an existing cap0 to enhance translation efficiency (Richner 2017).

Table 1 Cap modification

Name	Chemical structure	Modification	Reverse orientation	Capping efficiency	Effect in comparison to ARCA				References
					Half-life	Translation in RRL	Translation in cells	eIF4E binding	
mCap	m^7GpppG	–	30–50%	60–70%	-	– (2,3–2,6x)	– (15 × lower in JAWII) (2 × lower in DCs)	=	Pasquinelli et al. (1995), Mockey et al. (2006), Jemielity (2003), https://www.trilinkbiotech.com/cart/scripts/prodView.asp?idproduct=2800
ARCA	$m^{7\ 2'O}$GpppG	Methylation at P1	None	62%					Stepinski et al. (2001), Jemielity (2003), Grudzien-Nogalska et al. (2007)
ARCA	$m^{7\ 2'O}$GppppG	Extension of phosphate chain	None	55%		+ (1,1x)		+	Jemielity (2003)
ARCA	$m^{7\ 2'O}$GpppppG	Extension of phosphate chain	None			– (67%)		++	Jemielity (2003)
S-ARCA	$_m{}^{7\ 2'O}$Gpp$_s$pG	Sulfur substitution in phosphate chain	None		+ (1,2–1,6 × in HC11) (1–1,8 × in DCs)		+ (1,3–2,4 × in HC11) (1,3–1,5 × in DCs)		Grudzien-Nogalska et al. (2007), Kuhn (2010)
ARCA	$m^{7\ 2'x}$Gpp-x-ppG	Different substitutions in phosphate chain x = CCl_2 x = CF_2 x = CH_2	None		+ All Resistant to DcP2 degradation	= Only x = CCL_2	+ Only x = CCL_2 (1,18x)	+ (44–82%)	Rydzik (2017)
ARCA	bn^{2}m$_2{}^7$O2′GpppG	Benzyl-substitution at P2			+ (1,4x) HEKs	+ (1,44x)	+ (3,3x), HEKs	– (30%)	Kocmik (2018)

(continued)

Table 1 (continued)

Name	Chemical structure	Modification	Reverse orientation	Capping efficiency	Effect in comparison to ARCA				References
					Half-life	Translation in RRL	Translation in cells	eIF4E binding	
ARCA	(p-OCH_3bn)^2$m_2^{7O3'}$GpppG	p-metoxybenzyl-substsitution at P2			**No effect**, HEKs	+ (1,35x)	+ (2,42x)	+ (8x)	Kocmik (2018)
LNA (locked nucleic acid)	$m^{7(LNA)}$GpppG	Additional bond between O at P1 with the C connected to the phosphate chain	None	54%	+ (1,2x)	+ (2,2x)		+	Kore et al. (2009)
Ally-Cap	$m^{7\ 2'O-ally}$ GpppG	2′O-Allyl substitution at P1	None	59%		*+ in comparison to mCap (1,7x)*			Kore and Charles (2010)
Propagly-cap analog	$m^{7\ 3'O-propargyl}$GppppG	O'Propagyl substitution at P2	None	56%			*+ in comparison to mCap (3,1x), HeLa*	+	Shanmugasundaram et al. (2016)
ScriptCap			None	100%		+ (1,5x)			Meis et al. (2016)
CleanCap	m^7GpppNmN	–	None	99%			+		www.cleancapmrna.com

RRL rabbit reticulocyte lysate, *ARCA* anti-reversed cap analog, *eIF4F* eukaryotic initiation factor 4F; *DC* dendritic cell; *LNA* locked nucleic acid

2.2 *Untranslated Regions (UTR) of mRNA*

UTRs are an essential part of most eukaryotic mRNAs and all RNA viruses. They contain regulatory elements that recruit cellular factors to the mRNA 5′ and 3′ ends and with further optimization can improve translation efficiency and mRNA stability (Ahmed et al. 2011).

mRNA translation is initiated by eIF4E initiation factor interaction with the cap and assembly of the initiation complex 43S (Ahmed et al. 2011). TISU (translation initiation of short 5'UTRs, GCCAGAaug) and Kozak (GCCRCCaugG) sequences are translation initiation elements that allow binding of the ribosome 43S initiation complex which scans the mRNA for the first AUG start codon (Elfakess et al.2011; Kozak 1991). Weak AUG context sequences around the start codon can be skipped by the ribosome and translation initiated at the next AUG, resulting in shorter or different proteins, a process called *leaky scanning*/AUG skipping (Kozak 2005). Even though the Kozak sequence alone is sufficient to induce translation of the mRNA, a longer untranslated region upstream of the start codon can lead to higher translation efficiencies (Kozak 1991).

In humans, 5′UTRs, the regions upstream of the start codon, have a median length of 218 bp (Leppek et al. 2018). They can enhance the translation efficiency, e.g., the 5′UTR of Hsp70, β-globin, and tobacco etch virus increase protein expression level even when cloned upstream of a heterologous ORF (Schlake et al. 2012; Kozak 1991; Vivinus 2001; Schlake et al. 2019; Holtkamp 2006). UTRs can also decrease or even prevent protein expression. The iron responsive element (IRE), naturally found in the ferritin and the iron transporter ferroportin mRNAs, is bound by iron-regulatory proteins in low iron conditions. While the IRE in the ferritin mRNA is at the 5′ UTR, the interaction prevents association of the mRNA with the ribosome, causes translation inhibition and degradation, to reduce ferritin expression and storage of iron under iron starvation condition. Ferroportin mRNAs have the IRE on the 3′ end, which has the exact opposite effect and increases the expression of the transporter to maintain iron levels in the cell (Ahmed et al. 2011; Leppek et al. 2018; Muckenthaler et al. 2017).

Thus, therapeutic mRNA translation can be improved by adding a particular 5′ UTR. Variety of secondary UTR structures can be formed depending on length, GC content, and sequence, affecting translation efficiency. 5′ UTRs with a high GC content are more likely to have a complex secondary structures (Leppek et al. 2018), for instance stem loops which can favor 43S ribosome recruitment through the transacting factor eIF3. For example, the 5′ UTR of the interferon γ (IFNγ) mRNA forms a pseudoknot. In turn, this dsRNA structure activates the innate immune response locally. The activation leads to translation arrest and represents a negative feedback loop to prevent uncontrolled IFNγ production (Ben-Asouli et al. 2002). Other secondary structures such as stem loops, IRE, hairpins, and RNA G-quadruplexes might have similar impact on the translation efficiency (Leppek et al. 2018).

Interestingly, Trepotec et al. described a highly efficient minimal 5′ UTR of only 7–8 nucleotides (Trepotec et al. 2018). In combination with a Kozak sequence or a TISU element, these short sequences increased protein levels over the gold-standard 5′ UTR α-globin (30 bases).

3′ UTRs are located downstream of the ORF. They are usually longer than 5′ UTRs (median length 1200 nucleotides) and regulate the stability of the mRNA (Mayr 2008; Jan et al. 2011). Like 5′ UTRs, 3′ UTR sequences contain motifs that recruit RNA binding proteins (RBP) which act as linkers between the mRNA and functional proteins. Structural elements such as IREs and AU-rich elements (ARE) can induce repression of translation, deadenylation (shortening of the poly-A tail), decapping, or cleavage and lead to mRNA decay (Mayr 2008; Koh et al. 2019). Sequence motifs found only in the 3′UTRs are seed regions for miRNAs, which may also promote mRNA decay (Ahmed et al. 2011; Mayr 2008; Jia et al. 2013; Rabani et al. 2017). Some 3′ UTRs, like those from α- and β-globin, can increase mRNA stability (Holtkamp 2006; Wang et al. 1999). The α-globin 3′ UTR recruits the α-complex, which stabilizes the poly(A) binding protein (PABP) to protect the poly(A) tail and prevent deadenylation (Wang et al. 1999; Bernstein et al. 1989). In tumor tissues, it has been observed that 3′ UTR shortening significantly increases oncogene mRNA half-life (Mayr 2008). Additionally, 3′ UTRs can contain zipcodes, which serve as motifs for RBPs that interact with tubulin-associated motors and transport mRNAs closer to where the protein is needed, e.g., the leading edge of actin filaments for β-actin mRNA (Lawrence and Singer 1986). 3′ UTRs can also affect the function of the translated protein by influencing the location of the mRNA and its translation impacting protein interactions (Mayr 2008). For example, it has been demonstrated for the mRNA encoding CD47, that only the long 3′ UTR mRNA can lead to a cell surface expressed CD47 and interaction with SET, while the short 3′ UTR mRNA lead to intracellular CD47 expression. Cell surface expressed CD47 can prevent the cell from phagocytosis, while intracellular CD47 is involved in the induction of apoptosis. Even though both proteins have the same amino acid sequence, the 3′ UTR defines their functionality (Berkovits and Mayr 2015). Hence, UTR regulatory elements are able to strongly impact protein function.

For synthetic mRNAs, selection of suitable 3′ UTR can prolong expression and protein level. The inclusion of multiple 3′ UTRs can also increase stability and translation efficiency. A screen for stable mRNA constructs in dendritic cells identified a combination of the 3′ UTRs of mtRNR1 and AES mRNAs that increased protein expression in vitro and in vivo and led to higher T cell responses upon immunization (Orlandini von Niessen 2019). Until very recently, limited information on UTR motifs and their effects on translation efficiency was available, and choice of UTRs had to be evaluated empirically. Improvements in sequencing technologies and machine learning have identified a plethora of novel 3′UTR motifs and allow for predicting the impact of UTR sequences on ribosome loading and translation in human cells (Rabani et al. 2017; Sample 2019). The technology supports generation of 5′UTR sequences with targeted ribosome loading and protein expression levels (Sample 2019). It is likely that selection of UTRs will increase in the future mRNA designs.

2.3 3'End of the mRNA

The poly(A) tail forms the very 3′ end of an mRNA and is of varying length (Stewart 2019). Its intracellular shortening by deadenylases regulates transcript half-life. The poly(A) is also involved in the initiation of translation via interaction of poly-A binding proteins with the translation initiation complex at the 5′ end (Ford et al. 1997). Cellular mRNAs are polyadenylated in the nucleus independently of template and are subsequently exported to the cytoplasm (Stewart 2019).

Synthetic mRNAs can be polyadenylated as part of the IVT by encoding a defined number of As on the plasmid template, or by a poly(A) polymerase that adds poly(A) tails of varying lengths (30–200 nts) to the transcript after the IVT (Beverly et al. 2018; Kuhn et al. 2011). The plasmid encoded poly(A) is of defined length varying only in a few nucleotides (Beverly et al. 2018). In general, at least 30 As are needed for a functional tail, but longer tails confer higher stability (Ford et al. 1997). Sixty As seem optimal to induce high translation efficiency in most cell lines (Elango et al. 2005). Longer poly(A) tails led to decrease in translation efficiency in this context. Some immune cells seem to be exceptions. Translation efficiency can increase to lengths of 120 As for dendritic cells, while the maximal efficiency was reached at 300 As for primary T cells (Holtkamp 2006; Ford et al. 1997; Elango et al. 2005; Grier 2016). However, long A-stretches are challenging to produce. In bacteria, the poly(A) tail encoded on the plasmid template is shortened to approximately 70 nt as a result of recombination during amplification (Grier 2016). This can be avoided by segmenting the poly(A) tail into pieces of 40–60 nt separated by a few non-A nucleotides, without affecting mRNA stability or translation efficiency (Trepotec et al. 2019). Non-A nucleotides are tolerated within stretches of As. However, translation efficiency was ∼30% lower when dendritic cells were transfected with in vitro transcribed mRNA ending with five non-A nucleotides compared to a clean poly(A) tail (Holtkamp 2006). In primary T cells, up to 6 non-A nucleotides can be tolerated without pronounced reduction of translation efficiency if the extension contains exclusively GC (Grier 2016). An extension containing one or more Us strongly impaired the translation. This observation is especially relevant for the linearization of the template plasmid. Most commonly used restriction enzymes are of type II and generate a 3′end with 1–5 nucleotides at the end of the poly(A) (Elango et al. 2005). Recently, type IIS restriction enzyme has been employed for plasmid linearization to generate blunt A ends (Holtkamp 2006; Elango et al. 2005)

As an alternative to a poly(A) tail, a histone stem loop (HSL) can be used to terminate the mRNA. Histone mRNAs are usually not polyadenylated and end in a conserved 26 nt long stem loop structure that protects the transcript from degradation during the S-phase of mitosis (Kaygun and Marzluff 2005; Marzluff 1992). However, a few histone mRNAs are polyadenylated downstream of the stem loop in non-growing cells, allowing for better regulation of transcript levels. Thus, poly (A) and HSL are not mutually exclusive (Mannironi et al. 1989).

Synthetic mRNAs combining optimized 3′UTR sequences, poly(A) length, and a clean ending can improve transcript levels up to 100-fold, and peak protein levels and expression time fivefold (Holtkamp 2006).

2.4 ORF Optimization and Global Modifications

ORF sequences can have a big impact on mRNA half-life and translation. Each species has its own codon bias (Novoa and Ribas de Pouplana 2012), which can lead to increased protein expression up to 1000-fold (Gustafsson et al. 2004). The codon optimization of the synthetic mRNA ORF could strongly increase the expression of the respective encoded antigen. Moreover, the codon usage within the ORF may differ (Tuller and Zur 2015; Clarke and Clark 2010). Less structured 5′ end of the ORF supports ribosome scanning and binding, while the rest of the ORF has been described to benefit from higher structure that slows down the ribosome and improves translation and protein folding (Tuller and Zur 2015; Mauger 2019; Ding et al. 2014). The effect observed on cellular mRNAs can be leveraged to improve synthetic mRNA vaccine technology in the future. Currently, most mRNA platforms use a rather simple GC enrichment (Rauch et al. 2018).

Besides the optimization of the sequence itself, the nucleotides can be modified as well. In vitro transcribed (IVT) mRNAs are usually based on the classical natural nucleotides (A, T, G, C). Unfortunately, delivered ssRNAs are recognized as immunogenic and activate different antiviral innate immune mechanisms leading to cytokine release, translation breakdown, and RNA degradation. Even though an innate immune activation is necessary for a vaccine to induce an effective adaptive immune response (explained in more details in the "mode-of-action" section), a too strong innate immune reaction can be detrimental by interfering with the translation efficiency, impairing antigen expression, and as such inhibit the induction of an adaptive immune response.

A comparison of different host RNA species to IVT RNA or bacterial RNA showed that tRNA and rRNA, which contain a high amount of chemically modified nucleotides, are much less immune stimulatory (Karikó et al. 2005). Subsequently, several modified nucleotides have been analyzed for optimizing translation efficiency and reducing innate immune reaction. In this review, we focus on the five most commonly used modified nucleotides: pseudouridine [ψ, the most abundant nucleotide modification in host cell RNAs (Spenkuch et al. 2014)], N1-methyl-pseudouridine (m1ψ), 5-methylcytidin (5mC), N6-methyladenosine (m6A), and 2-thiouridine (s2U). The modified nucleotides have three major effects: reduction of innate immune stimulation, reduction of mRNA impurities, and effect on protein expression levels.

All modified nucleotides reduce activation of pattern recognition receptors (TRLs; RIG-I, and MDA) to prevent extensive cytokine release. In dendritic cells, m6A and s2U completely abolish the TLR activation (TLR 3, 7 and 8), while ψ and m5C only slightly reduce TLR activation and reach full suppression only in combination

(Karikó et al. 2005; Andries et al. 2015). Subsequently, it has been demonstrated that RIG-I signaling is impacted by modified nucleotides. m6A completely prevents interaction with RIG-I (Durbin et al. 2016). In contrast, other modification such as ψ, m1ψ, and 5mC RNAs bind to RIG-I, but inhibit signaling pathway activation by preventing the conformational change of RIG-I (Durbin et al. 2016).

In addition to their effect on innate immune receptors, modified nucleotides can influence the IVT reaction. Antisense RNA is a common side product of IVT. It creates dsRNA region that can activate MDA5, which usually detects long dsRNA (Kato 2008). RNA impurities are usually removed by HPLC purification (Karikó et al. 2011). Interestingly, incorporation of ψ, m1ψ, or 5mC (but not m6A) into synthetic mRNAs reduces the occurrence of such unintended products (Mu et al. Jun. 2018; Baiersdörfer 2019).

The inhibition of immune cell activation and cytokine release could be confirmed in several mouse studies after application of IVT RNA i.v. (Karikó 2008; Kormann 2011; Tusup et al. 2018), i.d., i.m. (Andries et al. 2015) or into the lung (Andries 2013). A systemic cytokine release of IFNα, IFNβ, TNF, and IL-6 was inhibited (Karikó 2008). Only few studies could not detect a difference between RNA with natural nucleotides or ψ-containing RNA (Kauffman 2016; Thess 2015).

Beside the evasion of immune activation, modified nucleotides can influence mRNA stability and translation efficiency. Because of the strong decrease of translation efficiency, m6A and s2U are not suitable modification for mRNA vaccines, (Karikó et al. 2005; Karikó 2008). Effects on translation efficiency by ψ, m1ψ, or 5mC containing mRNAs are debatable and seem to be cell type dependent. Ψ and 5mC RNAs have been described to increase translation efficiency in DCs, PBMCs, and MEFs (Karikó et al. 2011; Karikó 2008). On the other hand, ψ-containing mRNAs have the same or even lower translation efficiency compared to natural nucleotide mRNAs in macrophages, HeLa cells, and keratinocytes (Andries et al. 2015; Kauffman 2016; Thess 2015; Uchida et al. 2015; Loomis 2018). In HEK293 cells, reported results differ, probably depending on delivery methods and further differences in the RNAs (Karikó et al. 2011; Tusup et al. 2018; Svitkin et al. 2017). mRNA secondary structure influences protein expression level as well. Mauger and colleagues observed that certain modified nucleotides, especially m1Ψ, can stabilize the secondary structure to increase translation efficiency, whereas mo^5U destabilizes RNA structures (Mauger 2019). m6A destabilizes secondary structures such as hair pins (Kierzek and Kierzek 2003). Modified nucleotides can prevent the activation of intracellular antiviral defense mechanism, like PKR-induced phosphorylation of eIF2α, leading to a general translation inhibition and OAS-induced RNase L-mediated RNA degradation. Consequently, the inhibition of PKR and OAS ensures RNA stability and translation upon intracellular delivery (Svitkin et al. 2017; Anderson et al. 2011; Anderson 2010).

In vivo, most studies confirm a higher and prolonged protein expression by nucleotide-modified mRNAs (Baiersdörfer 2019; Kormann 2011; Karikó 2008; Tusup et al. 2018; Anderson et al. 2011; Karikó et al. May 2012). Andries et al. tested the three most potent modified nucleotides in mice by i.d. and i.m. application (most relevant for vaccines) and showed that m1ψ outperforms ψ and 5mC

by 13-fold in protein translation in vivo (Andries et al. 2015). In line with this observation, Drew Weisman and colleagues developed several m1ψ-containing mRNA vaccines against zika, HIV, and influenza (Pardi et al. 2017, 2018, 2019).

However, two studies could not confirm any improvements of modified nucleotides (ψ) over natural nucleotides in terms of cytokine induction and translation efficiency (Kauffman 2016; Thess 2015). Importantly, the mRNA used by Thess et al. does not have a traditional poly(A) 3′end but rather ends with a histone stem loop (Thess 2015). The secondary structure, and hence the function of this mRNA element, is potentially affected by incorporation of ψ, explaining the observed differences.

In most studies, uridine or cytosine has been replaced completely by the modified nucleotides (= 100% modification). However, based on the observation of host RNA species, where only a fraction of the nucleotides are modified, Kariko et al. observed that incorporation of only 10% modified nucleotides (ψ, 5mC, or m6A) is sufficient to reduce TNF release by 50% in DCs (Karikó et al. 2005). For complete inhibition, at least 90% of the nucleotides had to be modified. Similar results have been observed in RAW cells (macrophage cell line) (Uchida et al. 2015).

In summary, all properties of a synthetic mRNA can affect the mRNA efficiency and quality, including UTRs or choice of nucleotides.

3 Mode of Action of mRNA Vaccines

The general principle of any vaccine is the durable induction of a protective immune response against an antigen. For mRNA vaccines, this is achieved by delivering the antigenic sequence into the vaccinee's cells, so that they can express the encoded protein and present it to the immune system. mRNA vaccines have several advantages over recombinant protein and whole viral particle vaccines: ssRNA itself has an adjuvant-effect, which abolishes the need of an additional adjuvant (Edwards 2017). Furthermore, antigen expression by the host cells facilitates the correct folding of the antigen and enables the incorporation of transmembrane proteins presented on the cell surface in their native conformation. Viral antigens are expressed similarly during a viral infection. Like other vaccines, mRNA vaccines can be administered by different routes. For practical reasons, most vaccines are given intramuscularly (i.m.), even though various clinical trials suggest that intradermal (i.d.) administration induces stronger immune responses or needs less vaccine to induce the same response compared to i.m. or s.c. (subcutaneous) immunizations (Hickling et al. 2011; Zhang et al. 2015). It is believed that more professional antigen presenting cells (APC), such as dendritic cells (DCs), reside in the skin, and thus induce a stronger immune response. APCs take up the mRNA cargo, which stimulates innate immune signaling, and in response the cells migrate to the draining lymph node. There, the APCs present the antigen to B- and T cells and activate the adaptive immune response. We will discuss each of these steps in more detail in the following paragraphs.

3.1 Innate Immune Sensors Detecting mRNAs

Mammalian cells detect a variety of pathogen-associated molecular patterns (PAMPs) with the help of pattern recognition receptors (PRRs). Some PAMPs are molecules that only exist on pathogens, e.g., bacterial lipopolysaccharide or flagellin (Mogensen 2009). Others exist in the host (self) and the pathogen (non-self) and need to be distinguished by PRRs, e.g., unmethylated DNA of bacteria.

RNA as a PAMP is identified based on its location and structure. For example, mRNAs have no function outside of the cells. If cells take up extracellular RNA species via endocytosis, they are detected as non-self by PRRs in the endosome, specifically toll-like receptors (TLRs) 3, 7, and 8 (Georg and Sander 2019). TLR3 binds to dsRNA and activates TRIF (TIR-domain-containing adapter-inducing interferon-β), inducing a signaling cascade that leads to IRF3 activation, and the expression of type I interferons and IP-10. Both TLR7 and 8 sense ssRNA and signal through MyD88 and IRF7, but they show a cell type specific expression pattern (Patinote 2020). TLR7 is only expressed in plasmacytoid dendritic cells (pDCs) and B cells, where its activation leads to type I interferon production. TLR8, on the other hand, is found in monocytes and monocyte-derived DCs (mDCs) and induces TNF, IL-6, IL-12, and MIP-1α.

The cytoplasm contains a different set of PRRs, which have to distinguish non-self RNA from self RNA, especially mRNA. Retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated proteins 5 (MDA5) sense double-strand RNA (dsRNA) (Georg and Sander 2019; Brisse and Ly 2019). RIG-I prefers blunt end dsRNA ligands of at least 13 bp and with 5′-PPP—or Cap0-ends, to which it binds and oligomerizes (Devarkar 2016; Schmidt 2009). In contrast, MDA5 binds to dsRNA of at least 2000 bp, such as viral genomes, to which it binds at any position and starts polymerizing as well. RIG-I/MDA5 filaments then recruit and activate mitochondrial antiviral signaling protein (MAVS). MAVS activation leads to induction of IRF3 and IRF7 signaling, and expression of NF-κB and type I interferon. Furthermore, cytoplasmic NOD-like receptors like NRLP3 can also respond to dsRNA and to a lesser extent to ssRNA (Allen 2009). This induces the formation of the inflammasome. It is a major mechanism in macrophages and leads to a strong release of IL-1β and induction of apoptosis (Rajan et al. 2010).

Other dsRNA sensors like protein kinase RNA-induced (PKR) and 2′-5′-oligoadenylatesynthetase (OAS) are IFN-induced. In turn, they induce global translation arrest and RNA degradation upon activation. PKR is also an intermediary of TLR3 signaling and can stimulate the expression of IL-1β and IL-18.

Distinguishing self from non-self single-stranded RNA (ssRNA) in the cytoplasm depends as well on the mRNA cap structure. IFN-inducible proteins with tetratricopeptides (IFITs) detect ssRNAs and discriminate them based on their 5′ structure. IFIT1, for example, detects a missing 2′–*O*–methylation of the first nucleotide (i.e., cap0) and sequesters 5′-PPP-mRNAs (Fensterl and Sen 2015). Upon recognition of their ligand, IFITs associate with the RNA and prevent its translation or replication. As their name indicates, IFITs are induced by IFN signaling and are not constitutively expressed.

Downstream of all PRRs is the activation of antimicrobial signaling pathways and the expression and secretion of type I interferons, i.e., IFN-α and IFN-β. They can bind to their receptors (IFNAR) found on all cells and subsequently can induce paracrine activation of the "antiviral state" of neighboring cells through the expression of IFN-stimulated genes (ISGs). ISGs include PKR and OAS, which broadly inhibit protein translation. For example, PKR phosphorylates translation factor eIF2α in the presence of dsRNA, leading to a general inhibition of protein synthesis (Anderson 2010). OAS signaling leads to activation of RNase L, which degrades RNA (Anderson et al. 2011). Additional ISGs, like myxovirus resistance 1 (MX1) or apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC1), have more specific antiviral functions (Sadler and Williams 2008). More importantly, IFNα/β activate immature DCs and turn them into APCs by inducing the expression of i) MHC molecules, ii) co-stimulatory proteins, e.g., CD80 and CD86, iii) chemokine receptors, and iv) cytokines such as IL-12 (Kranz et al. 2016). This allows DC to present more antigens, better activate T cells, migrate to the lymph node where the T and B cells are located, and influence the adaptive immune response according to the antigen, i.e., IL-12 production drives T_H-1 differentiation (McNab et al. 2015). Other cytokines downstream of innate immune sensors includes pro-inflammatory cytokines, TNF, IL-1α/β, IL-6, and IL-8. Therefore, the early induction of type I interferons and cytokines is crucial for mounting a strong, long-lasting adaptive immune response against a pathogen.

3.2 Immune Activation by mRNA Vaccines (Adjuvancy vs. Overactivation)

Messenger RNA vaccines reliably stimulate the innate immune response independently of the encoded antigen. Consequently, the innate response induces a strong adaptive immune response. Thus, unlike subunit vaccines, mRNA vaccines do not require an adjuvant for immune stimulation but are innate stimulatory as such (Edwards 2017; Beverley 2002). After administration, cells at the injection site take up the mRNA vaccine. Most mRNA vaccines used today are formulated as lipid nanoparticles (LNP), which are taken up by endocytosis, and after endosomal escape, reach the cytosol where the mRNA is expressed (Edwards 2017; Kranz et al. 2016; Devoldere et al. 2016). Some of these cells will be APCs, especially dendritic cells (DCs), and to a certain degree, macrophages. They detect the non-self mRNA in the endosome via TLR7/8, mount an innate immune response, and produce pro-inflammatory cytokines (Kranz et al. 2016). Several studies confirm that TLR7 activation is predominantly responsible for the acute inflammatory response after mRNA vaccination (Kranz et al. 2016; Fotin-Mleczek 2011). Conversely, the T cell response is strongly impaired in TLR7–/– mice after vaccination with mRNA vaccines, showing the importance of TLR7 activation and subsequent cytokine release for T cell response (Kranz et al. 2016). When naked mRNA is delivered by electroporation, PRRs in the cytoplasm might be activated (Devoldere et al. 2016; Iavarone et al. 2017).

A broad range of pro-inflammatory cytokines has been detected at the injection site upon i.m. or i.d. injection, including the acute phase cytokines IL-6, IL-1β and TNF, leading to DC activation and migration, lymphocyte activation, and increased antibody production (Edwards 2017; Lutz 2017; Kowalczyk 2016). Additional cytokines and chemokines have been detected in the muscle after i.m. injection (Lutz 2017). MIP-1α, MIP-1β, CXCL9, MCP-1, and CXCL1 recruit more APCs, as well as NK-, B-, and T cells (Carr et al. 1994; Wolpe and Cerami 1989; Groom and Luster 2011; Tannenbaum et al. 1998). Dendritic cells, and to a certain degree macrophages, are the main actors in the cytokine production (Kranz et al. 2016). Overall, there is a local inflammatory reaction. However, the cytokine release has been reported to be short lived, peaking between 6 and 14 h post immunization and usually returning to baseline after 24 h (Edwards 2017; Lutz 2017; Kowalczyk 2016; Broos 2016). Generally, no or very low systemic cytokine release could be observed in comparison to the local reaction (Lutz 2017; Kowalczyk 2016).

Within 4 h of after immunization, high antigen levels are expressed in DCs and monocytes (Broos 2016; Liang 2017). Uptake and mRNA expression are concurrent with upregulation of activation markers, such as CD40, CD80, and CD86 as well as MHC for efficient antigen presentation (Kranz et al. 2016, Edwards 2017; Broos 2016; Liang 2017; Scheel 2004). Activated DCs have also been shown to migrate to the draining lymph nodes (dLN) and create a pro-inflammatory milieu (Kowalczyk 2016; Carralot 2004). After i.d. injection of protamine-formulated mRNA, increased chemokine (CXCL9 (MIG), CCL2 (MCP1), CCL3 (MPI-1α), CCL4 (MIP-1β)), and cytokine (TNFα, IFNα, IFNγ, IL-1) levels have been found in the dLN (Edwards 2017; Kowalczyk 2016). This results in recruitment and activation of lymphocytes and the induction of adaptive immune responses (Carr et al. 1994; Wolpe and Cerami 1989; Tannenbaum et al. 1998).

Taken together, i.m. or i.d. injections of mRNA vaccines induce a pro-inflammatory environment in the draining lymph nodes within 24 h, enabling DCs to present the antigen to the adaptive immune system and creating highly immune-stimulatory conditions to develop a strong immune response against the respective encoded antigen.

Unfortunately, due to the mRNA production process, incompletely capped or partially degraded mRNAs, as well as non-specific oligoribonucleotides, may appear as side products of RNA polymerase in in vitro transcription, can be contained in the vaccine, and can create unintended RNA PAMPs (Karikó et al. 2011). These would be detected by additional PRRs, result in an overstimulation of the innate immune system and an excessive inflammatory reaction, which can be detrimental to the adaptive immune response. The important role for type I interferons in connecting innate and adaptive immune systems has been described. IFNα is necessary for proper DC activation and maturation, leading to increased expression of co-stimulatory molecules (Kranz et al. 2016). Furthermore, it increases antigen cross-presentation and induces the release of chemokines for T cell recruitment (Crouse et al. 2015). The role of IFNα on the immune response upon mRNA vaccination seems to be double-edged. High levels of IFNα are associated with various symptoms of malaise. Prolonged IFNα expression has been linked to development of

autoimmune disease in a different context (Wills et al. 1984; Rönnblom 1991; Borg and Isenberg 2007). Locally, it upregulates antiviral effectors like OAS and PKR, potentially degrading the mRNA vaccine and preventing its translation and antigen presentation (Devoldere et al. 2016). Along those lines, a higher antigen expression was observed in IFNAR–/–DC after mRNA/lipoplex delivery (Pollard 2013). It is unknown to what extent a reduced antigen expression might affect vaccine efficacy. mRNA vaccine that is not expressed, or only at low levels, will not be able to induce an efficient adaptive immune response. In general, the role of IFNα and its effects on immune responses are incompletely understood (McNab et al. 2015). Depending on the timing, level, and environment of IFNα expression, it might stimulate or dampen T cell responses and regulate antigen expression in different ways (Kranz et al. 2016; Crouse et al. 2015; Pollard 2013; Beuckelaer 2016; Beuckelaer et al. 2017). Kranz et al. reported that IFNα induction is important for $CD8^+$ T cell effector function (Kranz et al. 2016). Intravenous immunization of IFNRA–/–mice with mRNA/lipoplex showed impaired and shortened DC activation and significantly lower levels of IFNα. Antigen-specific $CD8^+$ T cells were induced, but had a strongly impaired effector function lower Granzyme B, IFNγ and TNF expression. On the other hand, De Beucklear et al. claimed that the application of mRNA/lipoplex subcutaneously (s.c.) or i.d. in IFNAR–/–mice showed a much stronger cytotoxic T cell (CTL) response than in WT mice (Beuckelaer 2016). In a cytotoxicity assay, only a small portion of the antigen presenting target cells (peptide pulsed) could be eliminated by antigen-specific CTLs from WT mice, while there was a complete eradication of target cell by antigen-specific CTLs from IFNAR–/–mice. Based on these opposing observations, De Beucklear hypothesized that kinetics of IFNα and TCR signaling determine the positive or negative effect of IFNα release (Beuckelaer et al. 2017). When IFNα release and TCR stimulation occur simultaneously, like after i.v. injection, T cells are strongly stimulated, and IFNα serves as the third signal leading to differentiation and effector function (Kranz et al. 2016; Broos 2016; Le Bon et al. 2014). However, if the IFNα signal is induced before TCR stimulation, it might have the opposite effect leading to anergy and cell death/inhibition of proliferation. The hypothesis explaining these opposing IFNα effects is based on the ability of IFNAR to signal either through STAT4 (positive, pro-inflammatory, proliferative) or STAT1 (anti-proliferative, apoptotic) and is reviewed by Crouse et al. (2015). Upon TCR activation, T cells upregulate STAT4 while maintaining STAT1 level constantly low.

In conclusion, a balanced pro-inflammatory innate immune response is supportive of a subsequent adaptive immune response. The exact factors needed for optimal immune response to the mRNA vaccines, and especially the role of IFNα are subject to further studies. It is assumed that a potent but local (injection side and draining LN) pro-inflammatory environment is beneficial to the induction of both, a strong humoral and cellular immune response. Importantly, a substantial systemic cytokine release is not preferred, since it might be associated with side effects like fever, headache, chills, and fatigue induced by acute phase cytokine and type I IFNs. These reactions have been reported after mRNA vaccination in clinical phase 1/2 studies, including studies using modified and non-modified nucleotides (Gruys et al. 2005; Alberer 2017; Mulligan et al. 2020; Jackson et al. 2020; Bahl et al. 2017). Moreover, cytokine

release might lead to a decline of pDCs by the intrinsic apoptosis (Swiecki et al. 2011). Most mRNA platforms are describing only a transient local cytokine release without systemic inflammation (Lutz 2017; Pardi et al. 2017).

3.3 Adaptive Immune Response

Depending on the pathogen, different immune effectors are likely to confer protection. Most acute viral infections will be prevented by neutralizing antibodies as described for influenza, rabies, or measles viruses (Nigg and Walker 2009; Haralambieva et al. 2019; Padilla-Quirarte et al. 2019). RNA viruses with high antigen variability can rapidly escape neutralizing antibodies by mutations in surface antigens. In this case, a strong cellular immune response can limit the disease by killing cells actively replicating the virus, stopping viral spread to reduce pathology. Some therapeutic vaccines for HIV or other chronic viral infections aim to boost the cytotoxic T cell response to control viral replication (Ndhlovu 2015). For mRNA vaccines, both have been described, the induction of protective humoral as well as cellular immune responses.

3.3.1 Humoral Immunity

Indeed, mRNA vaccines induce strong antibody responses to a variety of viral infections, e.g., influenza (Pardi et al. 2018; Lindgren 2017; Petsch 2012), rabies (Lutz 2017), and HIV (Pardi et al. 2019). A nucleotide-modified mRNA/LNP vaccine encoding influenza virus hemagglutinin outperformed the inactivated influenza virus (IIV) vaccines and live attenuated virus vaccines in a mouse model (Pardi et al. 2018). The hemagglutination inhibition (HAI)-titer, a surrogate marker for neutralizing antibodies against influenza, was 40-times higher in mRNA vaccinated animals than in IIV-vaccinated mice after two i.d. immunizations and remained stable for at least 13 months. Similarly, a protamine-formulated mRNA also induced a strong antibody response with HAI-titers above the correlate of protection (1:40) even after a single vaccine dose (Petsch 2012). In a passive serum transfer experiment with a subsequent viral challenge, it was demonstrated that the induced antibodies are sufficient to confer full protection in mice. Impressively, this vaccine was not only efficient in adult mice, but could induce protective immunity in newborn mice and very old (18 month) mice (Petsch 2012). Similar results were obtained in newborn piglets for an mRNA vaccine encoding the rabies G protein (Schnee 2016). These findings show that mRNA vaccines might be efficacious in populations at increased risk of sever outcome such as newborns and aging individuals. Follow-up studies in non-human primates (NHP) using LNP-formulated mRNA confirmed the potency of mRNA vaccines to induce neutralizing antibody titers at least as high as licensed vaccines, e.g., influenza mRNA vaccine vs. Fluad, or rabies mRNA vaccine versus licensed inactivated Rabies vaccine (Lutz 2017).

Functional antibody titers in terms of HAI- titers in NHPs were stable for at least 1 year. The application route (i.m. vs. i.d.) does not seem to make a significant difference in this model. However, prime i.d. injections of modified mRNA vaccines induce a faster increase in antibody response (Lindgren 2017). Both immunization routes reach the same antibody titer plateau and total IgG avidity after the second injection.

B cell activation and maturation to antibody-secreting plasma cells take place in the germinal centers (GC) of lymphatic tissue. Messenger RNA vaccines efficiently promote the formation of germinal centers in vivo. In mice, the splenic GC showed a 10–20-fold increase of B cells upon nucleotide-modified mRNA/LNP immunization, significantly higher than after immunization with a recombinant protein or inactivated viral particles (Pardi et al. 2018). Enlarged GCs, pronounced increase in proliferating B cells and follicular helper T cells (TfH), were also observed in NHPs after immunization with nucleotide-modified mRNA/LNPs (Lindgren 2017). The frequency of antigen-specific TfH (4–8%) was significantly higher than observed after vaccination with protein (<1%) (Pardi et al. 2018). Since TfH cells are necessary for B cell affinity maturation and isotype switch, an increase in TfH cells should correlate positively with higher antibody titers (McHeyzer-Williams et al. 2009; Lindgren 2017). Finally, the first mature antibody-secreting plasma cells in the bone marrow were detected 2 weeks post prime immunization (Lindgren 2017). Low number of circulating memory B cells were also detectable. They significantly increased upon boost and remained stable for at least 25 weeks post immunization.

3.3.2 Cellular Immunity

In line with the ability to activate B cells, mRNA vaccines induce strong $CD4^+$ and $CD8^+$ T cell responses to various pathogens, e.g., rabies and influenza, including the establishment of effector memory $CD8^+$ T cells ($CD44^+CCR7^-CD62L^-$) (Fotin-Mleczek 2011; Lutz 2017; Petsch 2012). Several sequential immunizations boost T cell response further without a sign of T cell exhaustion or induction of regulatory T cells (Fotin-Mleczek 2011; Kowalczyk 2016). The original data used protamine-formulated mRNA, but new data with LNP-formulation confirmed efficient T cell induction by mRNA in mice and NHP, outperforming licensed vaccines (Lutz 2017; Petsch 2012).

The induction of a strong Th1 response is specifically preferred for vaccines against intracellular pathogens, since it not only leads to high antibody response but also induces cytotoxic T cells. T cells can eliminate infected cells and prevent further spread of a viral or different intracellular pathogen. Immunization with naked mRNA has been reported to induce Th2 responses, while LNP- or protamine-formulated mRNAs stimulate a Th1 response through the activation of TLRs and the signaling by MyD88 (Kranz et al. 2016; Fotin-Mleczek 2011; Scheel 2004; Carralot 2004; Pollard 2013). This is supported by the observation of higher IgG2a /IgG1 ratio in mice. After immunization with protamine-formulated mRNA, mainly IFNγ and IL-2 secreting $CD4^+$ T cells were detected (Fotin-Mleczek 2011),

which supports activation of $CD8^+$ T cells. Pardi et al. reported induction of multifunctional $CD4^+$ T cells expressing TNF, IFNγ, and IL-2 upon two i.d. immunizations of nucleotide-modified mRNA/LNP (Pardi et al. 2018).

mRNA vaccines induce cellular immunity more efficiently than standard licensed vaccines and stimulate multifunctional effector T cells of multiple subsets.

4 Conclusion

The first proof of concept for an mRNA vaccine was reported two decades ago (Martinon et al. 1993; Hoerr et al. 2000). Since mRNA vaccines against a multitude of pathogens have been developed and characterized in pre-clinical models. mRNA vaccines have several advantages over recombinant protein and whole viral particle vaccines. ssRNA itself has an adjuvant-effect, which abolishes the need of an additional adjuvant (Edwards 2017). The antigen expressed by the host cells facilitates correct folding and conformation and enables incorporation of transmembrane proteins to be presented on the cell surface. Viral antigens are expressed in situ similarly to expression during viral infection. Due to the lack of host gene integration risk, mRNA vaccines promise a better safety profile than DNA vectors. mRNA technology enables broad infectious disease application. It is independent of pathogen cultivation and inactivation and hence does not require specific biosafety environment. The basic manufacturing process for different vaccines is similar and does not need major adaptation to different pathogens (Schlake et al. 2012). The process can be streamlined and adjusted to global health threats. Recent advances in understanding the influence of untranslated mRNA sequences, formulations, and injection technologies suggest new and exciting developments in the field of mRNA vaccines in the next few years. New technologies, including machine learning and artificial intelligence, will provide new insights in mRNA vaccine designs for improved product candidates.

The proof of concept of mRNA vaccines was already demonstrated for several viral pathogens, e.g., influenza virus, rabies, CMV, ZIKA, HIV, tick-transmitted flaviviruses (Richner 2017; Pardi et al. 2017, 2018, 2019; Lutz 2017; Petsch 2012; Schnee 2016; John 2018; John et al. 2018).

Very high and long-lasting antibody titers are detected in both rodents and NHPs. The strong T cell response indicates the opportunity for therapeutic vaccination against chronic diseases such as life-long pathogenic infections or cancer. mRNA vaccines are immunogenic and protective in various animal models. Multiple clinical trials are ongoing, e.g., rabies virus (NCT03713086), CMV (NCT03382405), influenza (NCT03345043), as well as at least three mRNA vaccines for the ongoing SARS-CoV-2 pandemic outbreak (NCT04283461, NCT04449276, NCT04470427, NCT04405076, NCT04368728, NCT04380701). This highlights that especially in the case of a pandemic, mRNA vaccines are among the first vaccines manufactured for clinical use and subject to clinical testing. Already four months after the SARS-CoV-2 pandemic outbreak in

December 2019, an mRNA vaccine platform was the first vaccine used in a clinical trial starting 16th March (NCT04283461). The first clinical batch (https://www.modernatx.com/modernas-work-potential-vaccine-against-covid-19) was available 25 days after sequence selection. Efficacy was demonstrated for two mRNA vaccines within eleven months for two independent mRNA vaccines for SARS-CoV-2 in large phase III efficacy studies (NCT04368728, NCT04470427), and conditional marketing applications have been submitted by two companies to competent authorities. This unprecedented speed of development underscores that mRNA vaccines will have their share in preventing disease and addressing unmet medical need, including the ongoing Covid-19 pandemic and are proofing their value for future pandemic preparedness.

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Self-amplifying mRNA-Based Vaccine Technology and Its Mode of Action

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Contents

Author Contribution

GM, DY planned the manuscript. GM wrote and coordinated the draft. JBU, RR, DY reviewed the manuscript.

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Abstract Self-amplifying mRNAs derived from the genomes of positive-strand RNA viruses have recently come into focus as a promising technology platform for vaccine development. Non-virally delivered self-amplifying mRNA vaccines have the potential to be highly versatile, potent, streamlined, scalable, and inexpensive. By amplifying their genome and the antigen encoding mRNA in the host cell, the self-amplifying mRNA mimics a viral infection, resulting in sustained levels of the target protein combined with self-adjuvanting innate immune responses, ultimately leading to potent and long-lasting antigen-specific humoral and cellular immune responses. Moreover, in principle, any eukaryotic sequence could be encoded by self-amplifying mRNA without the need to change the manufacturing process, thereby enabling a much faster and flexible research and development timeline than the current vaccines and hence a quicker response to emerging infectious diseases. This chapter highlights the rapid progress made in using non-virally delivered self-amplifying mRNA-based vaccines against infectious diseases in animal models. We provide an overview of the unique attributes of this vaccine approach, summarize the growing body of work defining its mechanism of action, discuss the current challenges and latest advances, and highlight perspectives about the future of this promising technology.

1 Introduction

Synthetic messenger RNA (mRNA) has recently gained much attention because of its promise to revolutionize vaccination, immunotherapy, and treatment of genetic diseases. The concept of using mRNA to encode proteins of choice into target cells is rather straightforward, in that mRNA is the direct means of translating genetic information into proteins. From a theoretical standpoint, mRNA can prevent or treat any disease where administration or transient expression of a recombinant eukaryotic protein could be beneficial (Kowalski 2019; Schlake 2019; Pastor 2018; Zhang et al. 2019a, b; Warren and Lin 2019; Magadum et al. 2019; Trepotec 2019). Synthetic mRNA therapeutics are designed to share essential elements of eukaryotic host cell mRNA and utilize a simple, synthetic, rapid, generic, and cell-free process for the generation of the therapeutic molecules (Maruggi 2019; Hekele 2013; Ulmer et al. 2015; Geall et al. 2013; Kramps and Elbers 2017; Sahin et al. 2014).

Currently, two forms of mRNA vaccines have been developed: conventional mRNA encoding the antigen of interest flanked by 5′ and 3′ untranslated regions (UTRs), and self-amplifying mRNA.

Self-amplifying mRNA represents a unique form of synthetic mRNA. It is commonly based on the genome of positive-sense, single-stranded RNA (ssRNA) viruses (Tews and Meyers 2017; Lundstrom 2016b). It is engineered to encode a protein of interest and utilizes viral elements to drive self-amplification of the mRNA in the host cells, resulting in many copies of original mRNA, and high,

prolonged expression of the protein of interest (Geall 2012; Vogel 2018; Leyman 2018; Brito 2014). Since the demonstration that positive-strand RNA viruses are amenable to direct genetic manipulation and can be used for gene expression in target cells (Racaniello and Baltimore 1981; Semler et al. 1984; Xiong 1989; Liljestrom and Garoff 1991), a variety of vaccine strategies has been previously developed by utilizing virion packaged self-amplifying mRNAs (reviewed in Lundstrom 2016b, 2018; Ljungberg and Liljestrom 2015) or plasmid DNA (pDNA) to launch the self-amplifying mRNA genome (reviewed in Lundstrom 2016b; Singh 2019).

The first attempts to use exogenous naked self-amplifying mRNA for protein expression and vaccination in vivo was reported in the late 1990s with in vitro transcribed (IVT) RNA derived from positive-sense RNA virus sequences, overshadowed by the large body of work on pDNA and viral vectors (Zhou 1994; Mandl 1998). The pioneering work of Zhou et al. and Fleeton et al. showed that as little as 10 μg of naked self-amplifying IVT RNA could elicit immune responses against heterologous antigens at levels comparable to those induced by virally delivered RNAs (Zhou 1994; Fleeton 2001). Both reports highlighted that only a small amount of RNA was needed to induce immunity, due to the self-amplification nature of the RNA molecule. Despite the encouraging results, the developability of mRNA-based vaccines was perceived as too challenging, due to instability, innate immune activation, the difficulty of delivery, and the uncertain feasibility of large-scale manufacturing. Hence, the field primarily pursued DNA-, viral-, and protein-based approaches. Now, over 20 years later, these issues are no longer perceived as barriers as a result of technological advances in RNA biology, chemistry, and delivery systems.

Tremendous progress has been made in the last few years in the development of methods for long RNA molecule synthesis and of biomaterials and delivery strategies that can functionally transport RNA into cells, with a focus on encapsulation of large RNA molecules (Fig. 1) (Geall 2012; Brito 2014; Erasmus 2018; Chahal 2016; Demoulins 2017, 2016; Englezou 2018). Non-viral delivery of self-amplifying mRNA has shown considerable promise as a vaccine technology against a broad spectrum of viral, bacterial, and parasitical infections in preclinical models (Maruggi 2019). Self-amplifying mRNA vaccines are attractive because of the broad immune responses elicited (both humoral and cell-mediated), the precision and flexibility in antigen design, the potentially low dosage required to achieve high immunogenicity compared to non-amplifying mRNA and pDNA vaccines, and the potential for being rapidly produced with a scalable generic manufacturing platform (Ulmer et al. 2015; Kis 2020). The feasibility of this vaccine platform to fill the gap between antigen identification and vaccine availability against emerging pandemic infections was demonstrated by Hekele and co-workers, who generated a self-amplifying mRNA vaccine against pre-pandemic influenza eight days after the release of the viral gene sequence (Hekele 2013). The promise of rapid mRNA vaccine development has been recently shown with the demonstration of safety and efficacy in humans of a pair of optimized, conventional non-amplifying mRNA vaccine candidates for the global severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2) pandemic in less than 11 months from pathogen sequence identification (Polack 2020; Baden et al. 2020; Dolgin 2021).

To increase the efficiency and potency of self-amplifying mRNA vaccines, an efficient synthetic delivery system is crucial to induce a proper balance of innate immune and adaptive responses. Intracellular delivery of long IVT mRNA requires encapsulation into a delivery carrier (reviewed in Zeng 2020) to ensure protection from degradation, delivery across the cell membrane and, after cell uptake, usually by endocytosis, escape from the endosome, and release into the cytoplasm, where translation and self-amplification occur (Kowalski 2019; Houseley and Tollervey 2009). Once in the cell, the vaccine mimics viral RNAs and triggers a variety of cellular endosomal and cytosolic RNA sensors that induce a signaling cascade culminating in the release of type I interferon (IFN) and robust innate responses, including the production of chemokines and cytokines at the injection site (Pepini 2017; Pollard 2013; Zhong 2019). These responses are crucial for the successful induction of effective adaptive responses against the encoded antigen, although the precise mechanisms are largely unknown. However, excessive activation of type I IFN can have a detrimental effect on the launch of the self-amplifying mRNA via an antiviral response, thereby creating a potentially unfavorable environment for mRNA vaccine expression and potency, and also exerting deleterious effects on T cell responses (Pollard 2013; Chen 2017; Beuckelaer 2016). The mechanisms by which the complexity of type I IFN signaling, whether acting positively or negatively on efficacy and safety of self-amplifying mRNA vaccines and how to modulate it, requires further investigation.

This review outlines the current state-of-the-art of synthetic self-amplifying mRNA-based technology, supported by the growing body of work leading to our

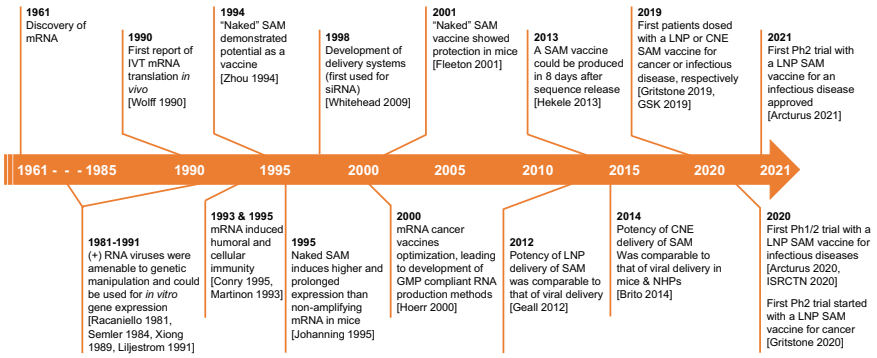

Fig. 1 Key milestones and findings in the development of synthetically delivered self-amplifying mRNA vaccines. (Geall 2012; Brito 2014; Racaniello and Baltimore 1981; Semler et al. 1984; Xiong 1989; Liljestrom and Garoff 1991; Zhou 1994; Fleeton 2001; Johanning 1995; Arcturus Therapeutics 2021; GRANITE-001 2019; Gristone Oncology 2020; GSK 2019; Wolff 1990; Conry 1995; Martinon 1993; Hoerr 2000; Whitehead et al. 2009). CNE, cationic nanoemulsion; GMP, Good Manufacturing Practice; IVT, in vitro transcribed; LNP, lipid nanoparticle; NHPs, non-human primates; SAM, self-amplifying mRNA

current understanding of how self-amplifying mRNA vaccines function. We discuss its unique attributes, strengths, key challenges, and the prospects for this transformative technology.

2 Self-amplifying mRNA: From Viruses to Synthetic Vaccines

2.1 Self-amplifying mRNA Biology and Mechanistic Clues from the Alphavirus Self-amplification Pathway

Self-amplifying mRNA vaccines have gained momentum as the next-generation approach for mRNA modality, due to their self-amplification properties, leading to higher and prolonged protein expression compared to conventional non-amplifying mRNAs (Geall 2012; Vogel 2018; Leyman 2018). Self-amplifying mRNAs are large mRNA molecules (~9–12 kb) with a type 0 cap (N7mGppp) at the 5′end and a poly(A) tail at the 3′end (Fig. 2). Upon entry into the cytoplasm, host cells can immediately translate them. However, the type 0 cap structure can be sensed by cellular innate sensors RIG-I and MDA5 and sensor-effectors IFIT1 and IFIT5 of the type I IFN pathway, thus stimulating innate immunity, and the cap 1 structure can be introduced to facilitate immune evasion of incoming RNA (Li 2019).

Self-amplifying mRNA vectors are commonly derived from the genome of positive-sense ssRNA viruses, such as alphaviruses, flaviviruses, and picornaviruses (Tews and Meyers 2017; Lundstrom 2016b). The most utilized and studied self-amplified mRNA molecules are derived from alphaviruses, such as Venezuelan Equine Encephalitis Virus (VEEV), Sindbis Virus (SINV), and Semliki

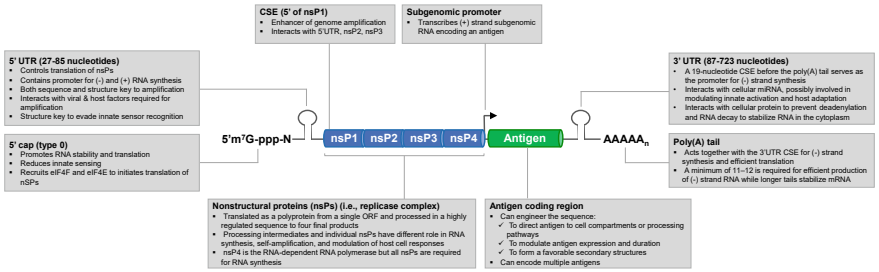

Fig. 2 Schematic representation of the sequence structure of alphavirus-based self-amplifying mRNA vaccines. In alphavirus derived self-amplifying mRNA, two open reading frames (ORFs) encode nonstructural proteins that are required for intracellular RNA amplification and the antigen of choice, respectively. Non-coding regulatory regions flank the ORFs and are also between them, which regulate gene expression, amplification, translation, and RNA-host interactions. Indicated are major RNA regulatory elements, ORFs, and length variation among the alphavirus 5′ and 3′ UTRs. ORF, open reading frame; nsPs, nonstructural proteins; UTR, untranslated regions; CSE, conserved sequence element; eIF4, eukaryotic initiation factor 4

Forest Virus (SFV) (Liljestrom and Garoff 1991; Ljungberg and Liljestrom 2015; Pushko 1997; Bredenbeek 1993). In all of these cases, the genome is a positive-sense, single-stranded RNA of ~11–12 kb, and contain two open reading frames (ORFs), which are expressed from two different mRNAs (genomic RNA and subgenomic RNA) and translated at different stages during the self-amplification process (Strauss and Strauss 1994). UTRs are present at the 5′ and 3′ ends of the genome, as well as between the ORFs. These noncoding regions contain distinct core promoter elements for minus-strand, plus-strand, and subgenomic RNA synthesis and, a combination of linear sequence and structural elements for the regulation of viral gene expression, replication, translation, and virus–host interactions (Frolov et al. 2001; Fayzulin and Frolov 2004; Kulasegaran-Shylini 2009; Kulasegaran-Shylini 2009; Hyde 2014, 2015). The first ORF is translated directly from genomic RNA and encodes a polyprotein that is subsequently processed into individual nonstructural proteins (nsPs) required for RNA synthesis, while the second ORF is expressed through the production of a subgenomic mRNA and encodes the structural proteins. To generate a vaccine, the viral structural genes are replaced with the antigen gene of interest generating an RNA termed replicon (Fig. 2), which mimics the virus RNA self-amplification cycle without producing infectious virions after vaccination (Fig. 3).

When alphavirus-derived RNA enters the cytoplasm, the replicase complex is directly translated from the incoming RNA as a polyprotein, which interacts with the 5′ and 3′ termini of the genomic RNA and with host factors to induce membrane invaginations, termed spherules. In spherule cavities, replication components are concentrated, double-stranded RNA (dsRNA) intermediates are likely protected by the membrane from host cell innate detection and disruption, and new genomic RNAs are synthesized (Frolova 2010; Spuul 2010; Jose et al. 2017; Pietila et al. 2018; Carey 2019). The viral replicase first uses the genomic RNA as a template to synthesize the complementary negative-strand RNA, which subsequently serves as the template for the synthesis of a very high copy number of two new distinct classes of capped and poly-adenylated positive-strand RNA species (Hellstrom 2016). The first positive-strand RNA species is a genomic-length RNA transcript, which is a direct copy of the initial full-length RNA transcript, while the second one is an abundant positive subgenomic RNA encoding the structural proteins or the exogenous gene of interest, in the case of replicons (Strauss and Strauss 1994; Sawicki 1978; Lundstrom 2005). This process is highly regulated in a sequential way at the level of the polyprotein processing into the individual nsPs (reviewed in Rupp 2015; Pietila et al. 2017). Processed intermediates and individual nsPs have a different role in RNA synthesis, self-amplification, and modulation of host cell responses. In the replicase complex, nsP1 is the viral capping enzyme and membrane anchor of the replication complex, nsP2 is both the protease responsible for polyprotein processing and the helicase unwinding RNA duplex during replication, while nsP4 is the RNA-dependent RNA polymerase (Ahola and Kaariainen 1995; Spuul 2007; Vasiljeva 2003; Gomez de Cedron 1999; Rubach 2009). nsP3 is an essential component of the RNA replicase, although its role is less defined, and

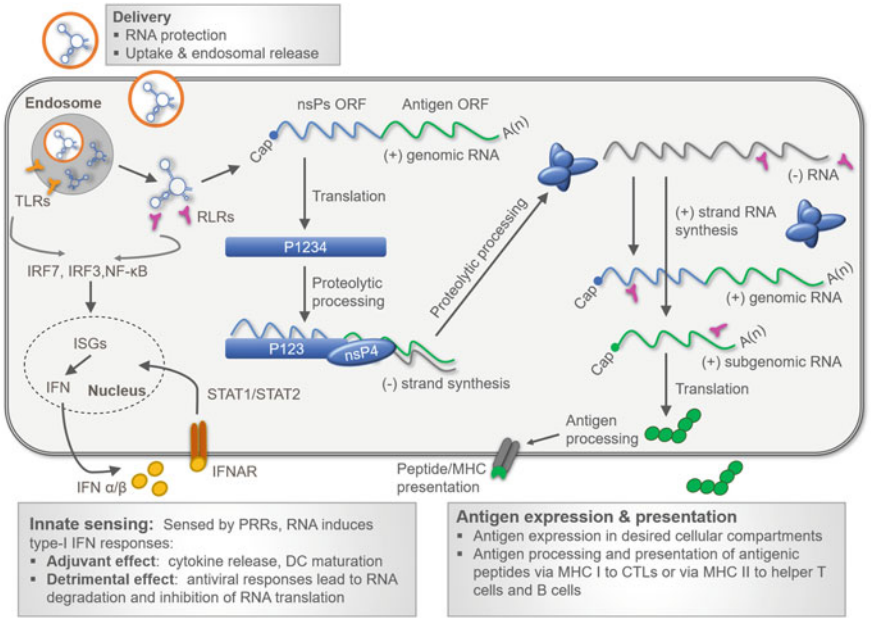

Fig. 3 Proposed mechanism of self-amplification and expression of synthetic self-amplifying mRNA vaccines. Upon cellular uptake of the self-amplifying mRNA encapsulated in the synthetic delivery system, the genomic positive-stranded self-amplifying mRNA is released in the cytoplasm and the nonstructural proteins (nsPs) are translated as a polyprotein. After cleavage of nsP4, the replicase complex synthesizes negative stranded RNA from the genomic RNA. Further proteolytic processing of all nsPs to individual proteins switches synthesis to both genomic RNA and positive stranded subgenomic RNA that encodes the antigen. Both incoming and intermediate RNAs during self-amplification are almost immediately sensed by pattern recognition receptors (PRRs) in the endosome and in the cytoplasm. This activate type I IFN response, which, depending the magnitude and kinetics, could adjuvant the vaccine response or negatively impact mRNA translation and T cell activation. Expressing antigenic proteins in situ within the cell allows processing and presentation of antigenic peptides via the intracellular MHC I pathway, or via the MHC II pathway, resulting in generation of antigen specific T and B cells. ORF, open reading frame; nsPs, nonstructural proteins; P, polyprotein; TLRs, toll-like receptors; RLRs, RIG-I-like receptors

appears to be linked to several important interactions with host proteins (reviewed in Lark et al. 2017; Gotte et al. 2018).

Similar to parental alphaviruses, self-amplifying mRNA replicons are expected to generate a very high copy number of genomic and subgenomic RNAs (up to 1×10^5 copies per cell), upon delivery in the cytoplasm of target cells (Lundstrom 2005). Full-length self-amplifying mRNAs can be readily produced by IVT from a pDNA template, with low batch-to-batch variability, using processes previously reviewed (Maruggi 2019; Brito 2015).

Replicons can be delivered as engineered viral particles when structural genes are provided *in trans* or as RNA formulated in synthetic delivery carriers. They have shown promise as vaccine vectors against infectious diseases, as vectors for

expression of therapeutic agents in cancer immunotherapy and of monoclonal antibodies against infectious diseases, or as a tool for high-sustained transient expression systems to reprogram cells (Lundstrom 2016b, 2003; Geall 2012; Brito 2015; Kim 2017; Yoshioka and Dowdy 2017; Miyagi-Shiohira 2018; Bernstein 2009; Wecker 2012; Morse 2010; Erasmus et al. 2020c). The attributes and efficacy of virally delivered self-amplifying mRNAs as prophylactic and therapeutic vaccines in various preclinical models and humans have been reviewed elsewhere (Lundstrom 2016a, 2018, 2019; Singh 2019) and are not the focus of this review, but provided the basis for the development of synthetic means to deliver replicon vaccines.

Non-viral delivery of synthetic self-amplifying mRNA is particularly attractive over viral vectored vaccines for their simple, flexible, rapid, and generic manufacturing processes, while maintaining the advantages of nucleic acid-based vaccines, namely the ability to enlist the human body to express the target antigen in the cell, but unlike plasmid DNA vaccines, without the need to cross the nuclear membrane barrier for protein expression. By expressing the proteins in situ, self-amplifying mRNA can achieve the production of antigens with proper folding and post-translational modifications (e.g., glycosylation), which plays an important role in eliciting appropriate immune responses to pathogens and in vaccine efficacy (Goddard-Borger and Boddey 2018; Wei 2010; Wu 2017; Zost 2017). Potentially any type of protein that can be properly expressed in eukaryotic cells, including cytosolic, intramitochondrial, transmembrane, and secreted proteins, can be encoded. Once the mRNA-encoded protein is produced, its subcellular localization is determined by signal peptides, which can direct the protein to specific compartments within the cell or targeted processing pathways (Maruggi 2017; Lundstrom 2003; Melo 2019). Finally, the protein sequence of the antigen encoded by the subgenomic RNA can be engineered to impact protein stability, half-life, and/or enzymatic activity with resulting modulation of its expression and duration (Farelli 2018). Codon optimization of the antigen and RNA secondary structure prediction tools, for example, can be used to engage ribosomes with high affinity and improve translation (Bell 2016; Zuber 2018; Latanova 2018; Mauger 2019). By directly producing the antigen in the human body, self-amplifying mRNA mimics a viral infection, resulting in potent humoral and cellular immune responses.

2.2 *Main Characteristics of Synthetic Self-Amplifying mRNA Vaccines*

Self-amplifying mRNA vaccines have several attractive features compared to conventional non-amplifying mRNA vaccines, including (1) increased magnitude and duration of expression resulting in a lower effective vaccine dose, (2) more robust intrinsic immune-stimulatory properties due to high levels of several ssRNA and dsRNA species formed during the amplification process, and (3) the possibility

to generate multivalent or complex vaccines by encoding multiple antigens in a single replicon molecule (Vogel 2018; Pepini 2017; Zhong 2019; Brito 2015; Magini 2016).

The first evidence that self-amplifying IVT mRNA expresses higher levels of antigens in vivo than conventional non-amplifying mRNA was reported by Johanning and coworkers in 1995 (Johanning 1995), one year after Liljestrom and his team had shown the potential for a naked, SFV-based mRNA to induce humoral responses in mice (Zhou 1994). Johanning et al. showed that a naked SIN-derived self-amplifying mRNA, administered by an intraglossal injection, expressed 24-fold more luciferase protein compared to an equal mass of conventional mRNA. Conventional mRNA-driven expression peaked at 8 h post-injection and returned to baseline within 3 days. In contrast, a single dose of self-amplifying mRNA transcripts generated luciferase activity peaking at 24 h post-injection and persisting for at least 7 days. The differential kinetics of antigen expression after mRNA vaccine administration were recently substantiated by Niek Sanders and co-workers. They compared luciferase expression of naked VEEV derived self-amplifying mRNA, N1-methylpseudouridine (m1Ψ) modified and unmodified conventional mRNAs after intradermal (i.d.) electroporation in pigs and mice (Leyman 2018; Huysmans 2019). In pigs, both forms of conventional mRNAs reached their peak expression at day 1, followed by a 2.5- to 5- fold decrease at day 6. Self-amplifying mRNA-driven expression was comparable to that of conventional mRNA at day 1 and maintained its high expression levels for at least 12 days. In mice, the expression of self-amplifying mRNA sharply increased during the first 24 h and, subsequently, steadily increased until the maximal expression levels were reached 8–10 days. In contrast, expression levels from both m1Ψ-modified and unmodified conventional mRNAs had almost reached background levels at 10 days post-administration. Calculation of the area under the curve over a 4-week period post-administration showed a significantly higher protein expression (≥ 1 log depending on dose) after self-amplifying mRNA injection compared to m1Ψ-modified and unmodified conventional mRNA. The m1Ψ modification induced more protein expression compared to the non-modified variant but less than self-amplifying mRNA.

Encapsulation of self-amplifying mRNA in non-viral delivery systems, such as cationic nanoemulsions (CNE) and lipid nanoparticles (LNPs), further improves the magnitude and persistence of the expressed antigen. RNA formulation with both LNP and CNE has been shown to enable expression up to six weeks after intramuscular (i.m.) administration in mice and for more than 14 days in non-human primates (NHPs) (Geall 2012; Brito 2014; Pepini 2017). Likewise, i.d. administration of LNP formulated self-amplifying mRNA results in a more rapid (24 h) and higher initial expression than electroporation in mice (Huysmans 2019). Interestingly, Blakney et al. quantified luciferase expression after i.d. injection of an LNP formulated VEEV derived self-amplifying mRNA in human skin explants, reporting that a signal was readily visible after 24 h, peaked at day 11, and persisted for at least 21 days (Blakney et al. 2019a). Importantly, Vogel and colleagues subsequently showed that while both self-amplifying mRNA and conventional

mRNA protected against infection with the homologous virus in an influenza challenge mouse model, 64-fold less self-amplifying mRNA dose was required (Vogel 2018). On a per gene basis, the self-amplifying mRNA dose was even lower considering that self-amplifying mRNA is much larger than mRNA (9.3 vs. 2.2 kb in this study). Recently, the quality of immune responses elicited by a single administration of LNP formulated self-amplifying mRNA and nucleoside-modified conventional mRNA expressing SARS-CoV-2 spike antigen was measured in mice (de Alwis et al. 2020). The self-amplifying mRNA vaccine-elicited significantly greater antigen-specific binding, neutralizing antibody, CD8+ T cell, and Th1 skewed responses than the conventional mRNA administered at comparable RNA doses. Interestingly, the highest tested dose of conventional mRNA (10 μg) induced comparable spike protein-specific antibodies as the lowest tested dose of self-amplifying mRNA (0.2 μg), but with lower avidity and neutralization activity. A single dose of the self-amplifying mRNA vaccine provided complete protection of hACE2 mice from a lethal SARS-CoV-2 challenge at a dose as low as 2 μg of formulated RNA. This SARS-CoV-2 self-amplifying mRNA is currently being tested in a Ph1/2 clinical trial in a single and prime/boost regimen (ClinicalTrials.gov: NCT04480957). Preliminary data show tolerability, SARS-CoV-2 spike specific antibodies, CD8+ and Th1 dominant cell responses in subjects receiving 7.5 μg single dose and 5 μg prime-boost regimens (Arcturus Therapeutics 2020). A phase 2 study has been recently approved based on these favorable results and will evaluate the safety and immunogenicity of both single 7.5 μg dose and two 5 μg dose priming in older and younger adults (ClinicalTrials.gov: NCT04668339) (Arcturus Therapeutics 2021).

The dose sparing quality of self-amplifying mRNA vaccines may be attributed both to the RNA amplification in the host cell and to the activation of adjuvanting immune stimuli. dsRNA intermediates are formed during the self-amplifying mRNA translation and amplification cycle. By mimicking viral genome and replication intermediates, both ssRNA and dsRNA molecules act as pathogen-associated molecular patterns (PAMPs) that are sensed by cytosolic or endosomal pattern-recognition receptors (PRRs), inducing robust type I IFN production and innate immune activation (reviewed in Chen 2017; Mesev et al. 2019). The role of type I IFN responses on the vaccine potency will be described in detail later.

The size of self-amplifying mRNA replicons creates challenges in the production process, stability, and internalization efficiency, requiring optimization. The success of RNA vaccines will depend primarily on the quality of the transcript. Known inhibitors of potency include process-derived double-stranded RNA species, aborted transcripts, as well as other degradants and contaminants that can trigger an innate inflammatory response and therefore need to be removed during the purification steps (Kariko 2011; Nelson 2020; Milligan 1987; Triana-Alonso 1995). Self-amplifying mRNA molecules are at least 10 kb in length and much larger than their conventional mRNA counterparts, for which purification methods have been established (Kariko 2011; Weissman 2013; Foster 2018; Baiersdorfer 2019). These long molecules offer additional challenges, due to size exclusion effects and poor

recovery. Available large-scale chromatographic purification, while effective in the purification of RNA molecules of 4–5 kb, is not yet an option for large RNA. Most of the preclinical in vivo data reported with self-amplifying mRNA vaccines utilized lithium chloride precipitation and ethanol wash or silica column as the purification method of IVT synthesized RNA, which are not compatible with Good Manufacturing Practice (GMP) production and do not eliminate shorter RNA contaminants. For the production of clinical-grade material, downstream purification of IVT RNA from reaction enzymes can be achieved via a series of tangential flow filtration steps, which can also be complemented by chromatographic purification techniques for purification of longer RNA species, although the level of enrichment in full-length self-amplifying mRNA molecules is not yet clear (Kis 2020). Hence, improved RNA purification methods that can enable cost and time-efficient purification of large RNAs at an industrial scale with high yield and pharmaceutical grade purity are needed.

Stability of the vaccine can also influence its potency, global distribution, and availability, with most RNA vaccine formulations requiring a cold chain infrastructure (Crommelin et al. 2020). Lyophilized or thermostable formulations have the potential to improve stability, scalability, supply chain logistics, and reduce cost per dose. Tailored assays are also needed to determine additional quality attributes of these long RNA molecules, such as stability, integrity, identity, purity and, homogeneity, and to identify the preferred product characteristics during in-process, release, and stability evaluation of the vaccine targets (Crommelin et al. 2020; Poveda 2019).

Polycistronic or co-delivered replicons are one of the key attributes of the self-amplifying mRNA technology that could potentially enable the development of multi-valent or combination vaccines from a single replicon RNA to simultaneously target multiple pathogens or to express multi-subunit complex antigens. In contrast to virally delivered nucleic acid-based vaccines, synthetically delivered self-amplifying mRNA vaccines are theoretically not limited by packaging constraints, offering a high degree of versatility in the type and number of proteins that can be encoded, although the true limit of their coding capacity has not yet been elucidated. Geall and co-workers reported that an LNP formulated self-amplifying mRNA co-expressing two antigens, either gH and gL from cytomegalovirus (CMV) or M1 and NP from influenza virus, effectively induces antibodies and CD4 + and CD8+ T cells against both antigens (Brito 2015; Magini 2016). The elicited immune responses were at levels comparable to those induced by an MF59-adjuvanted gH/gL protein complex (CMV) and sufficient to protect from an influenza virus challenge, after vaccination of mice with a low dose of RNA (1 and 0.1 μgrespectively). While polycistronic self-amplifying mRNAs might be useful to deliver multi-subunit antigens where co-expression in the same cell is critical for the generation of properly assembled protein complexes, co-delivery of multiple replicons might be an equally effective strategy to deliver multiple antigens that are not needed to form a complex. This approach is particularly useful for the generation of a cross-protective influenza single-vector vaccine able to induce both T cell- and B cell-mediated immunity, as shown by Démoulins et al. by using

polyethylenimine (PEI)-based polyplexes to co-formulate two classic swine fever virus-derived self-amplifying mRNA vaccines encoding influenza HA and NP, respectively (Demoulins 2016). Chahal and co-workers also showed that up to six VEEV-derived self-amplifying mRNA replicons are effective against lethal *Toxoplasma gondii* challenge when co-formulated in multiplexed dendrimer nanoparticles (Chahal 2016). An in-depth characterization of the quality, magnitude, and kinetics of T cell responses after vaccination with multiple self-amplifying mRNA vaccines, administered alone or in combination, is needed to understand the broad utility of this approach. Moyo and co-workers have recently reported such immune profiling after vaccination with two polymer formulated replicons (Moyo 2019).

Heterologous prime-boost regimens utilizing different vaccine technologies might be particularly powerful for induction of robust and durable humoral and T cell responses against difficult targets, such as complex infectious diseases and cancer (reviewed in Kardani et al. 2016; Excler and Kim 2019). Heterologous self-amplifying mRNA prime and adjuvanted recombinant protein boost have been shown to be effective in eliciting protective antigen-specific antibodies and T cell responses against intracellular pathogens, such as bacteria and parasites in mice, as well as against viruses in NHPs (Maruggi 2017; Duthie 2018; Bogers 2015). Heterologous prime-boost utilizing synthetic or conventional nucleic acid-based vaccines is attractive when strong and lasting T cell responses are needed, and this concept is currently being explored in two Phase 1/2 clinical studies. These studies are evaluating the safety, immunogenicity, and early clinical activity of a Chimpanzee adenovirus vector prime and nanoparticle-formulated self-amplifying mRNA boost encoding neoantigens, in combination with immune checkpoint inhibitors, for the treatment of patients with advanced solid tumors (ClinicalTrials.gov: NCT03639714 and NCT03953235) (2019). Preliminary data show tolerability of up to 300 μg of the self-amplifying mRNA vaccine boost in the prime/boost immunotherapy regimen and robust induction of neo-antigen-specific CD8+ T cells, expansion of those cells in the tumor microenvironment, and early evidence of clinical benefit (Drake et al. 2020; Webcast on GRANITE and SLATE 2020). Based on these promising Phase 1 data, the highest doses of the self-amplifying mRNA boosting vaccine have been advanced in Phase 2 to investigate the ability of this immunotherapy regimen to elicit robust immune responses against cancer (Gritstone oncology 2020). Results from this study will provide important information on the safety and potency of the heterologous prime-boost regimen and of repeated administration of increasing doses of LNP formulated self-amplifying mRNA. Given the promise of the mRNA technology, considerable preclinical research, summarized in Table 1 and reviewed in (Maruggi 2019; Bloom et al. 2020), has been conducted and shown induction of strong and protective immune responses elicited by self-amplifying mRNA against multiple infectious diseases in various preclinical models, including RSV in mice and cotton rats, HIV-1, HCMV, Influenza, HCV, Rabies, Ebola, Zika, VEEV, SARS-CoV-2, malaria and *Toxoplasma gondii* in mice, HIV-1 in rabbits, HIV-1, HCMV, and SARS-CoV-2 in NHPs (Hekele 2013; Geall 2012; Brito 2014; Erasmus 2018;

Table 1 Overview of preclinical in vivo studies using synthetic self-amplifying mRNA vaccines against infectious diseases[a]

Pathogen	Antigen	Delivery system	Route	Preclinical model	Elicited responses	References
Influenza virus	HA	CNE, LNP, MDNP, MLNP, LPP, naked, pABOL	i.m., i.d.	Mice Ferrets	Humoral, cellular, protection	Hekele (2013), Vogel (2018), Chahal (2016), Brazzoli (2015), Perche (2019), Blakney et al. (2020d), Beissert (2019), Goswami (2019)
	NP, M1	LNP	i.m.	Mice	Humoral, cellular, protection	Magini (2016)
	NP, GM-CSF	CNE	i.m.	Mice	Humoral, cellular, protection	Manara (2019)
	NP	LNP, naked	i.m.	Mice	Humoral, cellular	Zhou (1994), Lazzaro (2015)
	HA, NP	PEI, Chitosan	s.c.	Mice Rabbits	Humoral, cellular	Demoulins (2016), McCullough (2014)
HIV	gp140	CNE, naked, LNP	i.m.	Mice Rabbits NHPs	Humoral, cellular	Brito (2014), Bogers (2015), Cu (2013), Blakney et al. (2019b)
	gp120 eOD-GT8	LNP	i.m.	Mice	Humoral, cellular	Melo (2019)
	Gag-Pol mosaic antigens	PEI	i.m.	Mice	Cellular	Moyo (2019)
HCMV	gB, pp65-IE1	CNE	i.m.	NHPs	Humoral, cellular	Brito (2014)
	gH/gL	LNP	i.m.	Mice	Humoral	Brito (2015)

(continued)

Table 1 (continued)

Pathogen	Antigen	Delivery system	Route	Preclinical model	Elicited responses	References
VEEV	TC-83 VEEV	CNE	i.m.	Mice	Humoral, protection	Samsa (2019)
RSV	F	CNE, LNP	i.m.	Mice Cotton rats	Humoral, cellular, protection	Geall (2012), Brito (2014)
Rabies virus	GP	LNP, pABOL, CNE	i.m.	Mice Rabbits Rats	Humoral safety Biodistribution	Brito (2015), Stokes (2020), Blakney et al. (2020b)
Louping ill virus, RSV, Influenza virus	prM-E, F, HA	Naked	i.m.	Mice	Humoral, cellular, protection (partial)	Fleeton (2001)
Ebola virus	GP	MDNP,	i.m.	Mice	Humoral, cellular, protection	Chahal (2016)
Zika Virus	prM-E	MDNP, NLC Naked, CNE	i.m., i.d.	Mice Guinea pigs, NHPs	Humoral, cellular, protection	Erasmus (2018), Zhong (2019), Chahal (2017), Luisi (2020)
	NS3, prM-E	LNP	i.m.	Mice	Cellular, humoral protection	Elong Ngono (2020)
SARS-CoV-2	Spike	LNP, LION	i.m.	Mice NHPs	Humoral, cellular, protection	de Alwis et al. (2020), Erasmus et al. (2020a, b), McKay (2020)
	SLOdm,	CNE	i.m.	Mice		Maruggi (2017)

(continued)

Table 1 (continued)

Pathogen	Antigen	Delivery system	Route	Preclinical model	Elicited responses	References
Streptococci (A and B)	BP-2a				Humoral, protection	
Malaria	PMIF	CNE	i.m.	Mice	Humoral, cellular, protection	Baeza Garcia (2018)
L. donovani	F2, F3+	Naked	i.m.	Mice	Humoral, cellular protection	Duthie (2018)
Toxoplasma gondii	Six antigens[b]	MDNP	i.m.	Mice	Protection	Chahal (2016)
Chlamydia trachomatis	MOMP	CAFs	i.m.	Mice	Humoral, cellular	Blakney et al. (2019c)

[a]Abbreviations: *BP2a* Group B Streptococcus pilus 2a backbone protein; *CAFs* cationic adjuvant formulations; *CNE* cationic nanoemulsion; *F* fusion protein; *gB* glycoprotein B; *GM-CSF* granulocyte–macrophage colony-stimulating factor; *GP* glycoprotein G; *gp120 eOD-GT8* glycoprotein 120 germline-targeting engineered outer domain 60-mer; *HA* hemagglutinin; *HCMV* human cytomegalovirus; *HIV* human immunodeficient virus; *i.d.* intradermal; *i.m.* intramuscular; *i.v.*intravenous; *L. donovani* Leishmania donovani; *LION* Lipid InOrganic Nanoparticle; *LNP* lipid nanoparticle; *LPP* neutral lipopolyplexes; *NP* nucleoprotein; *M1* matrix protein 1; *MDNP* modified dendrimer nanoparticle; *MLNP* mannosylated LNP; *MOMP* outer membrane protein; *NHPs* nonhuman Primates; *NLC* nanostructured lipid carrier; *NS3* nonstructural protein 3; *pABOL* cationic poly(CBA-co-4-amino-1-butanol) polymer; *PC* pentameric complex; *PEI* polyethylenimine; *PMIF* plasmodium macrophage migration inhibitory factor; *prM-E* pre-membrane and envelope; *RSV* respiratory syncytial virus; *SLOdm* double-mutated Group A Streptococcus Streptolysin-O; *s.c.* subcutaneous; *VEEV* Venezuelan equine encephalitic virus

[b]AMA1, GRA6, ROP2A, ROP18, SAG1, and SAG2A from Toxoplasma gondii

Englezou 2018; Zhong 2019; Brito 2015; Melo 2019; Magini 2016; Moyo 2019; Bogers 2015; Brazzoli 2015; Baeza Garcia 2018; Samsa 2019; Chahal 2016; Chahal 2017; Erasmus et al. 2020b; Erasmus et al. 2020a; McKay 2020; Cu 2013; Stokes 2020; Blakney 2019b; Elong Ngono 2020; Luisi 2020). Different delivery systems, including CNE (Brito 2014), cationic lipids as part of LNP (Geall 2012; Englezou 2018; Erasmus et al. 2020b), PEI based polyplexes (Demoulins 2017), chitosan nanoparticles (McCullough 2014), neutral lipopolyplexes (Perche 2019), nanostructured lipid carriers (Erasmus 2018) and cationic polymers (Blakney et al. 2020d), have been tested, demonstrating an effective dose as low as 0.01 μg (Erasmus 2018). In parallel, the use of a dendrimer-based formulation has demonstrated the utility of vaccinating with multiple SAM constructs (Chahal 2016). Although the efficacy of synthetically delivered self-amplifying mRNA vaccines in humans is not available yet, the prospects are encouraging. A phase 1 clinical trial has been recently initiated to evaluate the safety, reactogenicity, and immunogenicity of a CNE formulated rabies glycoprotein G self-amplifying mRNA vaccine (ClinicalTrials.gov: NCT04062669) (GSK 2019). In response to the global SARS-CoV-2 pandemic, two different LNP formulated self-amplifying mRNAs expressing the spike antigen are currently being tested in humans in a phase 1/2 clinical trial (ISRCTN Register: ISRCTN17072692), assessing safety and immunogenicity of 0.1, 0.3, 1 μg dose administered twice, and in Phase 1/2 and Phase 2 clinical study (ClinicalTrials.gov: NCT04480957, NCT04668339), investigating safety and immunogenicity of a single and prime/boost immunizations regimens at 7.5 μg and 5 μg RNA doses respectively (Arcturus Therapeutics 2021; ISRCTN registry 2020). Results from these studies will provide important insights into the potency and safety of this technology.

In summary, self-amplifying mRNA is a versatile technology supported by extensive preclinical data and the clinical proof of concept is ongoing. It can be used to develop single- or multi-antigen vaccines, with the benefit of low effective dosage and of the versatility to be combined with other vaccine technologies.

3 Decoding the Balance Between Innate and Adaptive Immune Responses for Potent Self-amplifying mRNA Vaccines

The effectiveness of any vaccine is thought to be governed by the early interplay between the vaccine and innate immune cells, resulting in an immuno-stimulatory effect that can increase the magnitude and breadth of the subsequent antigen-specific adaptive immune response (Coffman et al. 2010). Efficient induction of immune responses by self-amplifying mRNA vaccination is dependent upon delivery into the cytoplasm (most likely through endosomal escape), cytosolic

amplification, host interaction, and expression of the antigen-encoding mRNA (Fig. 3). The nature of the delivery system and the route of administration also play an important role in determining the quantity and quality of local gene expression and innate immune stimulation, thus providing a synergistic adjuvant effect (Pepini 2017; Liang 2017; Kanasty 2012).

Upon entry into the cytosol, self-amplifying mRNA utilizes the host machinery for translation of the replicase complex, which generates several ssRNA and dsRNA intermediate species. These RNA intermediate species, together with the incoming RNA, are rapidly sensed by a variety of PRRs, which have evolved to detect foreign nucleic acids as the first line of defense. PRRs are present in different cell types and are particularly highly expressed in sentinel antigen-presenting cells (APCs). Through a complex cascade of intracellular signaling pathways, they regulate the differential expression of proinflammatory cytokines and chemokines. Two major classes of PRRs have been described to recognize mRNA: endosomal Toll-like receptors (TLRs), such as TLR3, TLR7, and TLR8, and cytosolic RIG-I-like receptors (RLRs), such as RIG-I,MDA-5, LGP2, PKR, and OAS (Chen 2017; Akira et al. 2006; Pippig 2009). The members of the TLR family are localized in the endosomal compartment of professional immune surveillance cells, such as dendritic cells (DCs), macrophages, and monocytes to recognize both ssRNA (TLR7 and TLR8) and dsRNA (TLR3) (Judge 2005; Ablasser 2009). TLR7 activation, in particular, can increase antigen presentation, promote cytokine secretion, and stimulate B cell responses (Hua and Hou 2013). RIG-I and MDA-5 recognize 5′-triphosphate short dsRNA and long dsRNA, respectively (Hornung 2006; Kato 2008, 2006). The activation of the majority of PRRs results in the expression and secretion of IFNs, which mediate pleiotropic and pro-inflammatory effects. The canonical pathway for IFN α/β signaling involves interaction with the IFN α/β receptor (IFNAR), activation of Jak1 and Tyk2 kinases, and phosphorylation and heterodimerization of STAT1 and STAT2 transcription factors. STAT1/2 heterodimers associate with IRF9, forming the ISGF3 complex, translocate to the nucleus, and bind to IFN-stimulated response elements, resulting in the upregulation of IFN-stimulated genes (ISGs). STAT1-independent IFN-α/β signaling and effects have also been documented (Gil 2001). Together, this results in an antiviral response to promote DC maturation and migration, antigen processing and presentation, T cell activation, and B cell modulation, all of which can enhance RNA vaccine efficacy. However, on the other hand, the IFN response can also lead to inhibition of cellular translation, RNA degradation, activation of pro-apoptotic pathways, and T cell exhaustion, which can also negatively impact the launch of the mRNA replicon and mRNA vaccine efficacy (reviewed in Boxel-Dezaire 2006). Therefore, the design of the mRNA sequence and delivery system should ideally achieve the proper balance to activate adequately the innate immune system while limiting its deleterious effects.

3.1 *Mechanistic Insights from Alphaviruses: Immune Recognition and Evasion of IFN-Mediated Defenses*

Among hundreds of ISGs, only a few have had their antiviral functions clearly defined, particularly for alphaviruses whose genomes were commonly used to generate self-amplifying mRNA vaccines (Zhang 2007; Karki 2012). During natural infection, IFN signaling does not interfere with attachment and entry of alphavirus virions into the cell or nucleocapsid disassembly, but it can inhibit translation and replication of viral RNAs, through IFIT1- dependent and independent mechanisms (Stokes 2020). Both PKR-dependent and independent pathways control alphavirus infection as well, highlighting highly redundant mechanisms (Zhang 2007; Atasheva et al. 2014; Reynaud 2015; Ryman 2005; Gorchakov 2004). This presents the challenge in delineating these mechanisms as in vitro results do not always correlate with the effect of each of these factors in alphavirus infection in mice (Zhang 2007; Atasheva et al. 2014; Reynaud 2015; Ryman 2005; Gorchakov 2004). Pepini et al. showed that the loss of TLR7 or MyD88 abrogates cell ability to respond to synthetic self-amplifying mRNA in culture but the loss of these functional PRRs did not result in any apparent changes in reporter protein expression in mice (Pepini 2017). Duthie et al. also observed no changes in total cell numbers within draining lymph nodes, as a readout of local immune activation, between wild type and TLR7–/– mice vaccinated with naked self-amplifying mRNA (Duthie 2018). Thus, given that an RNA molecule can activate multiple PRRs, the loss of a single nucleic acid sensor may not have a profound impact on antigen expression in vivo. Moreover, innate immune activation by mRNA molecules is also dependent on the route of administration, delivery system, and host species, hampering the interpretation of in vitro data. The development of robust animal models that can delineate the interaction between mRNA and innate immunity would be useful.

Inhibition of the IFN response is essential for alphaviruses to establish productive infections in mammalian hosts (Gardner 2010; Schilte 2010; White 2001). During infection, alphaviruses can suppress the induction of innate immune responses through multiple mechanisms, such as blocking host cell transcription and translation (Yin 2009; Trobaugh and Klimstra 2017). nsP2, for example, is a pleiotropic protein governing viral RNA replication, cytopathicity, and inhibition of interferon signaling in both the Old World and New World alphaviruses, but through different mechanisms (Garmashova 2007; Fros 2010, 2013; Frolova 2002; Breakwell 2007; Bhalla 2016; Simmons 2009; Fros and Pijlman 2016). SINV inhibits the expression of IFNs and ISGs through transcriptional host cell shut-off, while Chikungunya virus (CHIKV) or VEEV acts by combining early host translation shut-off and specific nsP2-mediated inhibition of JAK-STAT signaling. Alphaviruses can also reduce innate sensing by the induction of the unfolded protein response and the formation of stress granules, where various PRRs, including RIG-I and MDA-5, reside (Smith 2014; Fros 2015; White and Lloyd 2012; Varjak et al. 2010; Law 2019). During infection with some alphaviruses, the

activation of PRRs is also delayed likely due to sequestration of virus replicative structures into the immune-privileged side of membrane-bound vesicles to prevent their detection (Frolova 2010). Synthetically delivered self-amplifying mRNA might use similar mechanisms during amplification in the target cells while also being sensed in the endosome upon synthetic delivery. Furthermore, different alphaviruses have different levels of resistance to an IFNα/β induced, pre-established antiviral state, for example, with VEEV being more resistant than SINV (Yin 2009; Ryman and Klimstra 2008).

Alphaviruses can also escape innate recognition by evading the ISG protein IFIT1 through IFIT1 binding-resistant stem-loop secondary structures in the 5′UTR. IFIT1 binds to viral RNA cap structures lacking 2′-O methylation, which prevents the interaction of eukaryotic translation initiation factor 4E (eIF4E) with the cap structure (Habjan 2013; Kumar 2014). To overcome IFIT1 restriction of non-2′-O-methylated viral mRNA, many cytoplasmic viruses generate Cap 1 structures on their mRNA by encoding their own viral 2′-O-methyltransferases (e.g., flaviviruses, coronaviruses, poxviruses), stealing caps from host mRNA (e.g., orthomyxoviruses and bunyaviruses), or utilizing cap-independent translation (e.g., picornaviruses, caliciviruses, and hepatitis C virus) (reviewed in Diamond 2014). Alphaviruses lack 2′-O methylation at the 5′ UTR of their genomic and subgenomic RNAs, thus being susceptible to inhibition by IFIT1 (Dubin 1977). Despite this, some alphaviruses, including CHIKV, SINV, SFV, and VEEV can replicate more efficiently than other alphaviruses in cells producing IFIT upon stimulation with IFN (Reynaud 2015; Diamond and Farzan 2013). Recent studies indicate that the secondary structure of the alphavirus 5′UTR antagonizes the antiviral function of IFIT1 by inhibiting its binding to viral RNA (Hyde 2014). Mutations destabilizing the thermodynamic stability of the 5′ terminal stem-loop structure render VEEV and SINV mutants more susceptible to IFIT1 restriction. Collectively, these mechanisms highlight the diversity within alphaviruses, even though they all fall within a single genus in the *Togaviridae* family and can be potentially explored for synthetically delivered self-amplifying mRNA vaccines. It also remains unknown whether synthetically delivered self-amplifying mRNA replicons follow the same host interaction mechanisms as the parental viruses from which they are derived. Dissecting the interactions between the alphavirus genome of choice and the host is critical for the rational design of a potent self-amplifying mRNA vaccine.

3.2 Innate Immune Response to Synthetic Self-amplifying mRNA Vaccines

A systematic analysis of host responses after alphavirus infection and alphavirus replicon-based vaccination, either delivered as viral or synthetic particles, could elucidate the role of the delivery system and of the self-amplifying mRNA and guide future vaccine development. Host transcriptional responses to VEEV have

been reported in a few animal model systems (e.g., mice and NHPs after aerosol infection), in one in vitro study of human PBMC cells, and in unfractionated whole human blood of patients who were immunized subcutaneously (s.c.) with the live-attenuated TC-83 vaccine strain of VEEV (Erwin-Cohen 2012, 2017). For example, in PBMCs from patients infected with TC-83, transcription of chemokines (e.g., CXCL11, CCL3, CCL5, CCL7, and CCRL2) and a strong IFN-driven response (represented by transcription of IFN-β1, IFN-γ, IRF7, OAS, TLR3, TLR7, MX1, MX2, and STAT1) were induced. When comparing the transcriptomes of naïve individuals to those of individuals who received TC-83, Erwin-Cohen et al. showed significant transcriptional changes at days 2, 7, and 14 following vaccination. The top canonical pathways revealed at early and intermediate time points (days 2 and 7) included activation of PRRs, classic interferon response, and engagement of the inflammasome. By day 14, the top canonical pathways included oxidative phosphorylation, the protein ubiquitination pathway, natural killer cell signaling, and B and T cell development.

Similar to TC83 infection, i.m. LNP delivery of self-amplifying mRNA in mice also induces a strong local proinflammatory response with activation of innate immune, antiviral, and inflammatory signaling pathways (Pepini 2017). Within a few hours at the site of injection, RIG-I, MDA-5, and TLR7, but not TRL3, were up-regulated and an early and robust induction of type I IFN andIFN-stimulated responses were detected. Increase in gene expression of T cell markers CD8α and CD3ε was observed in the muscle by day 7, suggesting the presence of antigen-specific T cells infiltrating the site of injection. Genes in the type I IFN pathway were the most highly expressed in whole blood collected 1-day post-administration in mice inoculated with an LNP encapsulated SARS-CoV-2 Spike self-amplifying mRNA compared to conventional mRNA vaccine (de Alwis 2020). Similarly, in pigs vaccinated by i.d. electroporation of a naked VEEV based self-amplifying mRNA, innate immune markers, including genes involved in signaling downstream of TLRs, NOD-like receptors, RIG-1, and type I IFN signaling, were also up-regulated at the injection site 24 h after vaccination (Leyman 2018). Interestingly, in the pig model, self-amplifying mRNA triggered up-regulation of a panel of innate immune responsive genes at levels comparable to those stimulated by m1Ψ non-amplifying mRNA, which was lower than those by unmodified mRNA. Using IFN-β luciferase reporter mice, the same team compared the innate immune responses after i.d. electroporation and LNP delivery of a VEEV based self-amplifying mRNA, benchmarking them against pDNA, unmodified, or m1Ψ-modified non-amplifying mRNA (Huysmans 2019). All mRNA vaccines induced a clear IFN response (represented by IFN-β) in mice, which peaked at 8 h after injection and returned to baseline level 7 days later. Interestingly, the levels of IFN-β response induced by self-amplifying mRNA were not higher than those by either non-amplifying mRNAs despite its intracellular amplification. These data suggest that the induction of the IFN-β response could be the result of incoming, IVT self-amplifying mRNA rather than RNA species generated during amplification.

A complication in the aforementioned studies is possible contamination of the mRNA with dsRNA or short aborted mRNA species, which are known to induce innate immune responses (Kariko 2011). To address the impact of self-amplification on IFN activation, Zhong et al. vaccinated an IFN-β luciferase reporter in transgenic mice using a truncated non-replicative luciferase self-amplifying mRNA by i.d. electroporation (Zhong 2019). A dose-dependent IFN response, peaking at 24 h, was elicited with similar kinetics and magnitude to its amplification competent counterpart (Zhong 2019). Nonetheless, more studies are needed to dissect the contribution of incoming and amplifying mRNA to innate activation after vaccination. Importantly, it is critical to understand if the function of nsPs in antagonizing IFN responses is preserved upon synthetic delivery (Bhalla 2016; Simmons 2009; Carrasco et al. 2018). Intriguingly, i.d. LNP delivery of self-amplifying mRNA elicits a higher and more persistent IFN-β response compared to electroporation, possibly as a result of the LNP stimulatory effect or of the more intracellular delivery of LNP-formulated RNAs.

The innate immune responses triggered by the mRNA vaccine result in the production of chemokines and proinflammatory cytokines at the inoculation site, which can recruit innate immune cells, such as DCs and macrophages, facilitate trafficking of DCs toward the draining lymph nodes, and potentiate adaptive immune responses (Pepini 2017). McKay et al. have shown an RNA dose-dependent increase of IL-6, MIP-1β, RANTEs, IFN-β, and IP-10 in the sera 4 h after injection of mice with an LNP formulated VEEV based self-amplifying mRNA in comparison to the control groups (McKay 2020). Upon i.m. injection of CNE formulated self-amplifying mRNA, antigen expression mostly occurs in myocytes at the inoculation site (Brito 2014). Therefore, the transfection of myocytes may contribute to the magnitude and duration of antigen production, while the activation of cytosolic RNA sensors in these cells may lead to local inflammation and infiltration of cross-presenting DCs. However, it is also possible that other cells in the muscle and draining lymph nodes at the injection site, such as DCs, may internalize self-amplifying mRNA vaccine and express the antigen (Englezou 2018; Tonkin 2012) leading to direct presentation of antigen to lymphocytes. In either case, the launch of a self-amplifying mRNA molecule elicits proinflammatory cytokines, thereby providing an adjuvanting stimulus for induction of humoral and T cellular responses against the encoded antigen. High-resolution imaging studies tracking the spatial and temporal distribution of the delivery system and RNA in vivo, together with genetically engineered animal models, could delineate cytoplasmic delivery, target cells, and mechanism of action at single-cell and tissue levels. New imaging tools have been used to visualize non-amplifying mRNA vaccines at single-cell and tissue levels. This includes the generation of transgenic reporters mouse strains, such as the IFN-β reporter mice and Cre recombinase (mRNA) reporter mice, and the development of fluorescent imaging mRNA probes that can detect mRNA at high resolution by positron emission tomography (PET)-computed tomography (CT) in vivo imaging (Bhosle 2018; Blanchard 2019; Kirschman 2017; Lindsay 2019; Kauffman 2018; Rosigkeit 2018; Jeught 2018).

3.3 *Self-adjuvanting Effect of the Self-amplifying mRNA*

To generate an adequate adaptive immune response against a given vaccine antigen, the antigen must be expressed within or taken up by APCs, enter the endogenous route of antigen processing within the cell cytosol, and the processed peptides must then be loaded onto MHC molecules for presentation to T cells. The hypothesized mechanism for antigen presentation after self-amplifying mRNA vaccination is that T-cell priming is initiated by professional APCs rather than myocytes (Brito 2014; Lazzaro 2015). Cells recruited at the injection site after CNE delivery of self-amplifying mRNA in mice are prevalently monocytes, macrophage, and DCs (Brito 2014; Manara 2019). In mice, co-administration of a self-amplifying mRNA encoding the influenza antigen NP and one encoding an active form of granulocyte–macrophage colony-stimulating factor (GM-CSF), which was used as a model chemoattractant for APCs, induced a transient higher recruitment of DCs and macrophages in the muscle between 3 and 7 days post-administration, compared to co-administration with a self-amplifying mRNA encoding an inactive form of GM-CSF (Manara 2019). The higher recruitment of immune cells was associated with the improved magnitude of antigen-specific CD8+ T cell response in terms of both the frequency and activity in vivo.

Since APCs are not typically found in high numbers within the normal muscle tissue and migrate to the site of injection in response to inflammatory or chemotactic signals, it is hypothesized that the intrinsic adjuvanting effect of self-amplifying mRNA may play a positive role in this respect (Liu 2008). Conversely, the skin is a large, accessible organ containing many APCs, and therefore the mechanism of i.d. vaccination might differ. Profiling of cells expressing a reporter protein in primary human skin explants after i.d. delivery of LNP formulated self-amplifying mRNA has shown that while epithelial cells and fibroblasts were the majority of the resident skin cell population, the resident skin immune cells, including neutrophils, T, B cells and DCs, were found to preferentially uptake and express the RNA (Blakney et al. 2019a). By changing the formulation or the degree of mannosylation, the authors were able to increase expression of the self-amplifying mRNA in epithelial cells or adipocytes in this ex vivo human model, highlighting the interplay between formulation and route of administration for a productive response (Blakney et al. 2020a, c). It will be important to validate these data and the role of skin immune transfected cells in vivo, as these cells are able to migrate to the lymph node and modulate immunogenicity. Together, while more research is needed to determine the types of cells that are transfected and express the vaccine antigen after administration of self-amplifying mRNAs, these data highlight the positive effect of chemokine and proinflammatory cytokine production on vaccine potency.

The innate response elicited by self-amplifying mRNA needs to be appropriately balanced to potentiate adaptive immune responses, as excessive innate activation could have a detrimental effect on the launch of synthetic mRNA, viability of transfected cells, and consequently development of the adaptive immune responses

(Pepini 2017; Zhong 2019; Andries 2013; Chakrabarti et al. 2011; Vattem et al. 2001; Kepp 2010; Beuckelaer et al. 2017; Maruggi 2013). Consistent with previous reports on conventional mRNA, Pepini et al. found that blocking of type 1 IFN signaling via monoclonal antibody treatment or knockout mice resulted in a marked increase in the expression of a secreted alkaline phosphatase reporter protein or antibody titers to a respiratory syncytial virus (RSV) fusion protein encoded by an LNP formulated self-amplifying mRNA. Moreover, Zhong et al. have shown much lower antibody titers and CD8+ T cell responses to Zika antigen after i.d. electroporation of unformulated self-amplifying mRNA Zika vaccine in wild-type mice compared to IFNAR1 knock-out mice (Zhong 2019). However, based on relative magnitudes of luciferase expression and Zika-specific immune response, the authors concluded that the difference in antigen expression may not be the sole reason for the reduced antibody titers in wild-type mice. These data suggest that the vaccine-elicited IFN response might negatively modulate immunogenicity by preventing the development of long-lived antibody-secreting cells, as previously reported for the control of other viral infections (Laidlaw and Cyster 2016; Moseman 2016; Sammicheli 2016; Fallet 2016).

Excessive IFN activation could also negatively impact T cell responses and lessons may be learned from persistent viral infections. For example, chronic activation of the type I IFN pathway leads to the induction of regulatory DCs, deletion of virus-specific B cells, and strongly impaired T cell responses (Moseman 2016; Wilson 2013; Osokine 2014). Although type I IFNs can drive the differentiation of antigen-primed CD8 T cells into cytolytic effector cells, they may also promote T cell exhaustion (Beuckelaer 2016). It has been hypothesized that whether type I IFNs inhibit or stimulate the CD8+ T cell response to mRNA vaccines might depend on the duration, intensity, and relative timing between type I IFN signaling and T cell priming (Beuckelaer et al. 2017).

Many reports have described the potency of mRNA vaccines, based on both self-amplifying and non-amplifying mRNA, in eliciting potent and durable neutralizing antibody responses in preclinical models. Table 1 lists the examples of studies on self-amplifying mRNA vaccines. Long-lived and high-affinity antibody responses are driven by Tfh cells, which represent a subset of CD4+ cells that produce positive signals for B cell survival, proliferation, and differentiation and are required for the formation and maintenance of germinal centers (GCs) in secondary lymphoid organs (Crotty 2011; Suan et al. 2017). GCs are microanatomical sites where B cells that encounter antigens undergo multiple rounds of proliferation, somatic hypermutation, and differentiation into either memory B cells or plasma cells. Therefore, the magnitude, longevity, or quality of antibody responses induced by a vaccine is determined by its ability to induce Tfh cells (Havenar-Daughton et al. 2017). Immunization with a CNE-formulated self-amplifying mRNA vaccine that encodes Plasmodium macrophage migration inhibitory factor (PMIF) was shown to preserve the splenic GC architecture and B cell zonal expansion, which normally disrupted by Plasmodium infection (Baeza Garcia 2018). Compared to a control RNA replicon, PMIF self-amplifying mRNA vaccination led to a 2.5-fold increase in the number of CD4+ Tfh cells in infected mice with higher GC-derived

B cells and anti-Plasmodium antibody titers. Melo and colleagues recently measured GC B cell and Tfh cell responses in secondary lymphoid tissues of mice immunized with an LNP-formulated self-amplifying mRNA vaccine encoding HIV gp120 immunogen or with the equivalent recombinant protein vaccine (Melo 2019). RNA vaccine-elicited similar Tfh cell responses but approximately twice as many antigen-specific B cells compared to the recombinant protein vaccine, possibly because of in vivo self-amplifying mRNA-driven antigen expression. Sustained antigen availability during vaccination is critical to generate high antibody titers, GCs, and Tfh cell responses (Tam 2016; Pauthner et al. 2017; Cirelli and Crotty 2017). As self-amplifying mRNA vaccine drives persistent antigen expression over several weeks in vivo, this corroborates the critical role of antigen expression and suggests that its kinetics and magnitude should be considered for mRNA vaccine optimization and improvement.

Finally, potential interactions between the encoded nsPs and host factors, as well as their immunogenicity, merit additional investigation. Preliminary data generated by Melo et al. show that, with luciferase reporter gene expression, similar peak expression levels were observed post-prime and post-boost, but the duration of expression in the boost phase was shortened by $\sim$10 days (Melo 2019). Despite the shortened antigen expression post-boost observed with luciferase, homologous prime-boost with an LNP formulated self-amplifying mRNA expressing an HIV immunogen resulted in boosting of antigen-specific immune responses, in agreement with previous reports (Geall 2012; Brazzoli 2015). So far, there is no evidence in preclinical models that immune responses against the nsPs interfere with the immune response induced by subsequent booster doses (Uematsu 2012), but additional studies with self-amplifying mRNA vaccines are needed to validate the hypothesis.

4 Recent Advances and Optimization Approaches of Self-amplifying mRNA Vaccines

Recent progress has been made in developing synthetically delivered self-amplifying mRNA and understanding their molecular mechanisms of action, and further advancements in the mRNA rational design, formulation, and administration route are expected to exploit the full potential of this new vaccine technology.

The correct balance between self-amplifying mRNA-encoded antigen expression and adequate immune-stimulation, both of which are of crucial importance to the vaccine outcome, is a key factor. As previously described, although the mRNA-induced type I IFN response can potentiate the immunogenicity of the vaccine, it can also interfere with the encoded antigen expression and possibly even lead to counter-productive immune effects. Insights into the interaction between self-amplifying mRNA vaccines and intracellular innate immune pathways might

represent a key strategy that can guide the efforts to optimize the self-amplifying mRNA molecule and the delivery systems.

Incorporation of modified nucleosides to silence self-amplifying mRNA recognition by innate immune sensors is formally possible and could help delivered vaccine RNA escape from the recognition by endosomal PAMPs. Nonetheless, this seems to be a challenging option, as it could impair mRNA amplification in target cells and any effect would also be lost after the first round of amplification (Erasmus et al. 2020c; Huysmans 2019). To circumvent innate immune activation, lessons could be learned from viruses, as they have developed effective strategies to bypass innate immunity (Schulz and Mossman 2016). Co-expression of virally delivered IFN modulating proteins could be an effective solution for mRNA-based reprogramming approaches (Yoshioka and Dowdy 2017; Steinle 2019). To reduce PRR stimulation and consequent reduction of self-amplifying mRNA translation, Beissert et al. 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, a decoy receptor of vaccinia virus, which binds type I IFNs (Beissert 2017). These data are consistent with previous results showing an increase in translation from a virally delivered alphavirus self-amplifying mRNA co-expressing a dominant-negative PKR or co-delivered with a recombinant vaccinia B18R protein (Kim 2017; Gorchakov 2004). Similarly, Blakney et al. recently tested the impact of cis-encoded virally derived innate inhibiting proteins on antigen expression and immunogenicity of a VEEV derived self-amplifying mRNA (Blakney et al. 2020b). PIV-5 V protein blocks MDA5 and IRF3 and Middle East respiratory syndrome coronavirus ORF4a protein bind to long dsRNA to suppress activation of MDA5 and RIG-I. When these two proteins were co-encoded with a target antigen directly in the self-amplifying mRNA, they enhanced antigen expression in vitro (100- to 500-fold) in IFN competent cells, but not in mice. A tenfold increase in binding and neutralizing antibodies against a Rabies virus antigen encoded in the same RNA was found in rabbits, but not in mice or rats. An enhancement in resident cells in human skin explants and a slight but not statistically significant downregulation of IL-1α, IL-2, and MIP-3α in human PBMCs was observed with these replicons compared to a wild-type self-amplifying mRNA.

Introduction of on/off synthetic RNA circuits or switches might be beneficial for controlled expression of immunomodulators, for which timing of expression could be regulated via small-molecule drug administration to ensure their expression only when needed, and prevent cytotoxicity (Wroblewska 2015; Bell 2015; Wagner 2018; Mc Cafferty 2020). Additionally, antigen expression could be turned on and off as needed after one single vaccination, to enable controlled priming and boosting of the immune responses or IFN response modulation (Bell 2015).

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 in delivery to target various components of the IFN signaling cascade or other innate immune pathways (Kim 2007; Xu 2013; Blakney

et al. 2019c). To increase the effectiveness of a replicon RNA for immunotherapy, Li et al. have employed an in vitro evolution strategy to identify mutations in the nsPs of a VEEV-based self-amplifying mRNA that enhanced the strength and persistence of gene expression from the replicon's subgenome in human and murine cells (Li 2019). By subjecting the replicon to multiple rounds of selection for the highest subgenome expression of a fluorescent reporter protein, the authors generated mutations associated with prolonged and higher payload gene expression. Intramuscular or intratumoral administration of the best-performing and highly expressing mutant replicon in mice showed stronger transgene expression in vivo a week after administration compared to the wild-type replicon.

A different self-amplifying mRNA vaccine approach has recently been developed by Blakney et al. and Beissert et al. from VEEV and SFV, respectively (Blakney et al. 2018; Beissert 2019), which leverages the knowledge on the trans-replicon systems previously developed for mechanistic studies of alphavirus RNA replication or for the production of viral particles (Pushko 1997; Spuul 2011; Utt 2016; Bartholomeeusen 2018). In both cases, a bipartite RNA vector system was generated to encode viral nsPs and the gene of interest on separate RNA molecules, but still exhibit the self-amplification properties of the antigen encoding RNA. Blakney et al. developed a system where nsPs were expressed from the wild-type replicon, while the gene of interest was expressed by either a positive or a negative-strand RNA. Beissert et al. expanded this work to a more efficient bipartite vector system, whereby a trans-replicon was engineered from self-amplifying mRNA by deleting the replicase but carrying key regulatory elements for self-amplification and encoding the vaccine antigen. The replicase activity was provided *in trans* by a second molecule, either by a standard self-amplifying mRNA or a conventional mRNA, optimized for long RNA half-life and high translational efficiency. They showed that *in trans* amplification mediated by replicase activity encoded by conventional mRNA was superior to the one by a self-amplifying mRNA due to its higher translational efficiency and lack of interference with cellular translation. This bipartite vector system was also shown to induce influenza neutralizing antibodies and mount a protective immune response against live virus challenge in mice after i.d. vaccination of 50 ng of HA-encoding trans-replicon and 20 μg of replicase-encoding conventional mRNA. The response was comparable to that elicited by i.d. vaccination of 1.25 μg of HA-encoding, traditional SFV self-amplifying mRNA vaccine. Splitting the vaccine antigen and replicase components by encoding them in independent vectors might be favorable approach; each component can be independently manufactured and optimized, as invariable replicase encoding mRNA could be produced at a large scale and stockpiled, while the new production on demand would only be required for the shorter, variable, antigen-encoding trans-replicon RNA. This could be particularly useful in the case of rapidly evolving pathogens or newly emerging infectious diseases. Further studies of this system will be required to evaluate total RNA dosage, the efficiency of co-delivery to the same target cell in vivo, and impact on innate immune activation.

Directly targeting mRNA to APCs represents another strategy under investigation to enhance vaccine potency, due to the essential role of these immune cells in activating naïve T-lymphocytes as well as cross-presenting antigens to promote cytotoxic T-lymphocyte responses. Extensive studies have been performed for ex vivo mRNA loading of DCs and the first human trials evaluating mRNA delivery focused on an ex vivo approach, where monocyte-derived DCs were transfected with antigen-encoding mRNA and re-infused into patients as a cellular vaccine (reviewed in Gilboa and Vieweg 2004 and Benteyn 2015). However, in vivo targeting to DCs of synthetic self-amplifying mRNA vaccines against infectious diseases is still in the exploratory phases. In an effort to improve targeting of DCs via subcutaneous injection, McCullough and coworkers explored chitosan-nanoparticles (alginate-coated or hyaluronic-coated chitosan nanoparticles), PEI-based polyplexes (cationic biodegradable polymers), and lipoplexes (cationic biodegradable lipids) to increase the uptake, cytoplasmic release, and translation in DCs of a classical swine fever virus-based self-amplifying mRNA molecule (Demoulins 2016; Englezou 2018; McCullough 2014). The lipoplex, optimized for enhanced in vitro translation in DCs, elicited proinflammatory 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 (Englezou 2018). Another strategy recently reported to enhance APC targeting of LNP formulated self-amplifying mRNA vaccines is mannosylation of LNP (MLNP) (Blakney et al. 2020c; Goswami 2019). MLNP-formulated self-amplifying mRNA that encodes influenza HA exhibited the enhanced uptake from bone marrow-derived DCs in vitro, and more rapid onset of antibody responses in mice after vaccination i.d. or i.m (Goswami 2019). Despite these preliminary attempts, demonstration of in vivo increased uptake, self-amplification, and antigen expression in DCs has not yet been established. Formulation design, optimization, and combination with adjuvants or ligands targeting cell surface receptors on DCs might increase the success of DC-targeting after vaccination.

The delivery system is a key enabling factor in self-amplifying mRNA vaccines. The delivery system protects mRNA from RNases, enhances cytosolic delivery, possibly reduces endosomal PRR recognition of RNA and, for a productive stimulation of the innate immune system, it should favor mRNA delivery to immune cells including APCs, B cells, or T cells. Although many advances in mRNA delivery and formulation have been made in the past years in preclinical studies and clinical trials (reviewed in Kowalski 2019; Zeng 2020; Granot and Peer 2017), challenges remain, such as toxicity of some formulations, inefficient transport, and endosomal escape of mRNA, difficulty in reaching immune cells in dedicated secondary lymphoid organs, and the need to identify the optimal route of administration. The delivery system is extensively reviewed in Chapter 4 so will not be the focus of this chapter.

5 Conclusions and Perspectives

The field of mRNA vaccines is undergoing a major expansion, in large part due to its promise of broad application and streamlining vaccine development, and importantly the urgent need for an effective vaccine to control the current pandemic of COVID19. In this chapter, we reviewed and explored key factors that can determine the success of self-amplifying mRNA vaccine platform. A major strength of this platform is that, due to self-amplification of the vector in vivo, high-level and long-lasting protein expression, together with a built-in self-adjuvanting effect, is readily feasible upon vaccination. This technology has the ideal attributes for becoming a platform of choice for vaccine development, including efficacy at low dosages, one-dose vaccine regimen, faster and flexible manufacturing processes than conventional vaccines, all of which could enable rapid response to emerging infectious diseases.

Several key elements need to be further investigated to obtain a highly efficacious and safe self-amplifying mRNA vaccine. These include the design and modification of the most effective self-amplifying mRNA backbone sequence, robust process of IVT RNA production and purification producing high-quality full-length functional RNA, a nontoxic delivery system allowing efficient in vivo transfection of target cells, the proper route of administration, and, importantly, induction of a balanced innate activation leading to desired adaptive immune response, humoral, cellular, or both. Self-amplifying mRNA vaccine induces robust humoral and cellular immune responses in preclinical models, but its safety and effectiveness remain to be demonstrated in humans. Activation of PRRs by mRNA generally leads to strong production of type I IFN, the key player in modulating vaccine efficiency. Nonetheless, the levels of and kinetics of type I IFN response could determine if it is beneficial, via its stimulation for a productive immune response, or detrimental, via promoting apoptosis, inhibiting vaccine self-amplification and expression, and exhausting the immune response to the expressed antigen. The mechanisms governing the interaction of self-amplifying mRNA vaccines with host innate immunity, subsequent modulation of antigen-specific immune responses, and the differential outcome of type I IFN activation warrant further investigation. The intricate mechanisms of RNA sensing by PRRs in vivo and the impact of the route of administration account for the unpredictability of translating in vitro observations to in vivo results. Therefore, deciphering vaccine platform elements, host factors, and innate immunity that determine the potency of self-amplifying mRNA vaccines could pave the way for a more rational vaccine and regimen design and boost their clinical development. To date, there is no animal model that can reliably predict human immune responses to mRNA vaccines, underlying the importance of clinical trials and translational research.

mRNA technologies have broad applications beyond prophylactic vaccines and can be used to vaccinate against host disease targets (e.g., cancer), to deliver therapeutic molecules (e.g., molecular antibodies or chemokines), or as therapies to

compensate or correct for genetic defects (e.g., protein replacement). Robust immune activation is an attractive feature for a therapeutic vaccine, and the elevated magnitude and persistence of gene expression from self-amplifying mRNA makes it a promising platform to enable sustained in vivo expression for therapeutics. For the latter, strategies to reduce their inherent immune-stimulatory properties and increase immune evasion need to be further exploited.

The next 5 years will be important for the field of mRNA vaccines, especially for self-amplifying mRNAs. Developing and testing mRNA vaccines in humans is not always straightforward, as shown by the modest immunogenicity obtained in the first clinical trial with a protamine formulated sequence optimized non-amplifying mRNA vaccine (Alberer 2017), despite potency in animal models (Schnee 2016). However, this can provide insights on ways to optimize the vaccine, either at the sequence or at the delivery system level and generate more potent candidates (Lutz 2017; Aldrich 2021). Clinical data from prophylactic non-amplifying mRNA vaccines have been recently published showing an overall good tolerability profile and promising immunogenicity (Aldrich 2021; Bahl 2017; Feldman 2019; Walsh et al. 2020a, b; Sahin 2020; Mulligan 2020; Jackson 2020; Widge 2021; Anderson 2020). Importantly, the current pandemic has enabled the rapid development and emergency use authorization of two non-amplifying mRNA-based COVID vaccines developed by Moderna and Pfizer/BioNTech (Polack 2020; Baden et al. 2020). On the other hand, vaccine candidates based on synthetically delivered self-amplifying mRNA are in earlier stages of clinical testing, with data pending publication. Virally delivered alphavirus-based self-amplifying mRNA vaccines encoding CMV, HIV-1, or influenza antigens have been tested in humans and shown to be immunogenic (Bernstein 2009; Wecker 2012; Mogler and Kamrud 2015), which could serve as a proof of concept that the self-amplifying vector can be effective in humans. In preclinical models, synthetic delivery of self-amplifying mRNA vaccines has been shown to produce equivalent responses to those by viral delivery (Geall 2012; Brito 2014). The results from the ongoing clinical trials will inform if the current state of the art in self-amplifying mRNA vaccine development is sufficiently potent and safe, or if further optimization, including RNA engineering, new delivery systems, and modulation of elicited innate responses, among others, will be needed, which will provide a clearer understanding of the true prospects of the technology.

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Formulation and Delivery Technologies for mRNA Vaccines

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Contents

Abstract mRNA vaccines have become a versatile technology for the prevention of infectious diseases and the treatment of cancers. In the vaccination process, mRNA formulation and delivery strategies facilitate effective expression and presentation of antigens, and immune stimulation. mRNA vaccines have been delivered in various formats: encapsulation by delivery carriers, such as lipid nanoparticles, polymers, peptides, free mRNA in solution, and ex vivo through dendritic cells. Appropriate delivery materials and formulation methods often boost the vaccine efficacy which is also influenced by the selection of a proper

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administration route. Co-delivery of multiple mRNAs enables synergistic effects and further enhances immunity in some cases. In this chapter, we overview the recent progress and existing challenges in the formulation and delivery technologies of mRNA vaccines with perspectives for future development.

1 Introduction

Since the first use of in vitro transcribed messenger RNA (mRNA) to express an exogenous protein in mice in 1990 (Wolff et al. 1990), mRNA has evolved into a versatile platform spanning many therapeutic and prophylactic fields (Hajj and Whitehead 2017; Xiong et al. 2018; Li et al. 2019; Patel et al. 2019b; Pardi et al. 2020; Weng et al. 2020). In particular, numerous mRNA vaccines are being developed to tackle infectious diseases and various types of cancer, with many advancing to different stages of clinical trials (Pardi et al. 2018).

Several features of in vitro transcribed mRNA contribute to its vaccine potential. First, the development process of an mRNA vaccine can be much faster than conventional protein vaccines (DeFrancesco 2017). In response to the pandemic of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2020, an mRNA vaccine was administrated to the first volunteer in a phase 1 clinical trial within ten weeks after the sequence of the viral genome was revealed (Lurie et al. 2020). Second, in vitro transcription reaction is easy to conduct, has a high yield, and can be scaled up (Pardi et al. 2018). Advanced industrial setup can manufacture mRNA up to kilogram scales (Versteeg et al. 2019). Third, mRNA vaccine enables the synthesis of antigen proteins in situ, eliminating the need for protein purification and long-term stabilization which are challenging for some antigens. Fourth, transportation and storage of mRNA may be easier than protein-based vaccines, since RNA, if protected properly against ribonucleases (RNases), is less prone to degradation compared to proteins (Stitz et al. 2017; Zhang et al. 2019). Because of these advantages, mRNA vaccines have great potential to be manufactured and deployed in a timely manner in response to rapid infectious disease outbreaks.

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Despite mRNA's appealing features and advances in the field, in vivo delivery of mRNA remains challenging. The first challenge is the instability of mRNA mostly due to enzymatic degradation by RNases. RNases are present ubiquitously throughout the body to degrade exogenous RNAs (Gupta et al. 2012). And mRNA, consisting of hundreds to thousands of nucleotides, has to reach the cytosol in full length for active translation. Hence, protection against RNases is critical for most in vivo delivery strategies. Secondly, efficient intracellular delivery of mRNA is another challenge owing to the negative charge and large size of mRNA molecules. The negative charge prevents most mRNA from translocating across the negatively charged cell membrane. The large size makes efficient encapsulation and delivery more challenging than other payloads, such as small molecules, siRNAs, and antisense oligonucleotides (ASOs). Various delivery strategies have been investigated to address these obstacles with different delivery materials, formulation methods, and routes of administrations.

The mRNAs used as vaccines can be categorized into conventional mRNAs and self-amplifying mRNAs. Conventional mRNAs are similar to endogenous mRNAs in mammalian cells, consisting of a 5' cap, 5' UTR, coding region, 3' UTR, and a polyadenylated tail (Pardi et al. 2018; Kowalski et al. 2019). The typical size is 1–5 k nucleotides. When delivered to the cytosol, this type of mRNA is translated until its degradation without additional replication. On the other hand, self-amplifying mRNAs are derived from the genomes of single-stranded RNA viruses, such as alphaviruses (Brito et al. 2015). Besides encoding proteins of interest, self-amplifying mRNAs encode replication machinery consisting of several viral non-structural proteins (nsPs) to replicate themselves. Therefore, their typical size is approximately 8–12 k nucleotides, larger than the conventional mRNA vaccine. When delivered to the cytosol, self-amplifying mRNAs replicate themselves while expressing the designated proteins in a relatively large amount (Iavarone et al. 2017). More importantly, self-amplifying mRNAs are unique for vaccine applications because of their self-adjuvant nature (Maruggi et al. 2019). Many factors involved in their self-replication process, such as the double-stranded RNA (dsRNA) intermediate of replication (von Herrath and Bot 2003) and the nsPs in the replication machinery (Maruggi et al. 2013), could stimulate interferon-mediated immune responses (Pepini et al. 2017).

Three major types of proteins are encoded by mRNA vaccines: antigens (Grunwitz and Kranz 2017; Zhang et al. 2019), neutralizing antibodies (Stadler et al. 2017; Tiwari et al. 2018), and proteins with immunostimulatory activity (Bonehill et al. 2008; Manara et al. 2019). Antigens or neutralizing antibodies induce specific immune responses, while proteins with immunostimulatory activity, such as CD70 (Van Lint et al. 2012) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Manara et al. 2019) boost innate and/or adaptive immunity.

Advances in recent years made mRNA a promising vaccine platform. For example, chemical modifications of RNA using nucleotide analogs, such as pseudouridine, dramatically increased protein production in vivo by diminishing the translation inhibition triggered by the unmodified nucleotides (Kariko et al. 2008;

Warren et al. 2010). High-performance liquid chromatography (HPLC) purification further increased the purity and translation capability of mRNA by removing the byproducts from in vitro transcription, such as dsRNA, which could induce inhibition of mRNA translation (Karikó et al. 2011; Weissman et al. 2013). Lipid and lipid-derived nanoparticles (LNPs) were previously used to deliver small molecule drugs and siRNAs (Brito et al. 2015; Ickenstein and Garidel 2019). The adaptation of LNPs for mRNA delivery greatly enhanced the delivery efficiency of mRNA both in vitro and in vivo (Dimitriadis 1978; Malone et al. 1989; Martinon et al. 1993). The use of new formulation technologies, such as continuous-flow microfluidic devices, enabled reproducible production of nanoparticles at various scales with controllable sizes (Jahn et al. 2008; Valencia et al. 2012).

In this chapter, we summarize the routes of administrations for mRNA vaccines, discuss mRNA delivery carriers and their corresponding formulation methods, and overview the challenges and future development of mRNA vaccines. A comprehensive overview of recent advances in mRNA vaccine delivery may facilitate the future development of novel delivery strategies and effective mRNA vaccines.

2 Administration Routes for mRNA Vaccines

The administration route for mRNA vaccines plays an important role in determining vaccination efficacy (Eggert et al. 1999). Figure 1 depicts the most commonly used injection routes, including intradermal (ID), subcutaneous (SC), intramuscular (IM), intranodal (IN), and intravenous (IV) administration (Verbeke et al. 2019b). Other routes, such as intranasal injection (Lorenzi et al. 2010; Li et al. 2017a), intravaginal injection (Lindsay et al. 2020), and intratumoral injection (Scheel et al. 2006; Van Lint et al. 2016), were also tested. Since the immune cells and lymphoid organs are the common vaccination targets, the anatomical and physiological properties of the vaccination sites (skin, muscle, lymphoid organ, and systemic circulation) may affect the safety and efficacy of a vaccine (Johansen and Kündig 2015). Such information is useful for the selection of administration route when the type (conventional or self-amplifying) and the delivery format (carrier-mediated, naked, or cell-based) of the mRNA vaccine are chosen.

Intradermal (ID) injection delivers mRNA vaccines directly into the dermis region, which is dense connective tissue (Fig. 1a). Antigen-presenting cells (APCs) in the dermis tissue, such as dermal dendritic cells (DCs) and macrophages (Kashem et al. 2017), can internalize and process the mRNA vaccine. The vascular and lymphatic vessels in this layer of skin also help transport mRNA vaccines and APCs to the draining lymph nodes to activate T and B cells (Kashem et al. 2017; Melo et al. 2019). Because of these properties, ID injection was tested in clinical studies for delivering mRNA vaccine encoding rabies virus glycoprotein (Alberer et al. 2017). Their results showed ID vaccination by a needle-free device could induce better antibody response than IM injection. Although ID injection has preferential access to immune cells and lymphoid organs, and has shown vaccination efficacy, this method

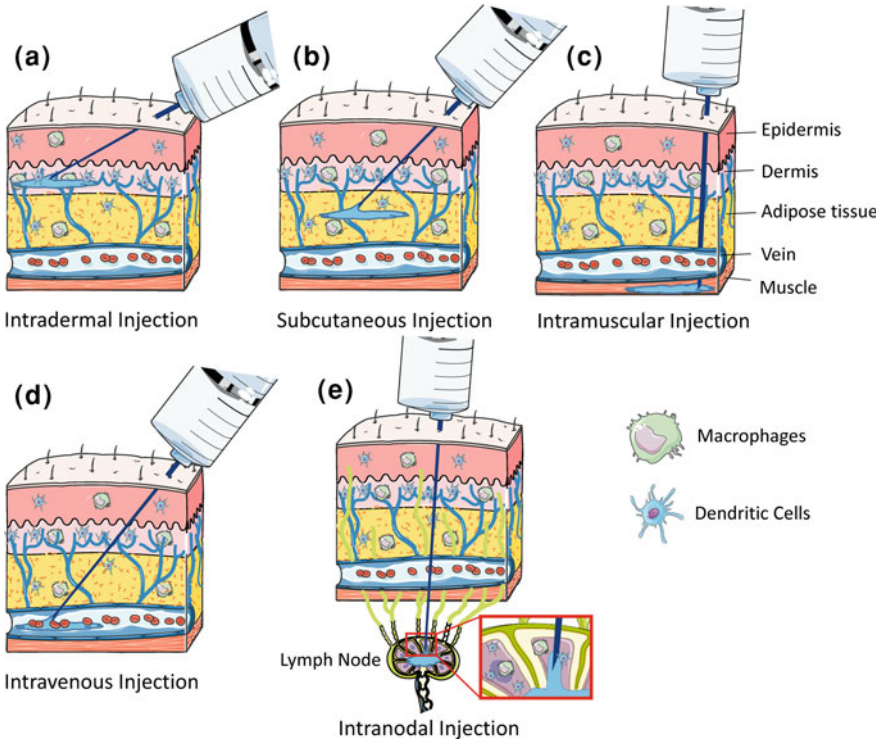

Fig. 1 Common routes for the delivery of mRNA vaccines

has been limited by its small injection volume and high risk of local adverse effect including swelling, pain, erythema, and pruritus (Engmann et al. 1998; Diehl et al. 2001; Rini et al. 2016; Sienkiewicz and Palmunen 2017). To increase injection volume and mRNA dose, patients received multiple ID injections at different sites per visit in a clinical trial (Sebastian et al. 2019).

Subcutaneous (SC) injection administers mRNA vaccines to the subcutis region under the epidermis and dermis (Fig. 1b). This layer of skin is mainly composed of a loose network of adipose tissues and few immune cells compared to the dermis (Ibrahim 2010). Comparing to ID injection, the loose adipose tissue at the SC injection site permits a larger injection volume (Sienkiewicz and Palmunen 2017), causing less pain and lower pressure (Johansen and Kündig 2015). In addition, the larger injection volume may compensate for the less efficient draining activity in this layer of skin (Johansen and Kündig 2015). It is noteworthy that the absorption rate in the SC area is slow, which may cause unintended degradation of the mRNA vaccine (Gradel et al. 2018).

Intramuscular (IM) injection delivers the vaccine into muscles, a deeper tissue under the dermal and subcutaneous layer (Fig. 1c). Muscles contain a large network of blood vessels that can help recruit and recirculate different types of immune cells,

such as the infiltrating APCs, to the injection site (Liang et al. 2017). A recent study indicated that after IM injection of LNPs-encapsulated mRNA, the radiolabeled mRNA was detected at the site of injection and draining lymph node for at least 28 h (Lindsay et al. 2019). Detailed flow cytometry analysis showed that APCs in muscle as well as APCs and B cells in draining lymph nodes contained the radiolabeled mRNA (Lindsay et al. 2019). The volume of IM injection is larger than that of ID injection in humans (Sienkiewicz and Palmunen 2017). Additionally, IM injection may cause milder local side effects compared with ID and SC routes. Thus, IM injection is the most widely used administration route for adjuvanted vaccines (Moyer et al. 2016).

Intranodal (IN) injection directly introduces mRNA vaccines to the peripheral lymphoid organs where APCs and primed T or B cells interact (Fig 1e). IN injection is considered to be an efficient way of vaccination, since the APCs in lymphoid organs can readily engulf the injected mRNA vaccine (Kreiter et al. 2010; Bialkowski et al. 2016; Joe et al. 2019). Even though studies reported increased vaccination efficacy by IN delivery route compared with other injection methods for DNA, peptide and protein vaccines (Senti and Kündig 2015), side-by-side comparison between IN delivery and other routes for mRNA vaccine remains limited (Kreiter et al. 2010). In addition, IN injection was seldom used mostly because of the relatively complicated procedure (Johansen and Kundig 2014; Senti and Kündig 2015). For example, IN injection needs ultrasound guidance in human (Senti and Kündig 2015).

Mucosal delivery of mRNA vaccines was studied because of the accessible APCs in lymphoid organs at the mucosal sites and their protective roles against various pathogens. Among the mucosal administration routes, intranasal and intravaginal administrations were utilized to deliver mRNA vaccines (Lorenzi et al. 2010; Li et al. 2017a; Lindsay et al. 2020). Intranasal injection delivers mRNA vaccines to the nasal mucosa and nasal associated lymphoid tissue (NALT), both of which contain rich APCs and related immune cells (Lobaina Mato 2019). As a result, the intranasal delivery of antigen-encoding mRNA was reported to induce humoral and cell-mediated immunity (Lorenzi et al. 2010; Zhuang et al. 2020). Intranasal delivery can also apply mRNA to the lung through the trachea. Similar to the nasal mucosa and NALT, the immature and activated APCs available in the lung can engulf and process the mRNA vaccine (Stehle et al. 2018). Additionally, an mRNA vaccine was delivered to the lung epithelial cells via intranasal injection and expressed neutralizing antibodies against virus infection (Tiwari et al. 2018). Intravaginal injection is another approach to deliver mRNA vaccines to the site of infection to express neutralizing antibodies. In one report, the intravaginal delivery of the mRNA vaccine encoding an anti-HIV antibody induced high levels of antibody expression in the reproductive tract of sheep and rhesus macaques (Lindsay et al. 2020). Immunofluorescence staining showed the expressed antigen was mainly found in cervical epithelial cells and stromal cells (Lindsay et al. 2020).

Intravenous (IV) injection delivers mRNA vaccines into the systemic circulation (Fig. 1d). The volume of IV injection is the largest among the delivery routes mentioned above (Diehl et al. 2001). The total amount of protein produced via IV

administration is often the highest compared to other routes (Pardi et al. 2015). Thus, the IV route was chosen for delivering mRNA encoding a neutralizing antibody when a functional concentration of the antibody was required in circulation (Kose et al. 2019). Generally, IV injected LNP delivers mRNA to the liver, and more specifically, to hepatocytes, Kupffer cells, and liver endothelial cells depending on different types of delivery carriers (Pardi et al. 2015; Conway et al. 2019). Moreover, IV injections may also allow the direct access of mRNA vaccines to immune cells and lymphoid organs in the circulatory system, which may enhance the vaccination efficacy (Kranz et al. 2016). For example, previous studies found IV injection of mRNA vaccine targeted spleen DCs and induced immune response against tumors in mice (Kranz et al. 2016). Despite the advantages of IV injection mentioned above, the plasma proteins, enzymes, and mechanical forces in the bloodstream may hinder the vaccine delivery (Reichmuth et al. 2016). Furthermore, the administration of mRNA and delivery carriers to the circulation may introduce systemic side effects, such as spleen injury and lymphocyte depletion (Reichmuth et al. 2016; Sedic et al. 2017).

In summary, the biological features of different administration routes may impact the safety and efficacy of vaccination. Table 1 summarizes the features of several major delivery routes of mRNA vaccines. For a given combination of mRNA-type and delivery carrier, a careful comparison of several administration

Table 1 Major delivery routes of mRNA vaccines

Delivery route	Access to APCs and lymphoid organs	Maximum injection volume per site		Advantages[3]	Challenges[4]
		Human[1]	Mouse[2]		
Intradermal	• Dermal DC • Lymph node DC • Lymph node	~0.1 mL	~0.05 mL	• Direct access to APCs	• Local side effect, • Limited injection volume
Subcutaneous	• Dermal DC • Lymph node DC • Lymph node	~1 mL (Adult), ~0.5 mL (Child)	~0.8 mL total at 2–3 sites[a]	• Larger injection volume (than ID) • Less local side effect	• Degradation of mRNA
Intramuscular	• DC • Lymph node	1–3 mL (Adult), 0.5–2 mL (Child)	0.05 mL per site, maximum of 2–4 sites	• Less local side effect • Dense blood networks	• Limited injection volume
Intranodal	• Lymph node DC • Lymph node	~0.2 mL	0.01-0.02 mL	• High delivery efficiency	• Complicated procedures
Intravenous	• Splenic DC • Lymph node DC • Spleen • Lymph node	~20 mL (bolus)	~0.1 mL (bolus)[a] ~0.5 mL (slow)[a]	• Large injection volume • Direct access to APCs and lymphoid organs	• Degradation of mRNA • Risk of systemic side effect

[a]based on a 20-g mouse

1, de Vries et al. (2005), Doyle and McCuteheon (2015), Sienkiewicz and Palmunen (2017)

2, Diehl et al. (2001)

3, Diehl et al. (2001), Moyer et al. (2016), Kashem et al. (2017), Liang et al. (2017), Sienkiewicz and Palmunen (2017)

4, Johansen and Kundig (2014), Reichmuth et al. (2016), Gradel et al. (2018)

routes will help determine the most appropriate injection method and promote the development of an effective mRNA vaccine.

3 Delivery Strategies for mRNA Vaccines

Researchers have investigated many methods to deliver mRNA vaccines. For example, delivery carriers, such as lipid-derived and polymer-derived materials, dramatically increased cellular uptake of RNAs, thus receiving tremendous attention in recent years (Reichmuth et al. 2016; Kowalski et al. 2019; Riley et al. 2019). mRNA vaccines were also delivered as free mRNA (Fleeton et al. 2001; Edwards et al. 2017). Additionally, dendritic cells were loaded with mRNA vaccines ex vivo and transferred to the hosts (Benteyn et al. 2015). In this section, we focus on the technologies for formulating and delivering mRNA vaccines in carrier-mediated, naked, and DC-based forms.

3.1 Delivery Carriers of mRNA Vaccines

3.1.1 Lipid-based Delivery

Lipids, lipid-like compounds, and lipid derivatives have been widely used to formulate lipid and lipid-derived nanoparticles (LNPs) for in vivo delivery of mRNA vaccines (Midoux and Pichon 2015; Reichmuth et al. 2016; Corthésy and Bioley 2018; Pardi et al. 2018; Li et al. 2019). LNPs are generally defined as nano-sized particulate systems that are composed of synthetic or physiological lipid materials (Ganesan and Narayanasamy 2017). Table 2 lists representative in vivo delivery of mRNA vaccines by LNPs. LNPs are developed for mRNA vaccine delivery for the following two main reasons. Firstly, LNPs can encapsulate RNA molecules, protecting RNA from enzymatic degradation (Midoux and Pichon 2015). The reported mRNA encapsulation efficiency by LNP was usually high, indicating the mRNA molecules were mostly encapsulated (Richner et al. 2017a, b). Secondly, LNPs can effectively deliver mRNA molecules into the cell cytosol through a series of endocytosis mechanisms (Sahay et al. 2010). For example, it was reported that the surface adsorption of apolipoprotein E (apoE) on LNP might facilitate its intracellular delivery via low-density lipoprotein receptor-mediated clathrin-dependent endocytosis (Basak et al. 2012). This endocytosis process transported the mRNA-loaded LNPs into cell membrane-bound vesicles, including endosomes and lysosomes (Sahay et al. 2010; Patel et al. 2019c). Eventually, the LNPs helped translocate mRNA cargos into the cytosol for protein expression (Midoux and Pichon 2015).

The LNPs usually contain one or more of the functional lipid components that are crucial for the intracellular RNA delivery described above (Midoux and Pichon 2015;

Kowalski et al. 2019; Verbeke et al. 2019b). The cationic or ionizable lipid materials, such as 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), N, N-Dimethyl-2,3-bis[(9Z,12Z)-octadeca-9,12-dienyloxy]propan-1-amine (DLinDMA), and N^1,N^3,N^5-tris(3-(didodecylamino)propyl)benzene-1,3,5-tricarboxamide (TT3) usually contain one or multiple amino groups (Semple et al. 2010; Jayaraman et al. 2012; Billingsley et al. 2020; Zeng et al. 2020). These lipid materials can be positively charged at a certain pH to encapsulate the negatively charged RNA molecules via electrostatic interactions and help interact with the cell membrane on target cells. Previous studies indicated the final step of RNA release from LNPs into the cytosol might involve the membrane disruption of endosomes (Cullis and Hope 2017). In this process, the ionizable cationic lipids were suggested to interact with anionic lipid on endosome membrane and form disruptive non-bilayer structures, which finally released the encapsulated RNA into the cytosol (Cullis and Hope 2017). Furthermore, the structure–activity relationship of the lipids head and tail for RNA delivery and endosomal escape was studied (Sato et al. 2019). The results indicated that the hydrophilic head group in lipid materials might determine the acid dissociation constant (pKa) and influence the delivery efficiency (Sato et al. 2019). Besides, modification of fatty acids structures in hydrophobic tails may also affect the delivery efficiency (Sabnis et al. 2018; Sato et al. 2019). Even though the membrane disruptive features of lipid materials improve the delivery efficiency, these synthetic materials may cause side effects in vivo (Pun and Hoffman 2013; Sedic et al. 2017). The helper lipids, such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol, stabilize LNPs structures and facilitate endosome escape (Cheng and Lee 2016). The PEG-lipid conjugates could stabilize the nanoparticles during preparation and provide a hydrophilic outer layer that prolongs the circulation time after in vivo administration (Ambegia et al. 2005; Heyes et al. 2005; Cheng and Lee 2016; Kowalski et al. 2019). In addition to these functions, the engineered ionizable lipid materials containing cyclic amino head groups, isocyanide linker, and two unsaturated alkyl tails were reported to provide adjuvant activities independent of the encapsulated mRNA (Miao et al. 2019). These cyclic amino head groups directly bound the STING (stimulator of interferon genes) protein and triggered the downstream signaling pathway, leading to an elevated innate response. After SC injection of an antigen-coding mRNA encapsulated by such LNP into mice, the researchers observed the upregulation of antigen-specific T cells and inhibition of tumor growth (Miao et al. 2019).

The formulation methods of lipid-based mRNA vaccines mainly include thin-film hydration (Akbarzadeh et al. 2013; Kranz et al. 2016), direct mixing (Borrego et al. 2017), ethanol injection (Geall et al. 2012), and continuous-flow microfluidic device (Chen et al. 2012; Kose et al. 2019). Among these methods, the continuous-flow microfluidic device emerges as a prevalent method to prepare RNA encapsulated nanoparticle, especially LNP, for in vivo use (Liu et al. 2018; Kowalski et al. 2019). These chip-based microfluidic devices mix two laminar flows, the RNA-containing aqueous phase and the carriers-containing solvent phase, through a confined microchannel equipped with chaotic mixers at a controlled speed, leading to rapid diffusion, change of polarity and self-assembly of

Table 2 Lipid-based nanoparticles (LNPs) delivery of mRNA vaccines in vivo

Formulation composition	Formulation method	RNA encoding	mRNA type	Delivery route	Target	Model tested	References
Cholesterol/DPPC/PS: 5/4/1, mol/mol	Thin-film hydration	Virus antigen	Conventional	IV, SC, IP	Virus infection	Mouse	Martinon et al. (1993)
PS/PC/cholesterol: 1:4.8:2, w/w	Thin-film hydration	Tumor antigen	Conventional	Direct injection into spleen	Tumor	Mouse	Zhou et al. (1999)
DLinDMA/DSPC/ cholesterol/ DMG-PEG: 40/10/48/ 2, mol/mol	Self-assembly by ethanol injection	Virus antigen	Self-amplifying	IM	Virus infection	Mouse Rat	Geall et al. (2012)
cKK-E12/DOPE/ cholesterol/PEG-lipid: 15/26/40.5/2.5, mol/ mol	Microfluidic device	Tumor antigens	Conventional	SC	Tumor	Mouse	Oberli et al. (2016)
DOTMA/DOPE:1/1, mol/mol	Thin-film hydration	Tumor antigen	Conventional	IV	Tumor	Mouse Human	Kranz et al. (2016)
DOTMA/DOPE:1/1, mol/mol	Mixing	Virus antigen	Conventional	IM, SC	Virus infection	Swine	Borrego et al. (2017)
Lipid shell: EDOPC/ DOPE/DSPE-PEG: 49/49/2, mol/mol Polymer-RNA core: PbAE	Thin-film hydration	Tumor antigen	Conventional	SC, IV	Tumor	Mouse	Persano et al. (2017), Guevara et al. (2019)

(continued)

Table 2 (continued)

Formulation composition	Formulation method	RNA encoding	mRNA type	Delivery route	Target	Model tested	References
ionizable lipid/DSPC/ cholesterol/PEG-lipid: 50/10/38.5/1.5, mol/ mol	Microfluidic device	Virus antigen, antibody	Conventional	ID, IM, IV	Virus infection, tumor	Mouse Ferret Non-human primate Human	Bahl et al. (2017), Liang et al. (2017), Pardi et al. (2017a, b), Thran et al. (2017), John et al. (2018), Awasthi et al. (2019), Kose et al. (2019), Roth et al. (2019)
A18/DOPE/ cholesterol/PEG-C14: 35/16/37.5/2.5, mol/ mol	Microfluidic device	Tumor antigen	Conventional	SC	Tumor	Mouse	Miao et al. (2019)
DOTAP/cholesterol: 2/ 3, mol/mol	Thin-film hydration	Tumor antigen	Conventional	IV	Tumor	Mouse	Verbeke et al. (2019a)
DOTAP/DOPE/ DSPE-PEG-Mannose: 50:50:1, mol/mol	Thin-film hydration	Virus antigen	Conventional	Intranasal	Virus infection	Mouse	Zhuang et al. (2020)
TT3/DOPE/ cholesterol/ DMG-PEG2000: 20/ 30/40/0.75, mol/mol	Microfluidic device	Virus antigen	Conventional	IM	Virus infection	Mouse	Zeng et al. (2020)

DPPC dipalmitoylphosphatidylcholine, *PS* phosphatidylserine, *PC* phosphatidylcholine, *DLinDMA* N,N-Dimethyl-2,3-bis[(9Z,12Z)-octadeca-9,12-dienyloxy] propan-1-amine, *DSPC* 1,2-distearoyl-sn-glycero-3-phosphocholine, *DMG-PEG* 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol, *DOPE* 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, *DOTMA* 1,2-di-O-octadecenyl-3-trimethylammonium propane, *EDOPC* 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, *DSPE* 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, *PbAE* poly-(β-amino ester) polymer, *A18* ethyl 1-(3-(2-ethylpiperidin-1-yl)propyl)-5,5-di((Z)-heptadec-8-en-1-yl)-2,5-dihydro-1H-imidazole-2-carboxylate, *DOTAP* 1,2-dioleoyl-3-trimethylammonium propane, *TT3* N^1,N^3,N^5-tris(3-(didodecylamino)propyl)benzene-1,3,5-tricarboxamide

mRNA-LNP at the interface (Belliveau et al. 2012; Yanez Arteta et al. 2018). The resulting lipid nanoparticles are relatively homogeneous formulation and usually show spherical and multilamellar morphology (Yanez Arteta et al. 2018). Compared to other preparation methods, the use of continuous-flow microfluidic devices increases reproducibility, improves molecular stability, reduces the chance of contamination, and is easily scaled up for preclinical and clinical studies (Damiati et al. 2018; Liu et al. 2018).

The delivery routes of lipid-based mRNA vaccines include IM, ID, SC, IN, and IV injection (Midoux and Pichon 2015). Delivery routes can affect the in vivo distribution pattern and expression kinetics of encapsulated mRNA vaccines (Pardi et al. 2015). Local injections, such as IM, ID, SC, and IN administrations, deliver LNPs mRNA vaccine to resident/infiltrating APCs and related immune cells, stimulating strong and prolonged local expression (Kreiter et al. 2010; Pardi et al. 2015; Hassett et al. 2019). Thus, local injections were utilized to deliver most LNP encapsulated antigen-encoding mRNA vaccines (see examples in Table 2). For example, TT3-LNPs were used to deliver mRNAs encoding the full-length spike protein or its receptor binding domain of SARS-CoV-2. After IM administration, the expression of both antigens was observed in muscle tissues (Zeng et al. 2020). The resident APCs in the skin, muscle, and lymph node can process the expressed antigens and capture mRNA nanoparticles (Moyer et al. 2016). The activated APCs can be recruited to the injection site to process the mRNA vaccine. Additionally, the lymph vessels may directly transport small-sized lipid nanoparticles to draining lymph nodes (Moyer et al. 2016). Systemic injection, like IV injection, often leads to liver accumulation of the LNP-delivered mRNA vaccine and can generate a relatively large amount of protein compared with local injection methods (Pardi et al. 2015). Therefore, IV injection is often used to deliver mRNAs encoding antibodies when the high functional concentration of neutralizing antibodies is required in the bloodstream (Kose et al. 2019). Furthermore, IV injection of LNPs-delivered mRNA may also target the spleen by changing the formulation ratio (Kranz et al. 2016).

Overall, LNPs-based mRNA vaccines have shown efficacy in preventing infectious diseases and treating cancers in preclinical and early-stage clinical studies (Bahl et al. 2017; John et al. 2018; Kose et al. 2019; Liang et al. 2017; Pardi et al. 2017a, b; Thran et al. 2017). In a recent phase 1 clinical trial, the safety, tolerability, and immunogenicity of LNPs-based Zika mRNA vaccine were evaluated after IM injection (NCT04064905). Another phase 2 clinical trial aimed at testing the efficacy of a personalized mRNA cancer vaccine through IM injection started in July 2019 (NCT03897881). With further improvement, LNPs may facilitate the development of more effective mRNA vaccines.

3.1.2 Polymer-based Delivery

Polymeric materials, including polyamines, dendrimers, and copolymers, are functional materials capable of delivering mRNA vaccines. Similar to functional lipid-based carriers, polymers can also protect RNA from RNase-mediated

degradation and facilitate intracellular delivery (Kowalski et al. 2019). However, the formulation of polymer-based mRNA nanoparticles tends to have high polydispersity (Kowalski et al. 2019). To stabilize the formulation and improve the safety profile, structural modification of polymer materials, such as incorporating lipid chains, hyperbranched groups, and biodegradable subunits, has been explored (Dong et al. 2016; Kaczmarek et al. 2016; Patel et al. 2019a).

Cationic polymers, such as polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimer, and polysaccharide, condensed and delivered negatively charged RNA molecules (McCullough et al. 2014; Chahal et al. 2016; Vogel et al. 2018; Blakney et al. 2020; Son et al. 2020). PEI was one widely used polymeric material for mRNA vaccine delivery. PEI formulations were often prepared by direct mixing PEI solution with RNA solution. For example, a PEI formulation delivered an mRNA encoding HIV gp120 and triggered specific antibodies against HIV infections after intranasal vaccination in mice (Li et al. 2017b). Later on, a PEI formulation of self-amplifying mRNA encoding the hemagglutinin antigens from several influenza virus strains stimulated high antibody titer after IM immunization in mice and protected mice against virus challenge (Vogel et al. 2018). More recently, a PEI-based formulation with mRNAs encoding HIV-1 Gag and Pol proteins induced specific CD8+ and CD4+ T-cell responses against HIV infections upon IM vaccination in mice (Moyo et al. 2019). Even though PEI formulation showed in vivo efficacy, the potential toxicity may impede its development (Kowalski et al. 2019). PAMAM dendrimer is another cationic polymer material. Antigen-encoding self-amplifying mRNAs formulated by PAMAM dendrimer protected mice from lethal challenge of Ebola, H1N1 influenza, Toxoplasma gondii, respectively, after IM administration (Chahal et al. 2016). IM vaccination of a similar dendrimer formulation with self-amplifying mRNAs encoding premembrane (prM) and envelope (E) proteins of Zika virus elicited specific IgG and CD8 + T-cell responses in mice (Chahal et al. 2017). Of note, the microfluidic mixing method was used by the above two studies to formulate the mRNA vaccines. Another report used chitosan, a polysaccharide material, to condense self-amplifying mRNAs encoding influenza virus hemagglutinin and nucleoprotein (McCullough et al. 2014). After SC injection, the expressed antigen was detected in DCs (McCullough et al. 2014). Moreover, a recent study reported that a cationic copolymer material co-delivering one mRNA encoding OVA and a CpG ssDNA adjuvant eliminated OVA-expressing lymphoma tumor from mice after either SC or IV administration (Haabeth et al. 2018).

Besides cationic polymer materials, anionic polymers, such as PLGA, were also used to deliver mRNA vaccines. Since an anionic polymer was not able to efficiently encapsulate the negatively charged mRNA molecules, cationic lipid materials were added to create lipid–polymer hybrid formulations (Yang et al. 2011; Islam et al. 2018; Kong et al. 2019). For example, a cationic lipid, N-bis (2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxy-carbonyl aminoethyl) ammonium bromide (BHEM-Chol), was mixed with a block copolymer poly(ethylene glycol)-block-poly(lactic-co-glycolic acid) (PEG-b-PLGA) and PLGA to form a lipid–polymer hybrid emulsion for mRNA delivery (Fan et al. 2018). This formulation

delivered OVA-mRNA and delayed OVA-expressing lymphoma growth in mice after IV injection (Fan et al. 2018).

In general, the mRNA vaccines delivered by polymer materials showed therapeutic effects in preclinical studies. New functional polymers, with improved biodegradability and delivery efficiency, are needed for clinical translation of the polymer-based mRNA vaccines.

3.1.3 Peptide-based Delivery

Various peptides are used as carriers to deliver mRNA vaccines. Peptides themselves are also a large class of vaccine agents, which have been reviewed in the literature (Li et al. 2014; Hos et al. 2018; Reche et al. 2018).

Peptides, when used as the primary carrier for RNA delivery, should be positively charged. Cationic peptides contain many lysine and arginine residues that provide positively charged amino groups, therefore enabling complexing with nucleic acids through electrostatic interactions (Grau et al. 2018; Qiu et al. 2019). The ratio of positively charged amino groups on the peptide to the negatively charged phosphate groups on the RNA affects nanocomplex formation. Increasing the ratio of charged amino to phosphate groups from 1:1 to 10:1 was reported to afford smaller particle size, larger zeta potential, and higher encapsulation efficiency (Udhayakumar et al. 2017).

Protamine is a cationic peptide used in many early studies for the delivery of mRNA vaccines. In solution, protamine and mRNA spontaneously form a complex, the size of which is dependent on NaCl concentration (Sköld et al. 2015). Protamine possesses two features beneficiary for mRNA vaccines. Firstly, protamine protects mRNA. In the presence of protamine, antigen-encoding mRNA was more resistant to RNase degradation, suggesting better stability in vitro (Hoerr et al. 2000). In another study, protamine maintained the vaccine efficacy in mice by protecting the mRNA encoding rabies virus glycoprotein during harsh storage conditions: long-term in high temperature or cycles of temperature variation (Stitz et al. 2017). Secondly, the protamine-mRNA complex has adjuvant activity. The protamine-mRNA complex is immunogenic through activation of TLR7, likely owing to its structural similarity with condensed viral RNA genome (Scheel et al. 2005; Fotin-Mleczek et al. 2011). When an irrelevant β-galactosidase mRNA was complexed with protamine and injected into glioblastoma tumor, the anti-tumor effect rivalled two uncomplexed nucleic acid adjuvants (CpG ssDNA and polyI:C dsRNA). The side effect of an enlarged spleen associated with CpG ssDNA was avoided (Scheel et al. 2006). However, mRNA was translated poorly when in complex with protamine (Scheel et al. 2004, 2005), limiting the expression of the encoded antigen and reducing its potential as an independent mRNA carrier. Therefore, further development used the protamine-mRNA complex as an adjuvant in combination with another naked mRNA to express an antigen in animal models and human trials (Fotin-Mleczek et al. 2011; Kallen et al. 2013). This method will be further discussed in Sect. 3.4, “Co-delivery of mRNA vaccines” below.

Cationic cell-penetrating peptides (CPPs) can complex with RNA. Although many CPPs were used in gene therapies [reviewed by (Kang et al. 2019)], only a few CPPs delivered mRNA vaccines. RALA peptide (sequence: N-WEARLARAL ARALARHLARALARALRACEA-C) is an amphipathic arginine-rich CPP with positively charged arginine residues on one side and neutral leucine residues on the other side (McCarthy et al. 2014). It condensed a modified OVA-mRNA into nanocomplex, transfected dendritic cells and induced OVA-specific cytotoxic T-cell activation upon intradermal injection into mice (Udhayakumar et al. 2017). The amphipathic feature enhanced the endosomal escape of mRNA as RALA peptide selectively disrupted the endosome membrane at low pH (McCarthy et al. 2014). Another amphipathic CPP, LAH4-L1 (sequence: N- KKALLAHALHLLALLAL HLAHALKKA-C) facilitated the binding of antigen-encoding mRNA to negatively charged polylactic acid nanoparticle (Coolen et al. 2019). The resulting nanoparticle induced innate and specific immune responses in primary human DC upon in vitro delivery. The mechanism study suggested mRNA complex was taken up by phagocytosis and clathrin-dependent endocytosis followed by endosomal escape (Coolen et al. 2019). In another report, a truncated 9-aa cationic CPP (N-RKKRRQRRR-C) derived from HIV Tat protein was fused to the C terminus of a tumor epitope antigen Melan-A (sequence: N-ELAGIGILTV-C) (Haenssle et al. 2010). The fusion peptide formed complexes with polyI:C dsRNA adjuvant. Transfection of immature DCs in vitro with the complex led to DC maturation and IL-12 secretion. The matured DC activated co-cultured antigen-specific lymphocytes from human donors (Haenssle et al. 2010). Furthermore, protamine-CPP fusion protein combines cationic and cell-penetrating features. A short CPP called Xentry (sequence: N-LCLRPVG-C) was fused to truncated protamine (sequence: N-RSQSRSRYYRQRQRSRRRRRRS-C) and delivered a protein-coding mRNA into several human cell lines in vitro (Bell et al. 2018).

Anionic peptides were also utilized to deliver mRNA vaccines in vitro. Anionic peptides cannot complex RNA due to their negative charges. Therefore, they were conjugated to positively charged polymers which served as scaffolds for RNA encapsulation. For example, an OVA-mRNA was first encapsulated with a random copolymer p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) (pHDPA) containing azide group (Lou et al. 2019). Next, an anionic peptide, named GALA (sequence: N-WEAALAEALAEALAEHLAEALAEALEALAA-OH-C), was conjugated to the azide groups on pHDPA by click chemistry through a BCN-PEG linker. The resulted particle showed similar delivery efficiency to macrophages and DCs as lipofectamine 2000 with lower cytotoxicity. Mechanism of action studies suggested the GALA peptide facilitated the cell uptake and release of mRNA into the cytosol through binding to sialic acid groups on the DC surface (Lou et al. 2019).

In summary, protamine was the only peptide carrier evaluated in clinical trials of mRNA vaccines. In these trials, the protamine-mRNA complex and a naked mRNA were injected simultaneously via ID or IM routes (Rausch et al. 2014; Alberer et al. 2017; Sabari et al. 2019). Although well-tolerated in patients, these mRNA vaccines did not induce sufficient immune responses against the designated vaccine

targets in the trials (Rausch et al. 2014; Kubler et al. 2015; Alberer et al. 2017; Sebastian et al. 2019).

3.1.4 Virus-Like Replicon Particle

Viral particles can package and deliver antigen-encoding self-amplifying mRNA into cytoplasm like a virus in a method called virus-like self-amplifying mRNA particle, i.e., virus-like replicon particle (VRP) (Lundstrom 2016). Self-amplifying mRNA then self-replicates and efficiently expresses the designated antigens. The viral structural proteins necessary for particle formation are expressed from packaging (helper) cell lines *in trans* to package self-amplifying mRNAs (Harvey et al. 2004; Li et al. 2017b). The viral particle and self-amplifying mRNA pair can be selected from either the same or different virus species (Dorange et al. 2004). Some VRPs are replication-competent but attenuated (Fuchs et al. 2015; Marzi et al. 2015), while other VRPs only engage in one cycle of transduction because the genetic regions encoding envelope and capsid proteins necessary for the viral infection are absent from the self-amplifying mRNAs (Lundstrom 2016). The advantage of VRPs arises from the efficient cytoplasmic delivery of RNA payload by viral vectors (Usme-Ciro et al. 2013). This ability is attributed to the fact that viruses have evolved to internalize and release their genomes into cells via many different pathways with high efficiency (Vázquez-Calvo et al. 2012). Many ssRNA viruses including alphaviruses, flaviviruses, measles viruses, and rhabdoviruses were used as VRP vaccines (Lundstrom 2016). For example, a Venezuelan equine encephalitis virus (VEEV) self-amplifying mRNA-based VRP expressing two dengue virus antigens was used to immunize non-human primates intradermally and protected them in the viral challenge (White et al. 2013). A Kunjin virus-derived VRP expressing GM-CSF was injected intratumorally, leading to complete removal of the primary tumor in more than half of the mice with colon carcinoma and OVA-expressing melanoma. Metastases to the lung were also reduced (Hoang-Le et al. 2009). Many more viral infections, bacterial infections, and various cancers were targeted using engineered VRP vaccines which were reviewed by Lundstrom (Lundstrom 2016).

However, there are two challenges for VRP-based mRNA vaccines. The first challenge is to scale up the production which is limited by the process of generating VRPs from packaging cell lines (Morrison and Plotkin 2016). Large-scale production of VRPs may require a special manufacturing process (Rauch et al. 2018). Another challenge is the antibody production against the viral vectors, which was reported in several clinical trials (Bernstein et al. 2009; Morse et al. 2010; Wecker et al. 2012; Fuchs et al. 2015). Although such anti-vector antibodies likely hindered two human trials of VRP-based anti-HIV-1 vaccines (Wecker et al. 2012; Fuchs et al. 2015), these antibodies did not prevent the development of specific immunity against the designated antigens in another two vaccine trials against cytomegalovirus (Bernstein et al. 2009) and cancer (Morse et al. 2010). Therefore, future development of VRP-based mRNA vaccines should improve efficacy and manufacture scale while minimizing the anti-vector immunity.

3.1.5 Cationic Nanoemulsion

Cationic nanoemulsion (CNE) combines nanoemulsion with cationic lipids for RNA delivery. Nanoemulsion utilizes hydrophobic and hydrophilic surfactants to stabilize the oil core in the aqueous phase, thereby generating particles. Nanoemulsion can be induced by various methods, such as vigorous agitation, ultrasound, and microfluidics. (Gurpreet and Singh 2018). MF59 is an FDA-approved oil-in-water nanoemulsion adjuvant used with inactivated Flu vaccine for elders (Vesikari et al. 2012). The components of MF59 include a naturally occurring oil (Squalene), sorbitan trioleate (Span 85), polyoxyethylene sorbitan monooleate (Tween 80) and citrate buffer (Podda and Del Giudice 2003; Cioncada et al. 2017). MF59 nanoemulsion enhances the efficacy of vaccines through MyD88-mediated release of cytokines/chemokines and recruitment of immune cells, without triggering TLRs (Seubert et al. 2011; O'Hagan et al. 2012; Calabro et al. 2013). Incorporation of cationic lipids, e.g., DOTAP, in the squalene-based formulation creates positively charged CNE particles that can absorb negatively charged nucleic acids to the outer shell (Ott et al. 2002; Brito et al. 2015). Such surface interaction still protected mRNA from RNase degradation (Brito et al. 2014). Squalene-based CNEs and MF59 are similar in structure and formulation and displayed equivalent recruitments of immune cells (Brito et al. 2014). CNEs delivered self-amplifying mRNA vaccines against several viral and bacterial infections (Brito et al. 2014; Davis et al. 2014; Brazzoli et al. 2016; Maruggi et al. 2017; Samsa et al. 2019). For example, MF59-based CNE delivered three chimeric self-amplifying mRNA vaccines derived from VEEV and Sindbis virus (SINV) (Brito et al. 2014). The three self-amplifying mRNAs expressed antigens against the respiratory syncytial virus (RSV), human cytomegalovirus (hCMV), and human immunodeficiency virus (HIV), respectively. After IM injection, the vaccines induced high antigen-specific IgG titer and efficient leukocyte infiltration in mice, rabbits, and rhesus macaques (Brito et al. 2014). In one recent study developing a vaccine against VEEV, CNE delivered an engineered replication-defective VEEV-based self-amplifying mRNA without the capsid gene (Samsa et al. 2019). Upon IM injection into mice, the self-amplifying mRNA-CNE stimulated neutralizing IgG production and protected mice from lethal VEEV challenge (Samsa et al. 2019). Overall, CNE has shown its potential for the delivery of mRNA vaccines in preclinical studies. Its vaccine efficacy in human awaits further evaluation by clinical trials.

3.2 Naked mRNA Vaccines

The mRNA vaccines can be delivered without any additional carrier, namely in a naked format. This method dissolves mRNA into a buffer and then injects the mRNA solution directly. The feasibility of naked RNA delivery in vivo was reported in an early effort in which a naked mRNA was delivered to mice by

intramuscular injection (Wolff et al. 1990). Although naked mRNA cannot diffuse across the membrane spontaneously, the mechanism(s) underlying its intracellular delivery remain debatable. Several studies proposed that naked mRNA was internalized via macropinocytosis (Diken et al. 2011; Selmi et al. 2016). Such a macropinocytosis pathway is highly active in macrophages (Redka et al. 2018) and immature dendritic cells (Kreiter et al. 2010; Diken et al. 2011; Lim and Gleeson 2011), both of which play critical roles in developing immune responses. Others speculated the cellular uptake of naked mRNA via mechanical forces. One possible force is the hydrostatic pressure formed after fast injection of a relatively large volume into small mammals. This pressure may disrupt the cell membrane and permit cytosolic delivery of nucleic acids (Stewart et al. 2018). Further study is needed to reveal the detailed mechanism(s) responsible for the delivery of naked mRNAs.

The naked mRNA vaccine has two prominent features. One feature is the ease to store and prepare. In the presence of a storage reagent, such as 10% trehalose, freeze-dried naked RNA remains stable in the refrigerator temperature (4 °C) for up to 10 months (Jones et al. 2007). Before administration, the naked mRNA vaccine only needs to be dissolved into a buffer. No additional formulation is needed. The other feature of the naked mRNA vaccine, especially those made of unmodified nucleotides, is its intrinsic immunogenicity, which serves as a double-edged sword. On one side, the immunogenicity might benefit vaccination by providing some adjuvant activity. The exogenous RNAs could be detected by RNA sensors, such as TLRs, RIG-I, PKR, IFIT1, leading to activation of NF κB and type I interferon signaling pathways, and release of cytokines (Schlee and Hartmann 2016). A naked mRNA vaccine was reported to trigger some RNA sensors and induce innate responses (Edwards et al. 2017). Unmodified RNA was considered a strong stimulator of TLR3/7/8 (Kariko et al. 2005) and PKR (Anderson et al. 2010). On the other side, the activation of certain RNA sensors may inhibit mRNA translation in cell cytosol (Pardi et al. 2018). For instance, activated PKR inhibits cap-dependent translation by phosphorylating eukaryotic translation initiation factor 2A (eIF2A) (Anderson et al. 2010). Therefore, detailed characterizations are necessary for each specific naked mRNA vaccine (Pardi et al. 2018).

When developing naked mRNA vaccines, the buffer is an essential component to be chosen carefully. Ringer's solution (Ringer 1882) and Ringer's lactate (Hartmann and Senn 1932; Lee 1981) are two commonly used buffers for dissolving and diluting naked mRNA vaccines before injection. Both buffers contain calcium which was suggested to trigger the uptake of mRNA into human cells via a calcium-dependent route (Probst et al. 2007). Ringer's solution was used in a clinical trial against melanoma (Sahin et al. 2017). In that trial, naked personalized mRNA vaccines were diluted in Ringer's solution, injected into patients' lymph nodes, and induced antigen-specific T-cell response (Sahin et al. 2017). Ringer's lactate was used to dissolve the mRNA encoding influenza A hemagglutinin antigen (Edwards et al. 2017). This mRNA solution expressed the antigen and stimulated innate response by triggering cellular RNA sensors in mouse models and primary human peripheral blood mononuclear cells (PBMC) (Edwards et al. 2017).

Naked mRNA vaccines are more susceptible to the delivery obstacles, namely, RNase degradation and intracellular delivery (Singer and Linderman 1990; Canton 2018). However, the obstacles might be partially alleviated by local administration of naked mRNA vaccines via intramuscular (Ying et al. 1999; Fleeton et al. 2001), intradermal (Edwards et al. 2017), intranodal (Kreiter et al. 2010; Bialkowski et al. 2016; Joe et al. 2019), intratracheal (Tiwari et al. 2018) or intranasal (Lorenzi et al. 2010) routes to minimize the contact of mRNA with RNases in the bloodstream. Direct exposure of immune cells with a higher dose of naked mRNA enhanced expression (Diken et al. 2011; Lorenz et al. 2011; Selmi et al. 2016).

In recent clinical trials, naked mRNA vaccines were administered via ultrasound-guided intranodal injection (Sahin et al. 2017; Leal et al. 2018). Repeated IN injection of naked mRNAs was well-tolerated and induced a various degree of specific immune responses against tumor or HIV-1 (Sahin et al. 2017; Leal et al. 2018).

3.3 Dendritic Cells-Based mRNA Vaccines

Therapeutic vaccination needs to effectively elicit the body's adaptive immunity. During the initial development of adaptive immune response, antigen-presenting cells (APCs) internalize, process and present antigens to functional lymphocytes. As the most efficient APCs, dendritic cells (DCs) can present antigens processed from various sources, for example, the captured microorganisms, virus-infected cells, and tumor cells (Wculek et al. 2019). Several special characteristics make DCs suitable vaccination targets, including their T-cell-oriented migration in the lymph nodes and high expression of major histocompatibility complex (MHC) molecules, co-stimulators, and cytokines (Garg et al. 2017). In addition, DCs can be loaded with various forms of antigens and stimulatory signals and are highly amenable to such modifications (Pardi et al. 2018). An early study revealed that the inoculation of antigen-pulsed DCs primed T-cell-dependent immune response (Inaba et al. 1990). A few years later, a DC-based mRNA vaccine was reported (Boczkowski et al. 1996). In that report, DCs were pulsed with the mRNAs expressing chicken ovalbumin (OVA). The tumor-bearing mice were then vaccinated with such mRNA-pulsed DCs and were protected against the subsequent challenge of OVA-expressing tumor cells (Boczkowski et al. 1996). From then on, preclinical and clinical studies began testing DC-based mRNA vaccines against infectious diseases and cancers.

Autologous DCs from primary human PBMC are the main sources for preparing mRNA-treated DCs for in vivo applications (Benteyn et al. 2015). For further stimulation and maturation, the DCs were transfected with mRNAs encoding specific antigens and maturation signals (Benteyn et al. 2015). Next, the mRNA-transfected DCs were validated with their phenotypes and functions, then re-introduced back to the patients to function as antigen-specific APCs (Benteyn et al. 2015).

To deliver mRNAs into DCs, several strategies, such as electroporation and lipid-derived carriers, were employed (Boczkowski et al. 1996; Van Tendeloo et al. 2001; De Temmerman et al. 2011). Electroporation is the most frequently used method for generating DC-based mRNA vaccines due to its high mRNA delivery efficiency (Van Tendeloo et al. 2001). Several DC-based mRNA vaccines prepared by electroporation were evaluated in clinical trials (Wilgenhof et al. 2013; Mitchell et al. 2015; Batich et al. 2017). Electroporation disrupted the cell membrane by an electric shock to enable intracellular nucleic acid delivery (Stewart et al. 2016). Important electrical characteristics, such as voltage, capacitance, and resistance, were adjusted to improve the delivery efficiency (Van Tendeloo et al. 2001; Derdelinckx et al. 2016). Other parameters, including electroporation solution, pulse time, cell number, density, and RNA quantity should also be optimized. Under the optimized condition, the mRNA-loaded DCs should maintain their biological properties, including cell phenotypes, maturation status, cytokine secreting ability, and antigen presentation function (Tuyaerts et al. 2002; Tateshita et al. 2019). Besides electroporation, lipid-derived carriers were also tested to deliver mRNA into DCs for the preparation of DC-based mRNA vaccines (De Temmerman et al. 2011; Tateshita et al. 2019). In one study, ionizable lipid-based LNPs were used to deliver tumor antigen OVA-mRNA into DCs. The resulting ex vivo DC-based mRNA vaccine showed prophylactic anticancer efficacy and inhibited the growth of OVA-expressing cancer cells in mice (Tateshita et al. 2019).

The routes for administration of mRNA-loaded DCs mainly include ID, SC, IV, and IN injections (Benteyn et al. 2015). These routes were chosen because they delivered mRNA-loaded DCs to where native DCs function in the body. Different routes of administration may exhibit different DCs distribution patterns in vivo. The distribution of mRNA-loaded DCs was compared in metastatic cancer patients after three ways of delivery: IV, ID, and SC injections (Morse et al. 1999). After IV injection, the DCs loaded with an Indium-111-labeled mRNA encoding a carcinoembryonic antigen localized to the lungs within one hour, followed by redistributing to other organs, including liver, spleen, and bone marrow. However, no DCs were found in local lymph nodes. After ID injection, a small number of DCs were detected in proximal lymph nodes in some patients. After SC injection, no radioactivity was observed in the draining lymph nodes. These results suggested IV and ID were superior to SC for administering mRNA-loaded DCs (Morse et al. 1999). Therefore, a combined IV and ID administration method was chosen in a clinical trial (Van Nuffel et al. 2012). The autologous DCs from one melanoma stage IV-M1c patient were electroporated with a mixture of three mRNAs encoding melanoma-associated antigens and three mRNAs encoding immunostimulatory proteins. After the combined IV and ID administration, the patient obtained a durable clinical response, including stable disease and partial response based on the response evaluation criteria in solid tumors (RECIST) (Eisenhauer et al. 2009). The tumor antigen-specific CD8+ and CD4+ T-cell response were also detected in peripheral blood and skin biopsies (Van Nuffel et al. 2012).

In summary, the DC-based mRNA vaccines have shown efficacy in many preclinical and clinical studies. In one recent clinical trial (NCT00639639), the

long-term progression-free survival (PFS) and overall survival (OS) were significantly increased in glioblastoma patients who were injected intradermally with autologous DCs pulsed with an antigen-encoding mRNA(Batich et al. 2017).

Taken together, the formulation and delivery of mRNA vaccines have been extensively studied. The delivery formats and delivery materials described above have advanced to various stages of preclinical and clinical studies. However, each delivery technology has its advantages and challenges which are summarized in Table 3. Their readiness for human use is also listed.

Table 3 Summary of the delivery strategies of mRNA vaccines

Delivery format	Advantages	Challenges	Readiness for human[a]
Lipid-based nanoparticles	• Protect mRNA from RNase degradation • Efficient intracellular delivery of mRNA • High reproducibility • Easy to scale up	• Potential side effects	Clinical trials
Polymer-based nanoparticles	• Protect mRNA from RNase degradation • Efficient intracellular delivery of mRNA	• Potential side effects • Polydispersity	Preclinical mouse model
Protamine	• Protect mRNA from RNase degradation • Protamine-mRNA complex has adjuvant activity	• Low delivery efficiency • mRNA complexed with protamine is translated poorly	Clinical trials
Other peptides	• Protect mRNA from RNase degradation • Peptides offer many functions to be exploited	• Low delivery efficiency	Preclinical mouse model
Virus-like replicon particle	• Protect mRNA from RNase degradation • Efficient intracellular delivery of self-amplifying mRNA • Strong expression	• Challenging to scale up • Antibody production against viral vectors	Clinical trials
Cationic Nanoemulsion	• Protect mRNA from RNase degradation • Squalene-based CNEs have adjuvant activity • Formulation can be prepared and stored without RNA for future use • Easy to scale up	• Limited delivery efficiency	Preclinical mouse model

(continued)

Table 3 (continued)

Delivery format	Advantages	Challenges	Readiness for human[a]
Naked mRNA	• Easy to store and prepare • Easy to scale up	• Prone to RNase degradation • Low delivery efficiency	Clinical trials
DCs	• Efficient APCs critical for innate/adaptive immunity • Biocompatibility	• Heterogeneous cell population • Complex process to manipulate and characterize DCs	Clinical trials

[a]See Chap. 7 of this book for clinical development

3.4 Co-delivery of mRNA Vaccines

Several mRNA molecules can be co-delivered to trigger synergic effects in vaccination. Co-delivery of mRNA vaccines enables either assembly of protein complexes, generation of multivalent mRNA vaccines, or better immune response against one specific target. The co-delivered mRNAs can be a combination of conventional mRNAs and/or self-amplifying mRNAs. There are many co-delivery options. Several mRNAs can be delivered naked or formulated, complexed together or individually, and injected through different routes at different times. In this section, we summarize the recent results for the co-delivery of mRNA vaccines, including delivery formats, dose ratios, formulation methods, and injection routes of the components. Table 4 lists representative examples for different applications of co-delivered mRNA vaccines.

3.4.1 Co-delivery of mRNAs to Assemble Protein Complexes

Antibodies, such as immunoglobulin G (IgG), and some antigens are assembled from more than one single-chain protein subunits. Co-delivery of mRNAs is an option to express these multi-subunit proteins to provide passive immunity or stimulate adaptive immune responses. All subunits need to be translated into one cell and assembled into a complex in the endoplasmic reticulum (ER), followed by translocation to their destinations (Ellgaard et al. 2016). Several methods were used to co-deliver multiple nucleic acids into the same cell. A cationic copolymer co-formulated an mCherry mRNA with FITC-CpG ssDNA and delivered them to the same cells in vitro, according to the flow cytometry results (Haabeth et al. 2018). Electroporation delivered into K562 cells two mRNAs each of which encoding half of an engineered IgG against a tumor-associated antigen, the tight-junction proteins claudin 6 (Stadler et al. 2017). The secreted whole IgG complex was detected in the supernatant by immunoblotting and induced better

Table 4 Co-delivery of mRNAs in vaccination

Purpose of co-delivery	Vaccine target	Co-delivered mRNAs	mRNA ratio	Carrier	Co-formulation method	Species	Route	References
Assemble protein complexes	Human CMV	Five mRNAs encoding five subunits of pentameric complex	1:1:1:1:1 (mass ratio)	LNP	Mix mRNAs before formulation	Mouse Monkey	IM	John et al. (2018)
	Respiratory Syncytial Virus	IgG light-chain mRNA IgG heavy-chain mRNA	1:4 (mass ratio)	Naked or two PEI derivatives	Mix mRNAs before formulation	Mouse	Intratracheal	Tiwari et al. (2018)
	Cancer	Two mRNAs each encoding half of an engineered IgG	1:1 (mass ratio)	Naked in electroporation buffer	Mix without formulation	Cell line	Electroporation in vitro	Stadler et al. (2017)
Express multiple antigens against the same pathogen/cancer	Herpes simplex virus type 2	Three mRNAs encoding glycoproteins C/D/E	1:1:1 (mass ratio)	LNP	Mix mRNAs before formulation	Mouse Guinea pig	IM ID	Awasthi et al. (2019)
	Toxoplasma gondii	Six self-amplifying mRNAs encoding antigens	1:1:1:1:1:1 (molar ratio)	Dendrimer nanoparticle	Mix mRNAs before formulation	Mouse	IM	Chahal et al. (2016)
	Cancer	Two mRNAs encoding ten cancer neo-antigens	1:1 (mass ratio)	Naked in Ringer's solution	No mixing, injected into separate lymph nodes	Human	IN with ultrasound	Sahin et al. (2017)
Develop multivalent vaccine against multiple pathogen strains	Influenza	Three self-amplifying mRNAs encoding hemagglutinins from three Influenza strains	1:1:1 (mass ratio)	PEI	N/A	Mouse	IM	Vogel et al. (2018)

(continued)

Table 4 (continued)

Purpose of co-delivery	Vaccine target	Co-delivered mRNAs	mRNA ratio	Carrier	Co-formulation method	Species	Route	References
Express antigen and immunostimulatory protein (s)	Influenza	Self-amplifying mRNA encoding nucleoprotein; self-amplifying mRNA encoding murine GM-CSF	1:1 (mass ratio)	Cationic nanoemulsion	Formulated separately, mixed before injection	Mouse	IM	Manara et al. (2019)
	HIV	TriMix mRNAs encoding CD40L, CD70, and TLR4; another mRNA encoding a fusion peptide with different epitopes	1:1:1[a] (mass ratio)	Naked in Ringer's lactate	Mix without formulation	Human	IN with ultrasound	Leal et al. (2018)
Express antigen while providing adjuvant activity	Cancer	RNActive: free mRNA and protamine-mRNA complex	1:1 (mass ratio)	Naked in Ringer's lactate Protamine	Add free mRNA after protamine-mRNA complex formation	Human	ID	Sebastian et al. (2019)

Routes: *ID* intradermal, *SC* subcutaneous, *IV* instantaneous, *IM* intramuscular and *IN* intranodal administration

[a]Indicates the mass ratio of the three TriMix mRNAs. Various mass of the antigen-encoding mRNA was tested

cytotoxicity against tumor cells in vitro than a single-chain bi-specific antibody expressed from one mRNA (Stadler et al. 2017). In another study, two mRNAs encoding heavy and light chains of one IgG, palivizumab, were administrated as an intratracheal aerosol to mouse lungs against the respiratory syncytial virus (RSV) (Tiwari et al. 2018). The two mRNAs showed similar in vivo distribution on tissue and single-cell levels. All three delivery formats tested as aerosol, naked mRNA or two PEI-derived formulations (Viromer RED and in vivo-jetPEI), reduced RSV infection after viral challenge (Tiwari et al. 2018). To develop an anti-hCMV vaccine, six conventional mRNAs encoding five subunits of the hCMV pentameric complex (PC) and one glycoprotein, respectively, were co-delivered by LNP in equal mass (John et al. 2018). Such a delivery enabled PC expression in vitro and induced specific anti-PC antibody production in mice and monkeys after IM injection. Notably, the five subunits of hCMV PC need to be expressed and assembled into a complex by the same cell to be immunogenic (Macagno et al. 2010; Gerna et al. 2017). Manufacturing, transporting, and storing a purified PC protein vaccine while maintaining its stability is challenging (Nelson et al. 2018). Co-delivery of mRNAs circumvents such a challenge by enabling the PC production in the cells (John et al. 2018), potentially lowering the logistical requirement and reducing the vaccine cost. This study also indicated that LNP had the potential to deliver multiple conventional mRNAs into one cell both in vitro and in vivo (John et al. 2018).

3.4.2 Co-delivery of mRNAs Encoding Multiple Antigens

Two or more independent antigen-coding mRNAs can be co-delivered to enhance and broaden immune responses. To enhance immunity against one target, six VEEV self-amplifying mRNAs each encoding one antigen from the same parasite, Toxoplasma gondii, were co-formulated in an equal molar ratio by a PEI-based monodispersed ionizable dendrimer nanoparticle. IM injection of the co-formulated self-amplifying mRNA vaccine protected mice from the lethal challenge (Chahal et al. 2016). In another example, Sahin and colleagues simultaneously delivered two mRNAs encoding a total of ten neo-epitopes from melanoma (Sahin et al. 2017). A variety of responses were observed in all patients, ranging from epitope-induced T-cell response, reduced metastasis to progression-free survival (Sahin et al. 2017). To broaden immunity with a multivalent mRNA vaccine, three self-amplifying mRNAs encoding hemagglutinin (HA) from three different influenza virus strains were formulated by a medium-length PEI in equal mass, co-delivered to mice intramuscularly, and protected mice against viral challenge (Vogel et al. 2018).

When co-delivering several antigen-encoding mRNAs, one challenge is to elicit potent specific immune responses to every antigen. The immunostimulatory activity of each antigen may be different. For example, two influenza virus antigens, nucleoprotein, and matrix protein 1 (M1) were expressed from two self-amplifying mRNAs (Magini et al. 2016). The two self-amplifying mRNAs were mixed in an equal amount, formulated together by LNP and delivered into mice intramuscularly. The mouse group immunized with two co-delivered self-amplifying mRNAs

showed similar immune response and protection as the group received the nucleoprotein self-amplifying mRNA alone. This was explained by the low immunostimulatory effect of the M1 antigen since the group injected with the M1 self-amplifying mRNA alone showed weaker immunogenicity and protection (Magini et al. 2016). A similar observation was reported in another study in 2018 (Vogel et al. 2018). One of the three hemagglutinins encoded by the self-amplifying mRNAs failed to reduce influenza viral RNA copies and elicit adequate protection in both monovalent and trivalent formats.

Even if each mRNA-encoded antigen triggers sufficient immune response when used alone, the co-delivery of several antigens may lead to competition in epitope presentation and diminished response. For example, one group generated seven antigen-encoding mRNAs in order to develop one anti-hCMV mRNA vaccine (John et al. 2018). One mRNA encoding a mutant pp65 antigen induced a strong specific cytotoxic T-cell response when used alone. However, after co-formulating this mRNA with six additional antigen-encoding mRNAs in equal mass by LNP and simultaneous intramuscular injection, the anti-pp65 response was barely above the negative control. Their further studies suggested the inhibition to pp65-specific response was likely due to the dominant response to the epitopes of the pentameric complex encoded by other co-delivered mRNAs. This epitope competition was alleviated by sequential injection of pp65-encoding mRNA on day 1 and all seven antigen-coding mRNAs on day 21 (John et al. 2018).

3.4.3 Co-delivery of mRNAs Encoding Antigens and Immunostimulatory Proteins

While antigen-encoding mRNAs trigger the adaptive immune response, co-delivery of mRNAs encoding immunostimulatory proteins boost innate response to enhance vaccine efficacy. For example, a recent vaccine study against influenza A virus employed two self-amplifying mRNAs: one encoding the influenza A virus nucleoprotein antigen and the other encoding murine immunostimulatory GM-CSF (Manara et al. 2019). The two self-amplifying mRNAs were formulated by CNE independently and mixed before simultaneous intramuscular injection to mice. Such injection enhanced immune cell recruitment to the muscle injection site, expanded the antigen-specific CD8+ T-cell counts and resulted in better survival upon the influenza challenge than other groups receiving a single antigen-encoding self-amplifying mRNA (Manara et al. 2019).

In one approach named TriMix, three protein-coding conventional mRNAs were used as immune-stimulators to enhance the dendritic cell-mediated immune response against cancer (Van Lint et al. 2012). The three proteins encoded by the mRNAs were CD40 ligand, constitutive active TLR4 and CD70. Co-electroporation of the three mRNAs in vitro outperformed any single-mRNA or two-mRNA electroporation for increasing the numbers of helper and cytotoxic T-cells (Bonehill et al. 2008). The three mRNAs were used in an equal mass ratio in mouse tumor models (Van Lint et al. 2012; Bialkowski et al. 2016; Van Lint et al. 2016; Guardo et al. 2017).

However, the total amount of the three mRNAs varied depending on specific applications and delivery routes. One mRNA encoding an antigen was commonly mixed with the three mRNAs and administered simultaneously to initiate specific immunity. The dose of the antigen-encoding mRNA was equal or several-fold larger than each of the three mRNAs encoding immunostimulatory proteins (Van Lint et al. 2012; Dewitte et al. 2014; Bialkowski et al. 2016; Van Lint et al. 2016; Guardo et al. 2017). The TriMix and antigen-coding mRNA mixture were co-delivered in vitro and ex vivo by electroporation (Bonehill et al. 2008; Van Lint et al. 2012; Guardo et al. 2017) or sonoporation (Dewitte et al. 2014), and in vivo in 0.8 Ringer's lactate through intradermal (Van Lint et al. 2012), intranodal (Van Lint et al. 2012; Bialkowski et al. 2016; Guardo et al. 2017) or intratumoral (Jeught et al. 2014; Van Lint et al. 2016) routes. The intranodal co-administration of the TriMix and antigen-encoding mRNAs was reported to be superior to the intradermal route for boosting antigen-induced specific tumor lysis (Van Lint et al. 2012).

Another method of co-delivering mRNAs vaccines was called RNActive (Fotin-Mleczek et al. 2011). This method utilizes one antigen-encoding mRNA: 50% was naked in Ringer's lactate to express an antigen and 50% was complexed with protamine as an adjuvant (Kallen et al. 2013). This vaccine was formulated in two steps (Fotin-Mleczek et al. 2011). First, protamine in Ringer's lactate was added to the mRNA in a 1:2 mass ratio to form a stable protamine-mRNA complex. Second, the naked antigen-coding mRNA was mixed with the complexed mRNA in a 1:1 mass ratio. The final mass ratio of free mRNA, complexed mRNA, and protamine was 2:2:1 in Ringer's lactate. Based on this co-delivery method, vaccines against various types of viral infection (Petsch et al. 2012; Schnee et al. 2016; Alberer et al. 2017), and cancers (Weide et al. 2009; Fotin-Mleczek et al. 2014; Sebastian et al. 2014; Kubler et al. 2015; Hong et al. 2016) were developed. Further development of this method used four-to-six mRNAs encoding different tumor antigens against non-small cell lung cancer or prostate cancer (Kubler et al. 2015; Hong et al. 2016; Papachristofilou et al. 2019; Sebastian et al. 2019). In these studies, every antigen-encoding mRNA was a mixture of its free and protamine-complexed formats. The mRNAs were formulated and injected separately through the intradermal route into patients. The cancer vaccines were well-tolerated and induced immune responses. Interestingly, the delivery approach appeared to influence the vaccine efficacy of this co-delivery method. In the clinical trial evaluating RNActive mRNA vaccine against rabies virus, the needle-free injection induced neutralizing antibody titers in some participates, while needle-syringe injection was not effective. And using the needle-free injection, the ID route performed better than the IM route (Alberer et al. 2017). Yet, multiple clinical trials for the RNActive mRNA vaccines had shown moderate efficacy, such as a weaker antibody titer than available vaccines in the clinic against rabies virus (Alberer et al. 2017; Fooks et al. 2019) and low anti-tumor activity against several types of cancer (Rausch et al. 2014; Kubler et al. 2015; Sebastian et al. 2019). Subsequently, their next generation of rabies mRNA vaccine delivered by LNP is being tested in a clinical trial (NCT03713086). Additional clinical trials against cancers combined the RNActive mRNA vaccine with other therapies, such as

radiation therapy (Sebastian et al. 2014; Papachristofilou et al. 2019) or checkpoint inhibitors (NCT03164772).

Overall, the co-delivery of multiple mRNAs is a promising vaccination strategy. However, optimization is essential to determine the appropriate antigens to be expressed, delivery material, formulation method, mass ratio of components, and administration route. It is also necessary to examine whether the antigens expressed from the co-delivered mRNAs interfere with each other. If such interference is detected, modification of vaccination procedure, such as injection time, is likely needed to improve immune response.

4 Current Challenges and Future Perspectives

While many carriers are effective in delivering mRNA vaccines in preclinical studies and clinical trials, there are still challenges to be addressed. The first challenge is delivery efficiency. During the delivery process, a large portion of RNA-loaded carriers is trapped in endosome/lysosome or recycled out of cells by exocytosis (Sahay et al. 2013; Sayers et al. 2019), reducing the effective amount of RNA reaching the cytosol. Future developments that enhance endosomal escape and reduce exocytosis of nanoparticles would likely improve delivery efficiency. The second challenge is targeting specific cell types in vivo. Current delivery technologies often deliver mRNA vaccines indistinguishably into many different cell types at the injection site, many of which contribute little to immune stimulation (Veiga et al. 2018). Active in vivo targeting to specific cell types of interest, e.g., dendritic cells, macrophages, B cells, and T cells, have the potential to enhance immunization efficacy (Fenton et al. 2017). The third challenge is the safety of the delivery vehicles. Delivery materials, such as cationic lipids and polymers, may induce high delivery efficiency through enhanced membrane fusion, disruption of the endosome, or other mechanisms that might be associated with cell stresses, leading to potential cytotoxicity (Lv et al. 2006; Xue et al. 2014). Although some approaches have been explored to reduce cytotoxicity, such as using biodegradable materials (Zhang et al. 2017) and masking cationic charges (Taratula et al. 2011), delivery systems with a broad therapeutic index are still in urgent demand. The fourth challenge is the applicability to human. Effective immunization observed in preclinical animal studies may or may not be applied to human. The mRNA dose necessary to induce sufficient immune response in mice and other animals might not be directly correlated to humans. The differences in the immune systems between human and other animal species may lead to distinct immune responses (Shay et al. 2013; Zschaler et al. 2014). Therefore, clinical trials are of paramount importance for assessing the efficacy of mRNA vaccines in humans. Chapter 7 of this book reviews the clinical development of mRNA vaccines in details.

Meanwhile, the molecular mechanisms of the delivery process demand further investigation (Sahay et al. 2013; Iavarone et al. 2017; Sayers et al. 2019). Regardless of the delivery formats, carrier materials, and administration routes, our

knowledge is limited regarding the factors and pathways responsible for cellular uptake, cytosolic release, endosomal escape, lysosomal degradation, and exocytotic recycling of mRNA vaccines. A more profound understanding of these biological processes will facilitate the development of delivery materials and administration strategies, leading to more effective immunization by mRNA vaccines.

5 Conclusion

mRNA has demonstrated its potential as a vaccine platform. In clinical trials, mRNA vaccines encoding antigen proteins from rabies virus, influenza virus, and cancers induced humoral and cellular responses in healthy volunteers and patients (Alberer et al. 2017; Sahin et al. 2017; Feldman et al. 2019). However, improvements are still needed to optimize the safety profile and to increase the vaccination efficacy. When delivering mRNA vaccines, a comparison of several administration routes will help determine the most appropriate injection method and promote efficacy. The progress in the development of various delivery carriers has enabled numerous preclinical studies and clinical trials. LNPs represent one of the most advanced platforms among various carriers for mRNA vaccine delivery in vivo. DC-based mRNA vaccines have been tested in many clinical trials and have shown acceptable safety profiles (Garg et al. 2017), while therapeutic efficacy needs to be further increased (Perez and De Palma 2019). Besides the improvement in delivery carriers, co-delivery of mRNAs in vaccination can enhance efficacy and/or enable expression of antigen complexes. As the delivery methods and the vaccine formulations further advance, mRNA vaccines will become an important class of medicine to effectively tackle diverse health issues, such as infectious diseases and cancers.

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Messenger RNA-Based Vaccines Against Infectious Diseases

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Contents

Abstract In vitro-transcribed, messenger RNA-based infectious disease vaccines have the potential to successfully address many of the weaknesses of traditional vaccine platforms, such as the lack of potency and/or durability of vaccine protection, time-consuming, and expensive manufacturing, and, in some cases, safety

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issues. This optimism is fueled by a great deal of impressive recent data demonstrating that mRNA vaccines have many of the attributes that are necessary for a viable new vaccine class for human use. This review briefly describes mRNA vaccine types, discusses the most relevant and recent publications on infectious disease mRNA vaccines, and highlights the hurdles that need to be overcome to bring this promising novel vaccine modality to the clinic.

1 Introduction

Conventional vaccine approaches such as live-attenuated, inactivated, and subunit vaccines have played a fundamental role in the eradication or control of numerous infectious pathogens (Plotkin and Plotkin 2011; Younger et al. 2016). Despite significant progress and the unquestionably positive impact on public health, current vaccines face several major challenges as they do not often provide broad and long-lived protection against many critical human pathogens such as influenza virus, hepatitis B virus, *Mycobacterium tuberculosis,* and others, and, most importantly, they cannot respond to pandemic threats with sufficient rapidity (Rauch et al. 2018). Radical changes in population density, travel habits, climate change, and the rise of antibiotic resistance have favored the emergence and rapid spread of dangerous pathogens (e.g., mosquito and tick-borne pathogens) (Rauch et al. 2018; Bloom et al. 2017) and have highlighted the necessity for a global pandemic preparedness and efforts to develop both prophylactic and therapeutic interventions. As vaccines are one of the simplest and most cost-effective means of addressing the abovementioned issues, development of more effective next-generation vaccines that can be rapidly deployed to prevent or contain outbreaks is one of the most critical unmet needs in the field of medical research.

The concept of RNA-based therapeutics was conceived three decades ago following direct transfer of mRNA encoding transgenes to mice and demonstrating their subsequent expression in muscle (Wolff et al. 1990). The seminal findings of this study were confirmed over the next decade, demonstrating that injection of viral or cancer antigen-encoding *in vitro*-transcribed (IVT) mRNA elicited antigen-specific immune responses (Martinon et al. 1993; Conry et al. 1995; Hoerr et al. 2000). Despite these promising results, nuclease sensitivity, instability, poor *in vivo* expression, and high inflammatory capacity of IVT mRNA were perceived as major shortcomings, and consequently, mRNA was minimally pursued further for the development of vaccines (Sahin et al. 2014).

Recent scientific and technological advances (i.e., incorporation of modified nucleosides (Andries et al. 2015; Kariko et al. 2005, 2008), removal of contaminants from IVT mRNA preparations (Kariko et al. 2011; Baiersdörfer et al. 2019), optimization of the antigen coding and untranslated regions (UTRs) (Asrani 2018; Thess et al. 2015), optimization of the length of poly(A) tail, development of improved capping analogs (Vaidyanathan et al. 2018), and the use of safe and efficient mRNA delivery materials (Kowalski et al. 2019; Maruggi et al. 2019;

Pardi et al. 2018a) resolved most of the aforementioned issues and enabled rapid advancement of mRNA-based vaccines into multiple preclinical studies and clinical trials (Sahin et al. 2014; Pardi et al. 2018a). Importantly, proof-of-concept studies demonstrated that mRNA-based vaccines induced potent and broadly protective immune responses against various pathogens in small and large animals, had an acceptable safety profile, and can be rapidly designed and cGMP-manufactured using cell-free production systems (Maruggi et al. 2019; Scorza and Pardi 2018) positioning this technology to be competitive to conventional platforms and other types of genetic vaccines.

In this manuscript, we will briefly review mRNA vaccine formats and provide a detailed overview of the most recent vaccine studies targeting infectious diseases. Throughout the manuscript, we highlight how these preclinical studies potentially contribute to disease prevention or treatment and provide perspectives on the future of this promising technology.

2 mRNA Vaccine Formats and Delivery Materials

2.1 Optimization of mRNA Production

Development of an mRNA vaccine is a fairly straightforward process that begins with the identification of the target antigen(s), followed by the *in silico* design of the optimized antigen-coding sequence, gene synthesis, and cloning of the antigen into an optimized mRNA production plasmid. Gene synthesis and cloning are not necessary when the gene(s) of interest(s) is (are) amplified using polymerase chain reaction (PCR). In most cases, Sp6 or T7 RNA polymerase has been used for IVT; thus, a Sp6 or T7 promoter should be incorporated into the mRNA production plasmid or the PCR product. The mRNA production plasmid is linearized using restriction endonucleases, purified and subjected to IVT after the addition of the four nucleotide building blocks of mRNA plus RNA polymerase. Addition of the 5′ cap to the mRNA is critical and a stringent purification (removal of double-stranded RNA and other abortive products) of the final mRNA product is also often included in the production process. Finally, the optimized mRNA is formulated into a stable, nontoxic, biodegradable pharmaceutical product that protects mRNA from degradation (Sahin et al. 2014; Kowalski et al. 2019; Maruggi et al. 2019).

RNA-based vaccines offer several advantages over conventional vaccine platforms (Maruggi et al. 2019; Pardi et al. 2018a; Liu 2019 and Table 1). Besides safety and potency (detailed in Table 1), one of the most important benefits is the enormous flexibility of mRNA vaccine design and production. The antigen-coding sequence (open reading frame, ORF) can be quickly modified at specific locations and/or codon-optimized to improve translation or engineered to direct the antigen to the desired compartment (i.e., soluble versus transmembrane antigen versions) (Kreiter et al. 2010, 2015; Maruggi et al. 2017), and/or devised to improve antigen

Table 1 Advantages and disadvantages of various vaccine types

Vaccine type	Vaccine subtype		Advantages	Disadvantages
Genetic vaccines	IVT mRNA	**Conventional and self-amplifying mRNA (SAM)**	Cell-free (animal-free) production	Product instability due to spontaneous degradation (autocatalytic degradation) and high sensitivity to nucleases
			Rapid and scalable production compared with other platforms (i.e., protein subunit, viral vector)	Potential endothelial toxicity from free extracellular RNA
			Generic manufacturing process	Potential for detrimental innate immune activation and high level of inflammation
			Non-integrating, noninfectious, biodegradable through natural RNA degradation pathways	Limited available immunogenicity and toxicity data in humans
			In situ expression to produce antigens with structure unaltered by *in vitro* manufacturing process	Requires efficient and nontoxic delivery systems to mediate the intended effect
			Flexibility; immunogens can be designed and modified for improved immunogenicity (i.e., codon-optimization, insertion of mutation, removal of glycosylation sites, fusion with other proteins)	Coadministration of drugs to treat certain condition may impact mRNA metabolism and consequently affect the potency of mRNA vaccines (or drugs)
		Conventional mRNA	Generally shorter length (lower chance for spontaneous breaks) and simple architecture compared to SAM	Higher effective RNA doses needed to mediate the intended pharmacological effect
			High yields during IVT	Shorter duration of expression compared to SAM
			Amenable to nucleoside modification to reduce the inflammatory capacity and improve the translation	–
			No risk of anti-vector immunity	–
			RNA's inherent adjuvant activity can be fine-tuned	–

(continued)

Table 1 (continued)

Vaccine type	Vaccine subtype		Advantages	Disadvantages
		Self-amplifying mRNA (SAM)	Enhanced and prolonged antigen expression compared to conventional mRNA; potential improvement in the therapeutic dose	Potential difficulties with production due to the length of SAM constructs (10-12 kb)
			Intrinsic adjuvant effect	The interaction between nonstructural proteins and host factors has to be determined and demonstrated to be safe
			–	Potential adverse events due to elevated inflammation; SAM is not amenable to modification with modified nucleosides
			–	Potential for inducing apoptosis of cells carrying the vaccine due to self-amplification and saturation of the cellular translational machinery
	DNA	**Plasmid DNA**	Similar advantages to IVT RNA such as rapid and scalable production processes, in situ expression, enhanced and prolonged antigen expression, design flexibility (i.e., codon-optimization, insertion of mutation, removal of glycosylation sites, fusion with other proteins)	Potential integration into the genome
			–	Cell-dependent production (different regulatory status compared to RNA)
			–	Poor immunogenicity in humans at high doses

(continued)

Table 1 (continued)

Vaccine type	Vaccine subtype		Advantages	Disadvantages
Conventional vaccine platforms	Viral vectored vaccines		Strong stimulation of immune responses including induction of potent antigen-specific T ($CD8^+$) and B cell immune responses in humans.	Induction of anti-vector immunity hindering repeated administration
			Broad tropism including dendritic cells (depends on the vector)	Difficulty and high cost of production, requiring highly trained personnel and advanced equipment
			High level of protein production from the transgene (depends on the vector)	
			–	Cell-dependent production (different regulatory status compared to RNA)
			–	Limited gene insert capacity (depends on the vector)
			–	Potential preexisting immunity may hinder the efficacy
	Recombinant protein subunit vaccines		Takes advantage of the recombinant DNA technology and its flexibility (same as genetic vaccines); immunogens can be designed and modified for improved immunogenicity (i.e., codon-optimization, insertion of mutation, removal of glycosylation sites, fusion with other proteins)	Difficulty and high cost of production, requiring highly trained personnel and advanced equipment
			Already on the market; extensive experience with these platforms helps further development	Solubility issues with some recombinant proteins, or modified proteins (e.g., transmembrane proteins (TM) insoluble unless TM removed)
			Production and quality can be controlled	Cell-dependent production (different regulatory status compared to RNA)
			–	Requires adjuvants to induce immune responses

(continued)

Table 1 (continued)

Vaccine type	Vaccine subtype		Advantages	Disadvantages
	Live-attenuated and inactivated vaccines			Potential reversion to pathogenic state of live-attenuated vaccines
			Extensive experience with these platforms helps further development	Toxicity issues with inactivated vaccines due to chemicals used during inactivation
				Requires repeated administration in most cases
				Not well characterized
			Intrinsic adjuvant properties	Difficulty and high cost of production, requiring highly trained personnel and advanced equipment
				Complicated and difficult release assay (e.g., yellow fever)
				Inactivation needs to be proven for regulatory acceptance and release (e.g., Poliovirus); complicated production depending on pathogenicity (e.g., BSL-2+ required for rabies inactivated vaccine)

presentation (Kreiter et al. 2010). Modifications such as point mutations, deletions, or removal of putative glycosylation sites can all potentially affect antigenicity, immunogenicity, and vaccine efficacy (Dowling et al. 2007; Richner et al. 2017a). Improved mRNA immunogens designed through reverse vaccinology (Rappuoli et al. 2016) can be readily designed and produced. Addition of exogenous sequences (for example ferritin domains) that permit the assembly of the mRNA-encoded antigen (or chimeric antigens) into viral-like particles (VLPs) or nanoparticles can likely improve immunogenicity and provoke robust immune responses (Melo et al. 2019). Although this strategy has not widely been used for mRNA vaccines, it has the potential to yield significantly improved vaccines, especially with regard to B cell responses. In addition to these modifications in the coding sequence, the half-life of mRNA, pharmacokinetics of protein expression (including magnitude and duration), and immunogenicity are all amenable to fine-tuning via modifications of the 5′ and 3′ UTRs, optimization of the length of the poly(A) tail, incorporation of modified nucleosides, utilization of various capping strategies, and purification of IVT mRNA (Vaidyanathan et al. 2018; Pardi et al. 2018a).

2.2 *mRNA Vaccine Types*

mRNA vaccines can be divided into non-replicating and self-amplifying mRNA (SAM) constructs; the pros and cons of these platforms have been extensively discussed in several recent publications (Sahin et al. 2014; Maruggi et al. 2019; Pardi et al. 2018a; Liu 2019 and Table 1) and will not be reviewed in detail in this manuscript. Both vaccine types have been successfully used against infectious diseases in various preclinical models, and some published clinical data using non-replicating mRNA vaccines are also available (Alberer et al. 2017; Bahl et al. 2017; Feldman et al. 2019); a review of the recent studies is presented in the next section.

Vaccines are typically administered through the intramuscular (IM), subcutaneous (SC), or intradermal (ID) routes to efficiently target antigen-presenting cells. As extracellular RNases are present in the skin and muscle tissues, it is critical to encapsulate or otherwise protect the antigen-coding IVT mRNA from rapid degradation. Apart from a limited amount of studies using naked mRNA for intranodal delivery (Kreiter et al. 2010; Van Lint et al. 2012; Van Lint et al. 2013; de Jong 2019; Guardo et al. 2017) or various mechanical methods such as electroporation or gene gun (Hoerr et al. 2000; Bugeon et al. 2017; Cu et al. 2013; Johansson et al. 2012; Sohn et al. 2001; Steitz et al. 2006), the vast majority of mRNA vaccine studies use carrier materials (Kowalski et al. 2019) to protect mRNA from rapid *in vivo* degradation, deliver mRNA to the cytoplasm of cells, and even to drive potent, protective immune responses against pathogens.

2.3 Delivery Materials for mRNA Vaccines

Delivery materials for mRNA have recently been discussed in detail (Kowalski et al. 2019; Hajj and Whitehead 2017; Li et al. 2019); therefore, in this review, we briefly summarize the general requirements for the development of efficient and safe delivery systems and concisely describe the formulations used in infectious disease mRNA vaccines.

The bench-to-bedside translation of mRNA vaccines relies on our ability to overcome technical difficulties related to the development of delivery systems that are able to encapsulate, protect, and deliver the cargo at high efficiency to its pharmacological site of action without overt toxicity. Other important attributes that need to be addressed for viable translation to human trials include rapid tissue clearance, potential adjuvant activity, targeted delivery to critical tissues and cell types (i.e., spleen, lymph nodes, or antigen-presenting cells), colloidal stability, and the development of manufacturing processes that ensure scalability, stability, and low cost of the mRNA drug product (Kowalski et al. 2019; Pardi et al. 2018a; Hajj and Whitehead 2017; Li et al. 2019; Yin et al. 2014; Yin et al. 2017).

The cationic polypeptide, protamine, has been used to condense mRNA owing to its high positive charge density and was shown to induce protective immunity in animals following the activation of the MyD88 pathway (Hoerr et al. 2000; Scheel et al. 2004; Scheel et al. 2005); however, mRNA-protamine particles demonstrated limited protein expression possibly due to the strong binding affinities between the two polyelectrolytes (Fotin-Mleczek et al. 2011; Schlake et al. 2012). This issue was subsequently resolved through the development of the RNActive® vaccine platform that utilizes the coadministration of noncoding RNA-protamine adjuvant particles (2:1 mass ratio) and an uncomplexed, unmodified (absence of modified nucleosides) sequence-optimized mRNA encoding the vaccine antigen (Rauch et al. 2017; Kallen et al. 2013). RNActive® vaccines induced protective immunity against lethal challenges with a variety of influenza virus strains and rabies virus in preclinical models (Petsch et al. 2012; Schnee et al. 2016; Stitz et al. 2017). Importantly, the RNActive® rabies vaccine was evaluated in Phase I clinical trial (Alberer et al. 2017). The vaccine offered short-lived antigen-specific antibody responses in the needle-free arm of the study (using an injector for vaccine delivery), and, surprisingly, no measurable effect after administration with syringe needles.

Polymers (non-lipid-based) have been extensively investigated as nucleic acid delivery systems and have made promising advances, but their clinical application remains uncertain due to potential toxicity, colloidal instability, relatively poor transfection efficiency, and high polydispersity (wide range of particle size). Chahal and colleagues chemically synthesized a modified dendrimer and formulated it with a lipid-anchored polyethylene glycol (PEG) and an antigen-encoding self-amplifying mRNA using a microfluidic device. The modified dendrimer formed monodisperse nanoparticles (MDNP) and elicited antigen-specific $CD8^+$ T cell and neutralizing antibody responses against several pathogens (Zika, Ebola,

and influenza viruses and *Toxoplasma gondii*) in mice after administration of a single intramuscular dose (Chahal et al. 2016, 2017). Multiplexing several self-amplifying mRNA-encoded antigens was protective in mice, highlighting the possibility of producing a single, multivalent vaccine formulation that can be used against different pathogens. Polyethyleneimine (PEI), a cationic polymer, can be synthesized with a linear or branched architecture, and its molecular weight (chain length), branching degree, or charge density can easily be fine-tuned. PEIs bind nucleic acids with high affinity and are endowed with the capacity to escape from the endosome, likely by means of the proton sponge effect (Akinc et al. 2005; Demoulins et al. 2016). Linear PEIs have demonstrated decreased *in vitro* toxicity and improved *in vivo* tolerability profile compared to their branched counterparts (Taranejoo et al. 2015). Linear PEIs, with or without modifications, have been used to develop preclinical RNA-based vaccines against influenza virus and HIV (Demoulins et al. 2016; Zhao et al. 2016; Li et al. 2016).

Lipid nanoparticles (LNPs) containing ionizable lipids represent the most advanced nucleic acid carrier enabling efficient *in vivo* delivery of mRNA (Kowalski et al. 2019; Hajj and Whitehead 2017). The development of LNP systems for mRNA delivery and vaccine applications leverages the extensive preclinical and clinical knowledge acquired during the development of nonviral delivery systems for clinical application of siRNA (Kowalski et al. 2019; Kanasty et al. 2013). LNPs have been extensively used to deliver both conventional and self-amplifying mRNA against infectious pathogens (Kowalski et al. 2019; Maruggi et al. 2019; Pardi et al. 2018a; Hassett et al. 2019); the efficacy results of these preclinical and clinical studies will be discussed in detail in the following section.

LNP formulations are typically composed of precise molar ratios of an ionizable cationic lipid (e.g., KC2, MC3, and C12-200), a zwitterionic lipid (i.e., dioleoylphosphatidylethanolamine or DOPE), cholesterol, and lipid-anchored PEG (Cullis and Hope 2017) (Fig. 1). Electrostatic interaction between the cationic/ionizable head group and the negatively charged mRNA generates lamellar structures with positively charged lipid bilayers separated from one another by sheets of negatively charged nucleic acids (Cullis and Hope 2017; Schroeder et al. 2010). Ionizable lipids have been rationally designed to improve potency and tolerability of LNPs (Cullis and Hope 2017; Semple et al. 2010; Sabnis et al. 2018). On the other hand, zwitterionic lipids are incorporated in LNPs to modulate vesicle fluidity, improve ion pairing with membrane phospholipids, and promote endosomal release of the cargo (Balazs and Godbey 2011). Inclusion of cholesterol enhances LNP product quality attributes (i.e., extended shelf life), decreases spontaneous dissociation in circulation (*in vivo*) through increased structural (physical) support of the nanoparticle membrane, and improves endosomal release through a thermodynamically favored process of nanoparticle–plasma membrane rearrangement (Takahashi et al. 1996). PEG-anchored lipids are formulated at low molar ratios (1–2%) to improve colloidal stability, reduce surface adsorption of proteins, slightly extend circulation half-life ($\sim$10 to 15 min), and improve surface hydration (Semple et al. 2010; Semple et al. 1998; Blanco et al. 2015).

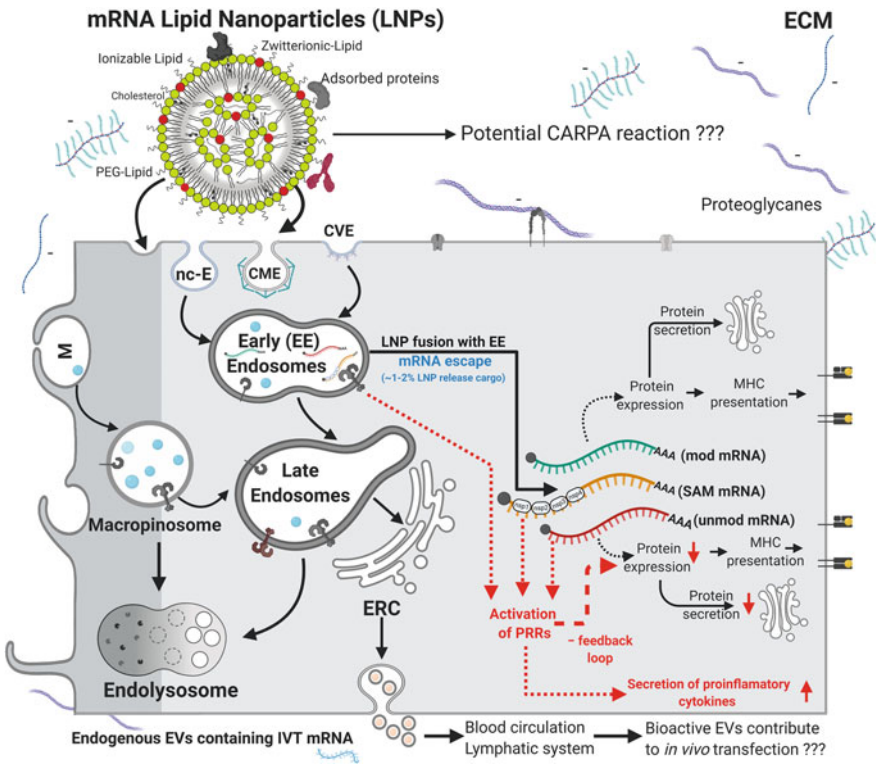

Fig. 1 Cellular uptake and endocytic pathways of mRNA-LNPs and mRNA release to the cytoplasm. mRNA-LNPs enter the cells through endocytosis (i.e., clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis), traffic via the endosomal network and exocytose in the form of endogenous endocytic vesicles (exosomes or extracellular vesicles). A small portion of mRNAs escape from the early endosome and enter the cytoplasm where they are translated into proteins that can be directed to various intra- or extracellular compartments (i.e., cytosol, extracellular space, or plasma membrane). Unmodified mRNAs (either non-replicating or SAM) are detected by pattern recognition receptors (PRRs). PRR activation induces secretion of pro-inflammatory cytokines (e.g., type I interferons) that decrease protein expression and secretion from the mRNA (arrow pathway in red). Green circles represent ionizable lipids while red circles represent zwitterionic lipids (i.e., DOPE) used to prepare lipid nanoparticles. Light blue circles in the macropinosome, early and late endosomes represent endocytosed lipid nanoparticles while light orange circles exiting the cells (exocytosis) represent endogenously formed extracellular vesicles (EndoEVs) containing IVT mRNA and ionizable lipids from endocytosed LNPs. mRNA—messenger RNA; CARPA—complement activation-related pseudoallergy; EE—early endosome; mod mRNA—nucleoside-modified mRNA; MHC—major histocompatibility complex, LNP—lipid nanoparticle; PEG—polyethylene glycol; M—macropinocytosis; ncE—non-classical endocytosis (not CME, not CVE); CME—clathrin-mediated endocytosis; CVE—caveolin-mediated endocytosis; EV—endocytic vesicles or exosomes; ERC—endosomal recycling complex; ECM—extracellular matrix; PRR—pattern recognition receptor; IVT—in vitro-transcribed; SAM—self-amplifying mRNA; nsp—nonstructural protein

LNPs enter the cells via a two-phase process that includes rapid internalization via clathrin-mediated endocytosis followed by macropinocytosis (Gilleron et al. 2013; Sahay et al. 2010; Sahay et al. 2013). In both cases, LNPs traffic through the endosomal pathway and a small fraction of the cargo (1–2%) enters the cytosol after disruption of the endosomal membrane (Gilleron et al. 2013; Martens et al. 2014; Wittrup et al. 2015) while remaining LNPs get degraded in the lysosomes (Rehman et al. 2013) or undergo exocytosis (Sahay et al. 2013; Maugeri et al. 2019) (Fig. 1). The precise mechanisms by which LNPs escape from endosomes are not well understood (Sahay et al. 2010, 2013; Patel et al. 2017; Vermeulen et al. 2018), and seem to involve less the proton sponge effect, and depend more on multiple factors including leakiness following fusion with the endolysosomal membrane (Sabnis et al. 2018; Patel et al. 2017; Vermeulen et al. 2018). A recent study from Maugeri and collaborators has shown that the exocytosis of mRNA-LNPs occurs by means of extracellular vesicles originating from endosomes (EndoEVs, exosomes) (Maugeri et al. 2019). EndoEVs were bioactive in mice and exhibited a biodistribution profile similar to LNPs, suggesting that part of the *in vivo* mRNA delivery (therapeutic effect) could be achieved by such EndoEVs (Maugeri et al. 2019).

Although LNPs are promising delivery systems, safety issues need to be addressed to enable proper clinical development of LNP-formulated mRNA vaccines. LNPs' potential toxicity could be complex and might manifest in systemic effects due to innate immune activation (induction of pro-inflammatory cytokine production), and/or in local, cellular toxicity due to accumulation of lipids in tissues (Hassett et al. 2019; Semple et al. 2010; Sabnis et al. 2018). Toxicity could potentially be abrogated, or reduced, by the administration of prophylactic anti-inflammatory steroids or other molecules and/or using biodegradable lipids (Hassett et al. 2019; Abrams et al. 2010; Tabernero et al. 2013; Tao et al. 2011). LNPs can also activate the complement system and might potentially elicit a hypersensitivity reaction known as complement activation-related pseudoallergy (CARPA) (Dezsi et al. 2014; Mohamed et al. 2019; Szebeni 2005, 2014), which can be alleviated using different strategies such as steroid and anti-allergic premedication (i.e., dexamethasone, acetaminophen, and antihistaminic drugs) or the use of low infusion rates during intravenous administration (Mohamed et al. 2019; Szebeni et al. 2018). Alternatively, co-delivery of regulatory cytokines (i.e., IL-10) using LNPs might be a viable strategy to reduce potential LNP-associated adverse events.

Published clinical data on the safety of mRNA-LNP vaccines are scarce, in comparison with siRNA, and are limited to local administration (ID and IM). A nucleoside-modified mRNA-LNP vaccine against H10N8 influenza virus induced immune responses (100% seroconversion) and displayed mild to moderate reactogenicity in most patients (20/23) (Feldman et al. 2019) after IM administration of a single dose of 100 μg vaccine, whereas strong local reactogenicity and systemic side effects were observed in three patients (3/23). Administration of the second dose of 100 μg vaccine induced moderately higher incidence of adverse events. The highest vaccine dose (400 μg) was administered only once due to concerns about higher incidence of grade 3 events. In line with the findings in

humans, Hasset and collaborators demonstrated in preclinical studies that the extended presence of MC3 [ionizable lipid used in the clinical study (Feldman et al. 2019)] at the site of injection, as well as in organs such as spleen and liver, was associated with increased toxicity (Hassett et al. 2019). Ionizable lipids with ethanolamine head groups and ester-containing lipid tails (biodegradable) were synthesized as an alternative to MC3-based LNPs to enable rapid *in vivo* metabolism (Sabnis et al. 2018) (reduce toxicity). These new LNPs displayed improved tolerability despite systemic IL-6 detection in some nonhuman primates after IM delivery with multiple mRNA-encoded antigens against influenza and Zika viruses (Hassett et al. 2019). These encouraging clinical (Feldman et al. 2019) and preclinical data (Bahl et al. 2017) warrant the investigation whether local cellular toxicities and/or CARPA are involved in the observed adverse events and highlight the need to further investigate LNPs in toxicological studies, elucidate the mechanism of induction of toxicity, and develop novel LNP materials for mRNA vaccine delivery.

3 Preclinical and Clinical Application of Infectious Disease mRNA Vaccines

The literature of infectious disease mRNA vaccines has been rapidly expanding. Multiple preclinical studies have demonstrated that this relatively new vaccine type has the ability to induce protective immune responses against viral, bacterial, and parasitic pathogens (Maruggi et al. 2019; Pardi et al. 2018a; Scorza and Pardi 2018). Importantly, comparative preclinical studies have shown that some mRNA vaccines are superior to conventional live-attenuated, inactivated pathogen, and adjuvanted protein subunit vaccines, further demonstrating the promise of this vaccine type. Although several clinical trials are underway or have recently ended, only a limited amount of clinical data has been published to date. In this section, we will review the current standing of the field of infectious disease mRNA vaccines and put the results in context by briefly discussing the potential requirements of protective vaccines against various pathogens and how mRNA vaccines may meet these requirements. As a seminal review paper has recently been published on this topic (Maruggi et al. 2019), we aim to mainly focus on new studies that have not been discussed elsewhere.

3.1 Influenza Virus mRNA Vaccines

Influenza virus infection causes significant morbidity and mortality every year. Currently, available influenza vaccines induce variable levels ($\sim$10 to 60%) of protection from the seasonal circulating strains and need to be reformulated

annually due to viral antigenic drift and shift. Production of currently used vaccine types requires eggs or cell culture systems that all have significant limitations and downsides such as the development of egg-adapted mutations by the virus that reduces vaccine efficacy, difficulties with protein purification, and concerns about optimal folding and glycosylation pattern of protein subunits produced in insect or mammalian cells (An et al. 2013; Settembre et al. 2014; Wu et al. 2017; Zost et al. 2017). Additionally, production of FDA-approved influenza vaccines may take several months, which is suboptimal for achieving protection from infection in the majority of the population. As the production of mRNA vaccines is fast (several weeks), requires only cell-free systems, and is completely sequence-independent, all major current problems related to influenza vaccine manufacturing can easily be overcome (Hekele et al. 2013).

Several early publications demonstrated that both self-amplifying and non-replicating mRNA vaccines induced strong, often completely protective immune responses against influenza virus infection in various preclinical models such as mice, ferrets, and pigs (Pardi et al. 2018a; and Scorza and Pardi 2018). Currently used clinical influenza vaccines (live or inactivated virus, adjuvanted protein subunits) display some level of protection from only the matched circulating strains; thus, it is worth emphasizing that some mRNA-based influenza vaccines have induced protective immune responses against antigenically distinct influenza virus strains (Petsch et al. 2012; Brazzoli et al. 2016; Joe et al. 2019; Pardi et al. 2018b). Brazzoli and colleagues demonstrated that two IM immunizations with A/California/07/2009 (A/Cal09) (H1N1) hemagglutinin (HA)-encoding SAM in an oil-in-water nanoemulsion elicited protection from homologous and heterologous (A/Puerto Rico/8/34, PR8) influenza virus challenge in mice and ferrets (Brazzoli et al. 2016). Petsch and coworkers showed that three immunizations with PR8 nucleoprotein (NP)-encoding protamine-complexed unmodified (not nucleoside-modified) mRNA vaccine induced protection from the homologous H1N1 and also from H5N1 (A/mallard/Bavaria/1/2006) viruses in mice (Petsch et al. 2012). In a recent manuscript, Pardi and colleagues demonstrated that a single immunization with A/Cal09 HA nucleoside-modified and purified mRNA-LNPs induced protection from homologous and heterologous (PR8) virus infection in mice (Pardi et al. 2018b). Most importantly, two immunizations with A/Cal09 HA mRNA-LNPs protected animals from heterosubtypic H5N1 virus challenge. The authors also demonstrated that nucleoside-modified HA mRNA-LNP vaccination induced antibody responses against the immunosubdominant stalk region of HA that is a potential target for broadly protective (or universal) influenza vaccines. Joe and coworkers took an unusual approach and injected mice with A/NL/18/94 H3N2 NP-encoding unformulated (naked) mRNA into the lymph nodes (Joe et al. 2019). Two injections (seven days apart) with 17 μg of NP mRNA resulted in some level of protection from the heterologous PR8 virus challenge eight weeks after the second immunization.

A general limitation of many currently used vaccines (including influenza vaccines) is that they cannot overcome the inhibitory effects of maternal antibodies on *de novo* antibody responses elicited by vaccination in infants (Halasa et al. 2008;

Mooi and de Greeff 2007; Voysey et al. 2017). An elegant recent study by Willis and colleagues has demonstrated that unlike inactivated or live influenza virus vaccines, a nucleoside-modified mRNA-LNP influenza virus vaccine has the ability to partially overcome the inhibitory effects of maternal antibodies in mice (Willis et al. 2020). If these findings translate to humans, it will allow us to induce protective immune responses against influenza virus infection in very young infants (a particularly vulnerable group to many infectious diseases) even in the presence of maternal antibodies.

As discussed above, mRNA influenza vaccines have demonstrated considerable potency and breadth in preclinical models and have important beneficial features over traditional vaccine formats; thus, they are extensively studied and are in the product pipeline of several pharmaceutical companies (Scorza and Pardi 2018). However, the available clinical data are still limited and create some level of uncertainty in the field. Two research papers have reported on the results of two Phase I clinical trials (NCT03076385 and NCT03345043) using nucleoside-modified mRNA-LNPs encoding full-length H10 and H7 HAs (Bahl et al. 2017; Feldman et al. 2019). Healthy adults received placebo or HA vaccine twice three weeks apart. Multiple doses (10–400 μg) were tested for IM, and 25 and 50 μg doses for ID delivery, and safety and immunogenicity were evaluated. The vaccines proved to be fairly safe; however, several grade 3 adverse events were noticed at higher doses (400 μg IM and 50 μg ID), and, as a result, the second dose was not administered to participants enrolled in these groups. The vaccines were moderately immunogenic, two IM doses with 100 μg H10N8 mRNA-LNPs were sufficient to induce HA inhibition (HAI) titers of ≥ 40 in all participants three weeks after the administration of the second dose, and HAI titers sharply decreased over time and were undetectable at the lower dose groups 140 days after the second immunization. Overall, the findings of this important clinical trial demonstrate that nucleoside-modified mRNA-LNP influenza vaccines have the ability to induce humoral immune responses in healthy adults. Decreasing adverse events and improving vaccine immunogenicity and durability will be important in further development steps. Additionally, the flexibility of mRNA vaccine production enables the generation of multivalent influenza vaccines and vaccines that use optimized immunogens that may induce greater efficacy and breadth [discussed in (Scorza and Pardi 2018)].

It is worth emphasizing that mRNA-based influenza vaccines elicited much more potent immune responses in preclinical experiments than in clinical studies (e.g., high HAI titers and durable humoral immune responses after vaccination of naïve animals and moderate, short-lived humoral responses in people). This could be due to preexisting host immunity to live/killed virus in people (Henry et al. 2018) that inhibits or modulates immunogenicity to mRNA or real biological differences between species in how they recognize and respond to mRNA. The former raises the appealing possibility of using mRNA vaccines in infants/very young children who do not have influenza virus-specific antibodies and thus do not face the detrimental effects of preexisting influenza virus immunity.

3.2 HIV-1 mRNA Vaccines

Human immunodeficiency virus type 1 (HIV-1) is certainly one of the most difficult vaccine targets. HIV has various strategies to evade protective immune responses: HIV envelope (Env)—the sole surface glycoprotein target for broadly neutralizing antibodies (bnAbs)—contains a large number of host glycans that make it a poor immunogen and help to hide neutralization epitopes, and HIV Env mutates rapidly such that host anti-HIV antibodies cannot evolve fast enough to effectively combat the virus (Wei et al. 2003). Application of traditional vaccine platforms (detailed in the previous section) and immunization schemes for HIV has so far never led to the generation of bnAbs in humans; thus, the development of new vaccine types that may successfully target HIV is a critical unmet need in the field.

Several preclinical and clinical mRNA-based HIV vaccine studies have been published to date. A small number of clinical studies used an *ex vivo* approach to target HIV: Human autologous dendritic cells (DCs) were electroporated with mRNAs encoding HIV antigens—and in some cases, an immunostimulatory molecule (CD40L)—and then reinfused into the patients (Allard et al. 2012; Gandhi et al. 2016; Gay et al. 2018; Jacobson et al. 2016; Routy et al. 2010; Van Gulck et al. 2012). This approach is expensive and labor-intensive and most of these studies resulted in only moderate T cell activation. To overcome the technical difficulties of *ex vivo* DC treatment, recent studies proposed an *in vivo* DC targeting strategy: Intranodal administration of naked mRNAs encoding critical HIV-1 target epitopes from Gag, Pol, Vif, and Nef and a combination of immunostimulatory molecules (TriMix: CD40L, CD70, and a constitutively active TLR4) (de Jong 2019; Guardo et al. 2017). Similarly, to the *ex vivo* approach, moderate antigen-specific T cell responses were elicited by the intranodally administered vaccine regimen in mice and humans.

Several studies evaluated the immunogenicity of directly injectable, formulated HIV mRNA vaccines in preclinical models (Zhao et al. 2016; Li et al. 2016; Bogers et al. 2015; Brito et al. 2014; Moyo et al. 2019; Pardi et al. 2019; Pollard et al. 2013). Non-replicating mRNA encoding HIV–1 Gag and complexed with 1,2–dioleoyloxy–trimethylammonium-propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) lipids elicited antigen-specific T cell responses after subcutaneous delivery in mice (Pollard et al. 2013). Others utilized PEI-complexed mRNAs in mice and found that the subcutaneously delivered HIV–1 Gag PEI-mRNA formulation elicited moderate T cell responses, and intranasally administered mRNA encoding the gp120 subunit of HIV–1 Env generated antigen-specific immune responses in the nasal cavity (Zhao et al. 2016; Li et al. 2016). Cationic nanoemulsion (CNE)-complexed self-amplifying mRNA vaccines also proved to be immunogenic in rabbits and rhesus macaques (Bogers et al. 2015; Brito et al. 2014). Moyo and colleagues utilized a PEI-based self-amplifying mRNA vaccine encoding conserved regions of the HIV-1 Gag and Pol proteins to induce potent $CD8^+$ T cell responses in mice (Moyo et al. 2019). A single immunization with this vaccine type induced polyfunctional $CD4^+$ and $CD8^+$ T cell responses that were maintained for at least 22 weeks

post-immunization. Administration of the second dose of PEI-mRNA (homologous prime-boost) did not significantly increase the numbers of HIV-specific T cells. Interestingly, booster immunizations with viral vaccines (heterologous prime-boost study) doubled HIV-specific T cell frequencies. These studies further demonstrate that self-amplifying mRNA vaccines can induce potent $CD8^+$ T cell responses that may be able to contribute to control HIV-1 infection.

As mentioned above, the generation of neutralizing antibodies against HIV-1 is extremely difficult even after the administration of multiple vaccine doses. Most HIV vaccine types induce some level of neutralizing antibodies against tier 1 (easy-to-neutralize) viruses but those antibodies have little relevance as they do not protect against HIV infection. An ideal antibody-driven HIV vaccine should induce long-lived antibody responses that neutralize multiple tier 2 (hard-to-neutralize) virus strains. This task has not been achieved by any vaccine to date. Some mRNA vaccines have been tested for the induction of neutralizing antibodies. CNE-complexed self-amplifying HIV Env mRNA immunization resulted in neutralizing antibody production against only tier 1 viruses in rabbits and nonhuman primates (Bogers et al. 2015; Brito et al. 2014). A more recent study evaluated nucleoside-modified mRNA-LNP HIV vaccines in rabbits and rhesus macaques (Pardi et al. 2019). Four intradermal immunizations with 50 μg of HIV Env mRNA-LNPs induced potent and durable Env-specific IgG responses but neutralization was limited to tier 1 MW965.26 strain in rabbits. Rhesus macaques were intradermally immunized five times with 50 μg of Env mRNA-LNP vaccine. High levels of Env-specific IgG titers were obtained, and beside tier 1 neutralizing antibodies, three out of six animals developed neutralizing antibodies against the autologous tier 2 virus. The latter finding is promising; however, the lack of durability of tier 2 neutralization activity somewhat diminishes enthusiasm. Of note, the authors found that the nucleoside-modified mRNA-LNP vaccines induced antibodies with potent antibody-dependent cellular cytotoxicity (ADCC) activity in both rabbits and rhesus monkeys. This finding is important because the ADCC activity of antibodies inversely correlated with the risk of infection in the RV144 (Thai) HIV vaccine trial (Yates et al. 2014). Melo and coworkers developed a DOTAP lipid nanoparticle-formulated SAM vaccine that expressed HIV Env gp120 protein fused with lumazine synthase enabling the formation of 60-mer protein nanoparticles (Melo et al. 2019). Two intramuscular injections (four-week intervals) with 3 μg vaccine induced antigen-specific IgG titers and a single dose induced germinal center T follicular helper (Tfh) cells and B cells in the draining lymph nodes. By performing single B cell sorting and B cell receptor sequencing, the authors demonstrated that the vaccine induced somatic hypermutation. Taken together, multiple studies have demonstrated that various types of mRNA vaccines induce HIV-specific T cell and B cell responses in preclinical models and humans. Some vaccines may be suitable for inducing cytotoxic T cells that help to control viremia; others may generate potent antibodies with tier 2 virus neutralization ability. The latter will be a critical feature for a prophylactic vaccine that could contribute to ending the HIV pandemic.

3.3 Zika and Other Flavivirus mRNA Vaccines

Zika virus (ZIKV) infection emerged as a global health problem as it started to cause congenital malformations and microcephaly in newborns in 2015–2016 (Richner and Diamond 2018). Companies and academic research institutions rapidly reacted to the ZIKV epidemic and developed various types of vaccines against the pathogen. Most of these vaccines induced protective immune responses in preclinical models, however, only a few of them demonstrated protective efficacy after a single immunization (Richner and Diamond 2018) that is one of the most critical features of an ideal vaccine for an epidemic scenario. mRNA vaccines targeting the pre-membrane and envelope (prM-E) surface glycoproteins of ZIKV were also quickly developed and demonstrated protective efficacy in small and large animals (Richner et al. 2017a, b; Pardi et al. 2017a). Pardi and colleagues developed a nucleoside-modified prM-E mRNA-LNP vaccine and evaluated it in mice and rhesus macaques (Pardi et al. 2017a). A single intradermal immunization quickly induced potent, protective antibody responses in both species. The durability of protective efficacy in mice and durability of neutralizing antibody responses in nonhuman primates were also demonstrated (Pardi et al. 2017a, 2018c). Richner and coworkers generated a similar vaccine that induced protection after two intramuscular immunizations in mice (Richner et al. 2017a). Importantly, the authors developed modified prM-E mRNA-LNP vaccines (with mutated fusion loop epitope in the E protein) that induced protective antibodies with significantly reduced antibody-dependent enhancement (ADE) activity against dengue virus (DENV) infection. This finding is critical as endemic subtropical and tropical areas for Zika and dengue viruses often overlap and a safe and effective Zika vaccine that induces antibodies with no ADE activity against DENV is highly desirable. A follow-up publication from the same team demonstrated that a nucleoside-modified prM-E mRNA-LNP vaccine elicited protective immune responses against ZIKV-induced congenital disease by inhibiting placental and fetal infection after vaccine administration in mice (Richner et al. 2017b). Initial publications suggested that nucleoside-modified mRNA-LNP prM-E Zika vaccines might outperform prM-E plasmid DNA vaccines but no direct evidence has been provided until recently. Jagger and coworkers compared the protective efficacy of these two vaccine types in mouse models of congenital infection (Jagger et al. 2019). These studies demonstrated that vaccination with mRNA elicited higher levels of antigen-specific long-lived plasma cells and memory B cells than immunization with plasmid DNA. Both vaccine types were effective against vertical ZIKV transmission. Although the nucleoside-modified prM-E mRNA-LNP vaccine has entered clinical testing (NCT03014089), no published data is available to date.

Self-replicating mRNA vaccines are also effective against ZIKV infection. Chahal and colleagues demonstrated that vaccination with an RNA replicon encoding ZIKV prM–E formulated into LNP-complexed ionizable dendrimer elicited antigen-specific antibody and $CD8^+$ T cell responses in mice (Chahal et al. 2017).

The study did not report on the protective efficacy of the vaccine. Another recent study utilized a vaccine with Zika prM-E self-amplifying mRNA formulated into a nanostructured lipid carrier (a hybrid between oil-in-water emulsions and solid LNPs) (Erasmus et al. 2018). Strikingly, a single intramuscular injection with as little as 10 ng vaccine formulation protected mice from ZIKV challenge 30 days post-immunization. A recent mouse study used naked self-amplifying Zika mRNA vaccine encoding prM-E (Zhong et al. 2019). Intradermal electroporation of BALB/c mice with two doses (four-week intervals) of 1 μg or 10 μg induced antigen-specific splenic $CD4^+$ and $CD8^+$ T cell and antibody responses. Interestingly, the lower dose induced more potent immune responses. Additionally, the authors found that the vaccine did not work very well in C57BL/6 mice, likely due to the more robust type I interferon (IFN) induction after immunization in this mouse strain. The latter finding is in agreement with other studies that found that the potent induction of type I IFN negatively affects protein production from mRNA and the strength of generated immune responses (Pepini et al. 2017).

As nucleoside-modified mRNA-LNP vaccines proved to be very effective against ZIKV infection, VanBlargan and coworkers aimed to develop other flavivirus vaccines using the same vaccine platform (VanBlargan et al. 2019). The authors generated vaccines against Powassan virus (POWV), an emerging tick-borne flavivirus, which potentially causes life-threatening encephalitis. A single intramuscular immunization with mRNA-LNPs encoding POWV prM-E induced protection from POWV infection in mice. Importantly, the modified mRNA-LNP vaccines induced cross-neutralizing antibodies against other tick-transmitted flaviviruses such as tick-borne encephalitis virus, Langat virus (LGTV), and Gadgets Gully virus and proved to be protective against LGTV infection.

Roth and colleagues have recently developed a T cell-based vaccine against dengue virus (DENV) infection using the nucleoside-modified mRNA-LNP platform (Roth et al. 2019). The vaccine encoded conserved and highly antigenic epitopes from the nonstructural (NS) proteins 3, 4B and 5 of DENV that are preferential targets of $CD8^+$ T cell responses. The authors demonstrated that two intramuscular immunizations (three or four weeks apart) with this vaccine induced potent antigen-specific $CD8^+$ T cell responses and some level of protection against DENV in various mouse models of DENV infection, indicating that a T cell-directed mRNA vaccine may be a viable strategy for flaviviruses.

3.4 Rabies mRNA Vaccines

Rabies is a zoonotic viral disease that causes severe neurological symptoms and is nearly always fatal after the onset of clinical symptoms. Currently used vaccines are fairly effective but three or four doses usually need to be administered within several weeks followed by booster injections at 3–5-year intervals (Hicks et al. 2012). Administration of multiple doses can be problematic due to poor infrastructure and high costs of vaccine production in many parts of Asia and Africa;

thus, the development of easy-to-produce and inexpensive vaccines that are protective after the administration of one or two doses is highly desirable. Schnee and colleagues evaluated the RNActive® vaccine platform (using non-replicating, sequence-optimized, unmodified, purified mRNA) encoding rabies virus glycoprotein (RABV-G) in mice and domestic pigs (Schnee et al. 2016). Two intradermal immunizations with 80 μg of mRNA vaccine induced antigen-specific T cell responses and protective antibody responses in mice. Three intradermal doses of 80 μg of RABV-G mRNA vaccine induced neutralizing antibody responses in the protective range in adult pigs (similar results were obtained in newborn pigs). After careful preclinical testing, the same vaccine regimen was evaluated in Phase I clinical trial (NCT02241135), and the results were shared with the public (Alberer et al. 2017). Various doses (80–640 μg) of RABV-G mRNA vaccine were administered intradermally or intramuscularly three times to healthy adults with no history of rabies vaccination. Importantly, not only needle–syringe but an intradermal/intramuscular injector was also used for vaccine administration. Surprisingly, the needle–syringe injection was almost completely ineffective, as there was no detectable immune response. Injection with the needle-free device elicited functional, boostable antibody responses but the immune responses declined one year after vaccine administration. Of note, adverse events—including systemic effects—were induced in 78% of the vaccinees. A more recent preclinical study from the same working group used LNPs for the delivery of RABV-G mRNA and demonstrated durable neutralizing antibody responses in mice and nonhuman primates (Lutz et al. 2017). Based on the promising preclinical data, a Phase I clinical trial using RABV-G mRNA-LNPs was initiated (NCT03713086). In summary, unmodified RABV-G mRNA vaccines induced potent immune responses in preclinical models (Schnee et al. 2016; Lutz et al. 2017). Unfortunately, the RNActive® rabies vaccine displayed limited and short-lived immunogenicity and concerning adverse events in Phase I trial (Alberer et al. 2017) indicating that further vaccine optimization is required for the development of an effective and safe human vaccine. Of note, GlaxoSmithKline has recently started a human trial (NCT04062669) using a CNE-formulated RABV-G SAM vaccine. As no published human data are available with SAM vaccines to date, this trial will likely provide critical information about the translatability of SAM vaccine safety and immunogenicity from preclinical models to humans.

3.5 Ebola Virus mRNA Vaccines

The unprecedented recent Ebola Virus (EBOV) epidemic in West Africa resulted in more than 11000 deaths and accelerated the development of vaccines against the virus (Meyer et al. 2019). The current lead vaccine candidate is a recombinant vesicular stomatitis virus-based vaccine (VSV-EBOV) that demonstrated protective efficacy in Phase III clinical trial (Henao-Restrepo et al. 2017). However, there are significant safety concerns about VSV vectors as they can cause severe human

disease (Quiroz et al. 1988). Non-integrating vaccine platforms such as mRNA likely have a safety advantage over VSV (or other viral)-based vaccines. Both self-replicating and non-replicative mRNA vaccines for Ebola have recently been evaluated in preclinical studies (Chahal et al. 2016; Meyer et al. 2018). Chahal and coworkers developed a dendrimer nanoparticle-based mRNA replicon encoding EBOV glycoprotein (GP) (Chahal et al. 2016). A single intramuscular immunization with 40 μg or two immunizations (three weeks apart) with 4 μg of EBOV GP vaccine elicited protective immune responses against lethal viral challenge in mice. Meyer and colleagues evaluated a nucleoside-modified mRNA-LNP vaccine in guinea pigs (Meyer et al. 2018). Two intramuscular immunizations (three weeks apart) with 20 μg of EBOV GP-encoding mRNA-LNPs induced high titers of GP-specific neutralizing antibodies and all vaccinated animals survived lethal virus challenge. Based on these preclinical results, mRNA-based EBOV vaccines are promising candidates for clinical testing.

3.6 Other mRNA Vaccines Against Viruses

The increasing enthusiasm about infectious disease mRNA vaccines also resulted in several proof-of-concept studies evaluating vaccines against herpes simplex virus type 2 (HSV-2), Human cytomegalovirus (HCMV) and Venezuelan equine encephalitis virus (VEEV). This section briefly summarizes the results of these novel studies.

HSV-2—the causative agent of genital herpes—is a common sexually transmitted pathogen with 11% global prevalence (Looker et al. 2008). As no clinical vaccine is available against the virus, once infected, people remain infected for life. Of note, HSV-2 infection increases the risk of HIV transmission by 3–4 folds, further underpinning the urgent need for an effective prophylactic HSV-2 vaccine (Wald and Link 2002). A recent study by Awasthi and colleagues evaluated a trivalent nucleoside-modified mRNA-LNP formulation in mice and guinea pigs (Awasthi et al. 2019). The vaccine encoded three HSV-2 surface glycoproteins: the entry molecule glycoprotein D (gD2) and two immune evasion molecules, glycoprotein C (gC2), and glycoprotein E (gE2). Importantly, the study demonstrated that the three different HSV-2 antigen-encoding mRNAs could be combined in a single vaccine and obtain the same level of neutralizing antibodies as when each was administered alone highlighting that the mRNA-LNP platform is suitable for the generation of multivalent vaccines that may have greater breadth. The authors included an alum/CpG-adjuvanted trivalent protein subunit vaccine (purified gD2, gC2, and gE2 antigens) as a direct comparator to mRNA-LNPs. Three intramuscular immunizations (two-week intervals) with the protein subunit vaccine and only two intradermal injections (four-week interval) with the nucleoside-modified mRNA-LNP vaccine induced potent T and B cell immune responses and completely prevented genital lesions in mice and guinea pigs. However, differences emerged in the protective efficacy between the two platforms when the authors investigated subclinical

infection. The trivalent protein vaccine prevented dorsal root ganglia infection and days 2 and 4 vaginal cultures were negative in 73% of mice compared with 98% in the mRNA-LNP vaccine group. In guinea pigs, 50% of the animals in the trivalent protein subunit group had vaginal shedding of HSV-2 DNA on 19/210 (9%) days compared with 2/10 (20%) animals in the mRNA group that shed HSV-2 DNA on 5/210 (2%) days. The trivalent nucleoside-modified mRNA-LNP vaccine was superior to trivalent proteins in stimulating serum and vaginal antigen-specific IgG antibodies, serum neutralizing antibodies, antibodies that bind to crucial gD2 epitopes involved in entry and cell-to-cell spread. Additionally, the mRNA-LNP vaccine induced potent Tfh and germinal center (GC) B cell responses confirming the results of previous publications (Pardi et al. 2018c; Lindgren et al. 2017). Based on the preclinical data, the trivalent HSV-2 nucleoside-modified mRNA-LNP vaccine is a very promising candidate for human trials.

A similar multivalent approach was used for the development of a nucleoside-modified mRNA-LNP vaccine against HCMV (John et al. 2018), a leading cause of congenital infection (Korndewal et al. 2017). The vaccine encoded HCMV glycoprotein B and the elements of the pentameric complex (PC): glycoproteins H and L and UL128, UL130, and UL131A. All six antigens are targets of neutralization antibodies. A second vaccine, targeting the pp65 and IE1 antigens, was also produced to broaden T cell responses. The hexavalent mRNA-LNP vaccine induced antigen-specific neutralizing antibodies after a single intramuscular administration in mice. Subsequent immunizations potently increased neutralizing antibody titers. Similar results were obtained in nonhuman primates: Cynomolgus macaques were intramuscularly immunized two times (three weeks apart) with 25, 100 or 400 μg mRNA-LNPs and all three doses induced neutralizing antibody responses that lasted for multiple months. The pp65-IE1 T cell vaccine induced potent $CD4^+$ and $CD8^+$ T cell responses in mice. Interestingly, these responses were inhibited when the hexavalent mRNA-LNP vaccine was coadministered with the T cell vaccine. The epitope competition was overcome by sequential administration of the pp65-IE1 and PC+gB+pp65-IE1 vaccine regimens. Based on the promising results, a Phase I clinical trial (NCT03382405) has been initiated to evaluate the safety and immunogenicity of the hexavalent nucleoside-modified HCMV mRNA-LNP vaccine in healthy adults.

VEEV is a significant acute disease in the Americas and also a highly developed biological weapon (Weaver et al. 2004). Investigational human vaccines such as TC-83 (a live-attenuated viral vaccine) are available but current formulations often induce suboptimal immune responses and elicit significant adverse events after administration (Paessler and Weaver 2009). A recent publication utilized the CNE-formulated self-amplifying mRNA platform to develop safer and more effective vaccines using modified VEEV genomes (Samsa et al. 2019). A live-attenuated TC-83-based formulation (LAV-CNE) and an irreversibly attenuated TC83 SAM formulation (IAV-CNE) were generated. Both vaccines induced high levels of VEEV-specific neutralizing antibodies in mice after one (LAV-CNE) or two (IAV-CNE) intramuscular immunizations. Not surprisingly, the LAV-CNE formulation performed much better than IAV-CNE and induced protection from lethal

VEEV infection after two immunizations with only 1 μg dose. Two immunizations with 10 μg of IAV-CNE protected 80% of the mice from lethal challenge. While the IAV-CNE formulation should certainly be safer than LAV-CNE or the investigational viral TC-83 vaccine, its protective efficacy is significantly decreased.

3.7 mRNA Vaccines Against Bacteria

Vaccine development against bacterial pathogens can be particularly complicated due to the high numbers of potential target antigens—as bacterial genomes are significantly bigger than viral genomes—as well as high antigenic variation (Telford 2008). A very limited amount of published work provided some evidence that mRNA vaccines might be suitable to target bacteria. An early study used intranasally administered unmodified naked mRNA to induce immune responses against *Mycobacterium tuberculosis* (MTB) (Lorenzi et al. 2010), one of the most critical vaccine targets, that infects over 1 billion people worldwide. The currently available MTB vaccine (bacillus Calmette–Guerin) has low efficacy, poor durability, and the ability to induce adverse events (Brewer 2000). Lorenzi and colleagues intranasally administered a single dose of 5 or 10 μg of heat shock protein 65 (Hsp65)-encoding mRNA to mice and challenged them intranasally with MTB thirty days later (Lorenzi et al. 2010). Four weeks post-challenge bacterial loads in the lungs were quantified and found to be significantly decreased in the mRNA-immunized animals compared to control mice demonstrating the viability of this approach.

Maruggi and coworkers utilized the SAM platform to target group A and group B streptococci (Maruggi et al. 2017). Three intramuscular immunizations with SAM vaccines encoding streptolysin-O and pilus 2a backbone protein induced partial protection in mouse models of bacterial infection.

3.8 mRNA Vaccines Against Parasites (Malaria, Leishmania, Toxoplasma)

Human eukaryotic parasites represent a diverse group of infectious pathogens affecting more than 1 billion people and cause more than 1 million deaths every year (Beaumier et al. 2013). Currently available vaccines have limited efficacy against these pathogens, largely due to the complexity of eukaryotic cells as vaccine targets and their ability to escape from innate and adaptive sterilizing immune responses. Importantly, several proof-of-concept mRNA vaccine studies have recently demonstrated some level of protective efficacy against toxoplasmosis, leishmaniasis, and infection with malaria (Chahal et al. 2016; Duthie et al. 2018; Garcia 2018; Luo et al. 2017). These studies have provided promising results and

can serve as the basis of further mRNA vaccine development against parasitic diseases.

Toxoplasma gondii is an obligate intracellular parasite and the causative agent of toxoplasmosis, which is believed to elicit severe symptoms in infected immunocompromised individuals (Saadatnia and Golkar 2012). Chahal and colleagues applied their dendrimer nanoparticle-encapsulated mRNA replicon to develop a hexavalent vaccine encoding conserved antigens (GRA6, ROP2A, ROP18, SAG1, SAG2A, and AMA1) from *T. gondii* (Chahal et al. 2016). A single intramuscular immunization with 40 μg vaccine induced protective immune responses against lethal protozoan infection in mice. Long-term (six months) follow-up of challenged animals noticed no clinical symptoms, demonstrating that the vaccine completely cleared the parasite. A second study evaluated an LNP-formulated SAM vaccine encoding nucleoside triphosphate hydrolase-II in mice (Luo et al. 2017). Two intramuscular immunizations (three weeks apart) with 10 μg of this mRNA vaccine formulation induced antigen-specific cellular and humoral immune responses that were associated with partial protection from lethal *T. gondii* infection.

Leishmaniases are a group of insect-transmitted often deadly infectious diseases elicited by protozoan pathogens in the genus Leishmania. Approximately, 350 million people live in countries endemic for Leishmania species (Schroeder and Aebischer 2011). Duthie and colleagues developed unformulated SAM vaccines encoding the LEISH-F2 and LEISH-F3+ fusion antigens (Duthie et al. 2018). Two intramuscular immunizations (three weeks apart) with the LEISH-F2 RNA replicon induced measurable but not strong immune responses. Interestingly, heterologous prime-boost immunizations with the LEISH-F2 RNA replicon and SLA-SE (oil-in-water TLR4 agonist)-adjuvanted LEISH-F2 protein subunits induced potent antigen-specific $CD4^+$ T cell and antibody responses. Heterologous prime-boost immunizations with the LEISH-F3+ antigen induced potent $CD4^+$ and $CD8^+$ T cell responses. Most importantly, unlike homologous prime-boost, heterologous LEISH-F2 RNA replicon prime and LEISH-F2 SLA-SE protein boost immunizations resulted in a significant reduction of parasite numbers in the livers of *Leishmania donovani*-infected mice, providing proof-of-concept for the feasibility of this approach.

Malaria is one of the world's leading health problems, responsible for more than 200 million clinical cases and hundreds of thousands of deaths every year (Beeson et al. 2019). Although the past several years have brought significant progress in the field of malaria vaccine development, no current vaccines induce potent and durable immune responses against *Plasmodium falciparum* and *Plasmodium vivax*. A recent study evaluated a SAM-CNE vaccine in mice (Garcia et al. 2018). The replicon encoded PMIF, a macrophage migration inhibitory factor, which was shown to inhibit germinal center formation and decrease the magnitude of Tfh cell responses during malaria infection leading to inadequate antibody responses against the pathogen. Mice were immunized intramuscularly two times (three weeks apart) with 15 μg of PMIF-encoding SAM-CNE and T and B cell immune responses and protective efficacy were evaluated. The vaccine induced antigen-specific $CD4^+$ T cell responses and antibody responses that were associated with better control of

parasitemia after sporozoite challenge. Interestingly, the vaccine elicited complete protection from reinfection, suggesting the induction of memory responses after vaccination. Moreover, PMIF SAM-CNE immunization was shown to induce elevated levels of antigen-specific memory $CD4^+$ and liver-resident memory $CD8^+$ T cells. Adoptive transfer experiments demonstrated that $CD4^+$ and $CD8^+$ T cells were associated with protection from infection with sporozoites. This elegant study demonstrated the potency of the PMIF SAM-CNE vaccine in a lethal murine model and laid the ground for further development.

4 Passive Immunization with mRNA-Encoded Monoclonal Antibodies Against Infectious Diseases

Passive immunization against infectious diseases can be critically important when there is insufficient amount of time to develop antibodies against a given pathogen after prophylactic vaccine administration. The past ten years have brought a breakthrough in this area as new technologies allowed scientists to identify multiple potent human monoclonal antibodies (mAb) from single B cells of appropriate donors (Walker et al. 2011). Improving the half-life and potency of these antibodies by genetic engineering further increased their therapeutic value. Currently, the high cost of recombinant protein production and the need for frequent administration are critical limiting factors for the widespread use of mAb therapy. Several alternative delivery platforms, such as plasmid DNA and various types of viral vectors, have been developed for antibody gene transfer but they have significant limitations such as induction of inflammatory responses and anti-vector immunity, toxicity, and concerns about the regulation of antibody production (Hollevoet and Declerck 2017). mRNA-based approaches could overcome many of the issues associated with recombinant protein, plasmid DNA, or viral vector-based delivery systems and, indeed, proof-of-concept publications have demonstrated that mAb therapy with mRNA-encoded antibodies could be a safe and effective means of passive immunotherapy (Pardi et al. 2017b; Thran et al. 2017).

Pardi and colleagues described nucleoside-modified mRNA-LNPs encoding the light and heavy chains of the broadly neutralizing anti-HIV antibody, VRC01 (Pardi et al. 2017b). A single intravenous injection with 1.4 mg/kg of VRC01 mRNA-LNPs resulted in 170 μg/ml antibody concentration in the plasma of treated mice 24 h post-vaccine administration. Weekly injections with 1 mg/kg of VRC01 mRNA-LNPs maintained plasma VRC01 antibody levels above 40 μg/ml. Most importantly, a single treatment with 0.7 mg/kg of VRC01 mRNA-LNPs completely protected humanized mice from intravenous HIV-1 challenge.

Thran and coworkers published an elegant study demonstrating that administration of antibody-encoding unmodified mRNA-LNPs induced protection against lethal viral or toxin challenges (Thran et al. 2017). Mice were treated with a single intravenous dose of 40 μg of rabies or influenza virus-specific mAb-encoding

mRNA-LNPs and then intramuscularly challenged with a lethal dose of rabies virus. All animals in the anti-rabies mAb mRNA-LNP-injected group survived the challenge, while animals in the control group succumbed. Importantly, the authors demonstrated that therapeutic treatment with anti-rabies mAb-encoding mRNA-LNPs two hours post-exposure of rabies virus resulted in 100% survival of the treated animals. In the second model, the authors generated camelid heavy-chain-only V_H domain (VHH)-based neutralizing agent (VNA)-encoding mRNA-LNPs against botulinum toxin. A single intravenous treatment with 40 μg of VNA-encoding mRNA-LNPs six hours after lethal botulinum toxin challenge resulted in 100% survival of the treated animals, confirming the therapeutic utility of mAb-encoding mRNA-LNPs.

Tiwari and coworkers generated nucleoside-modified mRNAs encoding various types (whole IgG and VHH) of membrane-bound or secreted mAbs against respiratory syncytial virus (RSV) (Tiwari et al. 2018). As RSV primarily targets the lungs, it is reasonable to deliver mAb-encoding mRNAs to the lungs to provide effective prophylactic or therapeutic treatment against the virus. Thus, the authors used a microsprayer to intratracheally deliver the aerosolized mRNA preparations. Administration of 40 or 100 μg of unformulated (naked) anti-RSV mAb-encoding mRNA significantly reduced RSV titers in both the prophylactic and therapeutic settings in mice. Membrane-anchored VHH displayed increased half-life and was detectable in the lungs of mice 28 days after mRNA administration. Interestingly, formulation of mRNAs with PEI-derivatives did not increase the therapeutic efficacy of the treatment.

A recent study provided valuable large animal data about the feasibility of treatment with mRNA-encoded mAbs (Kose et al. 2019). Kose and coworkers isolated potent neutralizing antibodies from a survivor of natural chikungunya virus (CHKV) infection and encoded the light and heavy chains of the most potent antibody (CHKV-24) using nucleoside-modified mRNAs. The mRNAs were formulated with LNPs and tested in mice and cynomolgus macaques. A single intravenous injection of immunocompromised AG129 mice with low doses (0.02–0.5 mg/kg) of CHKV-24 mRNA-LNPs resulted in relevant levels (up to ~14.9 μg/ml) of mAb production 24 h post-treatment. Animals were then challenged with CHKV: The highest dose (0.5 mg/kg) of mRNA-LNP treatment resulted in 100% survival, 0.1 mg/kg of mRNA-LNPs yielded 40% survival, and there was no survival with the lowest dose (0.02 mg/kg). Therapeutic treatment of immunocompetent mice with a single dose of 10 mg/kg of CHKV-24 mRNA-LNPs diminished clinical symptoms (arthritis and musculoskeletal disease) and viral titer. Next, cynomolgus monkeys were treated with a single intravenous dose of 0.5 mg/kg of CHKV mRNA-LNPs and the kinetics of serum CHKV mAb concentration was followed over time. Animals produced variable levels of mAb (mean IgG titers of 10.1 and 35.9 μg/ml in two independent experiments) that persisted for multiple weeks with a half-life of 23 days. Moreover, the authors demonstrated that two intravenous injections (seven days apart) of 3 mg/kg of CHKV mRNA-LNPs to monkeys resulted in 16.2 and 28.8 μg/ml serum mAb concentrations 24 h after administration of doses 1 and 2, respectively. Importantly, no severe or irreversible adverse events were observed

after administration of the second CHKV mRNA-LNP dose demonstrating the safety of this approach in nonhuman primates. Moderna Therapeutics has recently announced the positive results of a dose-escalation trial (NCT03829384) using CHKV-24 in healthy individuals (https://investors.modernatx.com/news-releases/news-release-details/moderna-announces-positive-phase-1-results-first-systemic). The interim report claimed that administration of CHKV-24 mRNA-LNPs resulted in detectable level of antibody production (1-14 μg/mL) in all study participants. Although adverse events were induced in some study subjects after mRNA-LNP infusion, they were transient and resolved without treatment. This critical study represents the first successful application of a systemic mRNA therapeutic that showed the production of a secreted protein in humans.

5 Conclusions and Future Perspective

mRNA vaccines represent a novel, promising vaccine platform with the beneficial features of rapid deployment, low cost, cell-free production, flexibility, safety, and potency. Considerable progress has been made toward demonstrating the feasibility of various types of mRNA vaccines against critical human pathogens (Maruggi et al. 2019; Pardi et al. 2018a). Importantly, published data from Phase I clinical trials using prophylactic mRNA vaccines against rabies (Alberer et al. 2017) and influenza viruses (Feldman et al. 2019) and the interim analysis of an ongoing passive immunization study against chikungunya virus are also available and provide proof-of-concept for the applicability of this vaccine type to humans. However, it is important to note that the published human data are somewhat disappointing in light of the very impressive results from multiple preclinical studies. Thus, further optimization of mRNA vaccine immunogens, delivery vehicles, or immunization schemes seems to be critical to improving the potency of this vaccine type in humans. Of note, recent publications revealed that application of mRNA vaccines including several different antigens for a given pathogen in a single formulation could also be a straightforward strategy to increase vaccine breadth and protective efficacy (Awasthi et al. 2019; John et al. 2018). In addition, this would also allow the development of combined formulations similar to MMR (measles, mumps, and rubella) that can be injected as a single vaccine regimen targeting several different infectious microbes. Due to the enormous flexibility of genetic vaccines, new findings from antigen optimization studies can be readily applied to mRNA vaccines. Finally, a deeper understanding of the mechanisms of action of mRNA vaccines and the influence of its various properties will likely be critical to increase the efficacy of vaccine formulations; these features may include the stringency of mRNA purification after the IVT reaction, the delivery vehicle used for mRNA encapsulation, the site of vaccine administration, and other aspects of the mRNA design.

Taken together, the data reviewed here highlight the exciting advances being made to demonstrate the viability of mRNA vaccines. The coming years will be particularly exciting as more human data will be available and new clinical trials using more advanced formulations will be initiated.

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Advances in Development of mRNA-Based Therapeutics

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Contents

Abstract Recently, mRNA-based therapeutics have been greatly boosted since the development of novel technologies of both mRNA synthesis and delivery system. Promising results were showed in both preclinical and clinical studies in the field of cancer vaccine, tumor immunotherapy, infectious disease prevention and protein replacement therapy. Recent advancements in clinical trials also encouraged scientists to attempt new applications of mRNA therapy such as gene editing and cell programming. These studies bring mRNA therapeutics closer to real-world application. Herein, we provide an overview of recent advances in mRNA-based therapeutics.

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1 Introduction

Great progress has been made in the development of nucleic acid-based therapeutic agents. Many drugs have already been approved for clinical applications by the regulatory bodies in the USA and in Europe. Gene therapy drugs Glybera (Watanabe et al. 2015), Strimvelis (Aiuti et al. 2017), Luxturna (Ginn et al. 2018) and Zolgensma (Rao et al. 2018), antisense oligonucleotide (ASO) drug Spinraza (Dolgin 2017), and small interfering ribonucleic acid (siRNA) drug Onpattro (Hoy 2018) are just a few examples of approved nucleic acid drugs. Messenger RNA (mRNA)-based therapeutics have a great potential in human applications. The mRNA molecule can be constructed to not only express conventional proteins for protein replacement therapy (Kormann et al. 2011) but also produce therapeutic antibodies (Kose et al. 2019). In addition, it can be tailored to encode chimeric proteins that are used for cell engineering and antigen proteins or peptides in vaccine development. Despite the great potential, clinical application of mRNA-based therapeutics has been lagged comparing to other types of nucleic acid drugs. One of the bottleneck issues has been difficulties in delivering mRNA molecules into the target organs and/or cells inside the body. Comparing to the ASOs and siRNA oligos, mRNA molecules are usually much bigger in size and are very sensitive to enzymatic degradation. Thus, technology platforms designed for ASO and siRNA cannot always be applied directly for mRNA delivery. Another technical challenge is synthesis of a large quantity of mRNA molecules for large-scale drug production. In this chapter, we will introduce recent progress in platform development and in vitro synthesis of mRNA molecules. In addition, we will review potential clinical applications of mRNA therapy, with primary focus on cancer immunotherapy and protein replacement therapy.

2 Delivery Platforms for mRNA Therapeutics

There has been a long history in the development of platforms for RNA transfection in vitro and RNA delivery in vivo. Verma and colleagues applied a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), to prepare liposomes and applied it to transfect luciferase mRNA into NIH3T3 cells back in 1989 (Malone et al. 1989). Hoerr and colleagues used protamine-condensed mRNA and liposome-coated protamine/mRNA complex to enhance the stability of mRNA and uptake of mRNA into cells in vivo in 2000 (Hoerr et al. 2000). Multiple lipid-based platforms have been described since then. Among the variety of technology platforms, lipid nanoparticle (LNP) is one of the most widely used

formulations for in vivo applications (Semple et al. 2010; Genc et al. 2011; Owen et al. 2018). Although initially designed for delivery of small molecule drugs, LNPs have been adapted to deliver siRNA oligos (Akinc et al. 2010), plasmid DNAs (Vijayanathan et al. 2002), and most recently mRNAs (Thess et al. 2015; Guimaraes et al. 2019). LNPs are usually less than 100 nm in diameter and are composed of structural phospholipids, cholesterol, and cationic lipids. DlinMC3DMA is one of the cationic lipids that have been successfully applied in mRNA delivery (Yanez Arteta et al. 2018). Many laboratories and companies have developed various forms of LNP-based platforms for mRNA delivery. Rosigkeit, S and colleagues developed a LPX delivery platform that was composed of DOTMA and dioleoyl phophotidylethanolamine (DOPE) and applied it to deliver mRNA and induce immune responses by targeting lung or spleen through the adjustable surface potential of nanoparticles (Rosigkeit et al. 2018). Kowalski, P. S. and colleagues applied liposome consisting of lipid and lipid-like polymer to deliver mRNA for protein replacement therapy (Kowalski et al. 2018). Patel, S. and colleagues applied LNP with novel lipid structures to increase protein expression efficiency (Patel et al. 2017; Hassett et al. 2019). Since most lipids can be dissolved in ethanol, the lipids can be mixed with nucleic acids in water in a microfluidic setting (Belliveau et al. 2012). This allows for controllable and standardized large-scale production. Indeed, precision nanosystems (www.precisionnanosysytems.com) have developed microfluidics-based instruments to prepare LNPs at different scales including one for GMP-grade drug manufacturing.

Other delivery platforms have also been optimized to achieve maximum delivery efficiency and protein expression. They include lipoplex (Koynova et al. 2007), cationic peptide (Yang et al. 2009), cationic polymer (Green et al. 2008), and micelle (Zheng et al. 2013). Persano, S. and colleagues applied a lipopolyplex (LPP) delivery platform which is composed of a polymer-condensed mRNA inner core surrounded by a lipid shell (Persano et al. 2017). Preliminary results suggest effective delivery of mRNA molecules to dendritic cells and potent protein expression.

Despite the tremendous advances in the field, there is still a big demand for new delivery platforms that are applicable for different routes of administration routes such as intravenous, intradermal, subcutaneous, intramuscular, and intratumor injections (Pardi et al. 2015). These platforms are expected to provide an optimal biodistribution pattern and to enable effective endosomal escape of mRNA molecules so as to improve protein expression. In addition, new platforms should also have an ideal biocompatibility.

3 In Vitro Synthesis of mRNA Molecules

Production of a large quantity of mRNA molecules is vital for the success of development of mRNA therapeutics. Although there are a few commercial entities that sell mRNA molecules, the list of mRNAs on their catalogs is not long. Thus, most laboratories and biopharmaceutic companies rely on their own facility or contract research organizations (CROs) to produce mRNAs, most through in vitro

transcription (IVT). Although the technology has been improved dramatically in the last three decades, bacteriophage-derived RNA polymerases have served as key enzymes for in vitro mRNA synthesis throughout the time (Sarnow 1989). A single strand DNA that contains a 5′ untranslated region (UTR) including a T7 promoter, an open-reading frame of the gene-of-interest, and a 3′ polyA tail is used as the template in the IVT. An anti-reverse di-guanosine cap analog is included in the IVT to generate a 5′ cap (Warren et al. 2010). However, it was found that the synthetic mRNA molecules may cause undesirable immune responses (Linares-Fernández et al. 2020). High performance liquid chromatography (HPLC) has been applied to purify the IVT product so as to mitigate the side effects (Kariko et al. 2011). In addition, pseudouridine and other modified nucleosides have been incorporated into the synthesized mRNA molecules to suppress immune responses, to increase translational efficiency, and to enhance mRNA stability (Warren et al. 2010; Kariko et al. 2008).

4 Clinical Application of mRNA Therapeutics

4.1 mRNA in Cancer Immunotherapy

Cancer immunotherapy has gained much attention in recent years due to its huge success in patient care with therapeutic antibodies, T cell-based therapies, and cancer vaccines (Mellman et al. 2011). Although no approved mRNA drug is available for now, mRNA-based therapy has played a big role in the field of cancer immunotherapy (Foster et al. 2019; Diken et al. 2017). Owing to their versatile applicability, mRNA molecules have been used to generate tumor-associated antigens and neoantigens in cancer vaccine development (Tanyi et al. 2018), in therapeutic T cell manipulation (Beatty et al. 2014), in antibody production (Stadler et al. 2017), and in expression of therapeutic cytokines (Hewitt et al. 2019). Comparing to proteins and peptides, mRNA may offer unprecedented advantages, such as generating diversified tumor antigens from one single molecule as a result of alternative mRNA splicing or intron retention (Frankiw et al. 2019; Smart et al. 2018). In Sects. 4.2–4.4, we will describe mRNA-based cancer immunotherapeutics in detail. In the meantime, mRNA vaccines have also been successfully applied in the fight against infectious diseases, such as the Zika virus (Richner et al. 2017a, b). Details will be described in Sect. 4.5.

4.2 Cancer Vaccine

Applying mRNA encoding tumor antigens has unique advantages over the traditional protein or peptide-based vaccine strategies. mRNA molecules serve as self-adjuvants (Ziegler et al. 2017). There is no limitation on human leukocyte antigen (HLA)-type restrictions. Comparing to DNA-based therapies, mRNA

vaccines do not integrate into the genome and therefore do not generate the risk of gene mutation. In addition, mRNA vaccines are adaptable to both dividing and non-dividing cells. Mechanistically, mRNAs encoding cancer antigens are delivered to the antigen-presenting cells (APCs) where they are translated in the cytoplasm. The newly synthesized protein is then processed into peptides, and the generated antigen peptides are presented by major histocompatibility complex (MHC) class I or MHC class II molecules which then activate T cells (Fiedler et al. 2016). Both MHC-TCR and B7-CD28 interactions are needed to generate antigen-specific T cells and to promote T cell proliferation. In order to develop a potent therapeutic cancer vaccine, it is essential to select the proper tumor antigen and adjuvant(s).

4.2.1 Tumor-Associated Antigen (TAA)-Based Cancer Vaccine

TAAs are antigens that are overexpressed in tumor cells. They are the primary choice for cancer vaccine development. In some of the pilot studies, scientists in CureVac demonstrated antigen generation from intradermally injected naked mRNA, cationic liposomal mRNA or protamine complex-encapsulated mRNA, and these mRNA vaccines induced both antigen-specific cytotoxic T lymphocytes (CTLs) and IgG antibodies (Hoerr et al. 2000). In addition, they found that T helper 2 (Th2)-type immune responses could be induced by intradermal vaccination of naked β-globin untranslated region (UTR)-stabilized mRNA, and that Th2-biased response could be shifted to a Th1-type response by co-delivering granulocyte–macrophage colony stimulating factor (GM-CSF) (Carralot et al. 2004). Early clinical trials demonstrated that intradermal injection of protamine-complexed mRNA or mRNA combined with GM-CSF was feasible, safe and effective, and treatment successfully induced antigen-specific T cell and antibody immune responses (Rittig et al. 2011; Weide et al. 2008). The strategy was further optimized by packaging two mRNA components in one formulation: a naked mRNA to encode the tumor antigen and a protamine-complexed mRNA to stimulate Toll-like receptor 7 signaling. The two-component mRNA vaccines with self-adjuvant property induce balanced adaptive immune responses and significantly better antitumor activity compared to single-component mRNA vaccine (Fotin-Mleczek et al. 2011). Apart from that, it is important to note that the strategy of using mRNA encoding a single antigen may not have enough immunogenicity to break central immune tolerance (Vansteenkiste et al. 2016). Therefore, strategies based on antigen cocktail were applied to maximize the immunogenicity of vaccines. Recent clinical results showed that CV9201, a RNA cancer vaccine encoding five non-small cell lung cancer antigens (NSCLC) (Sebastian et al. 2019), exhibited a well-tolerated safety profile and significantly improved immune responses against TAAs in patients who received a dosage of 1600 μg (NCT00923312). In another study, they combined BI1361849 (CV9202), a self-adjuvanted vaccine formulation consisting of protamine-complexed mRNA encoding six antigens (Papachristofilou et al. 2019), with local radiotherapy to reverse the immunosuppressive tumor microenvironment through induction of immunogenic tumor cell death and to

enhance recruitment and stimulation of T cells in patients with stage IV NSCLC. Results from a phase Ib trial showed that treatment was well tolerated and induced antigen-specific response in 84% patients. In addition, 46.2% patients achieved stable disease after vaccination (NCT01915524). Current clinic trial is ongoing to evaluate the effect from CV9201 in combination with the checkpoint blockade antibody durvalumab (NCT03164772).

4.2.2 Neoantigens Antigen-Based Cancer Vaccine

Neoantigens are generated when mutations are introduced in cancer cells (Schumacher and Schreiber 2015). Compared with non-mutated self-antigens, neoantigens may contribute more significantly to tumor control, since T cells stimulated by neoantigens tend to avoid central immune tolerance (Gilboa 1999). In order to identify neoantigens, DNA samples from both tumor and normal tissues are sequenced, and results are used to predict binding affinity from mutant proteins to patient's HLA alleles. Mutant antigen peptides are then ranked, and the information is applied to synthesize peptides or neoantigen-encoding mRNAs that will be used for vaccine preparation (Grabbe et al. 2016). DNA sequencing technology has been advanced so dramatically in recent years, and machine learning algorithms are being used to predict mutated peptides binding with HLA molecules (Linnemann et al. 2015; Abelin et al. 2017; Fritsch et al. 2014; Bulik-Sullivan et al. 2018). Applying mass spectrometry has also greatly advanced the field of tumor antigen identification (Creech et al. 2018). Consequently, personalized vaccines for cancer immunotherapy have been remarkably promoted. Preclinical studies from BioNTech AG revealed that one-third of mutated epitopes identified from B16F10 murine melanoma were immunogenic (Castle et al. 2012). Intravenous administration of neoantigen-encoding mRNA in lipoplex induced interferon-α secretion by plasmacytoid DCs and macrophages and promoted strong immune responses including maturation of DCs, and proliferation of antigen-specific effector and memory T cell. The immune responses subsequently mediated potent IFNα-dependent rejection of progressive tumors (Kranz et al. 2016). There have also been reports that showed an important role from CD4+ T cells in remodeling the tumor microenvironment after MHC class II-restricted epitopes administration (Sebastian et al. 2015). Proof-of-concept personalized cancer immunotherapy with mRNA cancer vaccine was first demonstrated in 2017 in patients with melanoma (Sahin et al. 2017). T cell immune responses to multiple neo-epitopes were induced in the patients who received treatment with mRNA cancer vaccines. Neoantigens-specific T cell responses were detected after vaccination in two patients with resected metastases, and one of them achieved a complete response after treatment with vaccine in combination with PD-1 inhibition therapy (Vallazza et al. 2015). Multiple clinical trials using mRNA-based cancer vaccines are currently being conducted in multiple cancer types, such as melanoma, colorectal cancer, glioblastoma, non-small-cell lung cancer, esophageal cancer, and bladder cancer (NCT03480152) (Li et al. 2014).

4.2.3 Dendritic Cell Vaccine

Since DCs are professional APCs, they can be loaded with tumor antigens to induce anti-cancer immune responses. mRNA in DCs can serve both as the source for antigen production and a potent adjuvant to stimulate TLR7/8 signaling (Laurent et al. 2012). First DC vaccine pulsed with mRNA was reported in 1996, and results showed that exposure of DCs to antigen-encoding mRNA or total mRNA extracted from tumor cells could induce significant T cell immune responses and inhibit growth of established tumors (Boczkowski et al. 1996). Many clinical trials using mRNA-based DC vaccines have been performed in cancer patients since then, and feasibility and safety of this treatment strategy have been well established (Gerold 2010; Daphné et al. 2015; Krug et al. 2014). In addition, efficacy from mRNA-transfected DCs can be further enhanced by combining cytokines and checkpoint blockade inhibitors in treatment (Mu et al. 2005; Kyte et al. 2006). It is important to point out that the cytokines and antibodies in combination treatment can also be produced by mRNA molecules inside the cells. DCs displayed a strong stimulatory potential after transfection with mRNAs encoding IL-12, IL-18 or other proinflammatory cytokines (Bontkes et al. 2007; Bontkes et al. 2008). Introduction of mRNA encoding the soluble extracellular part of PD-1 or PD-L1 resulted in elevated levels of CD80 (a DC maturation marker) and a group of cytokines and induced multifunctional T cells and cytokines secretion (Pen et al. 2014). Since DCs activation is mediated by several pathways, combination treatment of DCs to stimulate these pathways may achieve even better outcomes. TriMix-DC vaccine, a DC vaccine with mRNA molecules that encode TLR4, CD40L and CD70 (Pen et al. 2013), showed superior stimulatory capacity and suppressed the activity of regulator T cells (Treg), thus lifting CD8+ T cell activity.

4.3 Therapeutic mRNA Encoding Cytokines and Other Immune-Modulating Factors

mRNAs encoding therapeutic cytokines, checkpoint blockade antibodies, and immune agonists have the potential to convert an otherwise non-inflamed tumor (that lacks T cell infiltration, also known as immunologically "cold") into an inflamed tumors (Galon and Bruni 2019). This group of reagents is under extensive investigation in both preclinical and clinical studies. Hewitt and colleagues designed a triple combination therapy for intratumor injection of mRNAs encoding IL-36 γ, IL-23, OX-40L to turn "cold" tumors into "hot" tumors. Animal studies showed that mRNA mainly expressed in tumor tissues and triple therapy successfully modified the tumor microenvironment. The reagents stimulated both the innate and adaptive immune system by stimulating production of cytokines including IL-6, IL-22, TNF-α, IFN-γ, and IL-1β, promoting proliferation and infiltration of immune cells (DCs, NK cells, CD4+/CD8+ T cells) in both tumor

tissue and proximal lymph nodes without affecting Treg cells. As a result, treatment of MC-38 colorectal tumor-bearing mice with cytokine-encoding mRNA via intratumoral injection dramatically inhibited tumor growth (Hewitt et al. 2019). The result showed that intratumoral triplet mRNA therapy may avoid systemic toxicity and drive in vivo immune activation against tumor antigens and obtain a long-term therapeutic effect. Clinic trials have been initiated to evaluate potential toxicity from mRNA encoding IL-12, OX 40L monotherapy (NCT03323398) and mRNA encoding IL-36 γ, IL-23, OX-40L triple therapy (NCT03739931). Intratumoral delivery of mRNA has also been applied to produce other cytokines and chemokines including a fusion protein composed of interferon-β and the extracellular binding domain of the TGF-β receptor II (Van der Jeught et al. 2014). In addition, the strategy has been used to produce antibodies against cytokines IL-6 and TGF-β (Bialkowski et al. 2018). Furthermore, intratumoral delivery of mRNA has been applied to produce recombinant bacteriophage MS2 virus-like particles (VLPs) (Harper and Sardh 2014), mAbs targeting checkpoint molecules (PD-1, TIM-3, LAG-3), and necroptosis executioner mixed lineage kinase domain-like (MLKL) protein (Van Hoecke et al. 2018).

4.4 CAR-T Cells

Clinical trials showed that engineering T cells with chimeric antigen receptors (CARs) or T cell receptors (TCRs) have a significant therapeutic benefit on patients with relapsed or refractory hematological malignancies (Cummins and Gill 2018). The first CAR-T (Kymriah) product was approved in 2017 for treatment of patients with acute lymphoblastic leukemia. Transfection of T cells with mRNA to express CAR has the potential to temporally limit the targeting capacity of genetically modified T cells due to transient CAR expression and therefore to reduce the potential of sustained killing of normal cells that express the targeted TAAs such as mesothelin (Hung et al. 2018), EGFR (Caruso et al. 2016), CD19 (Caruso et al. 2016), CD20 (Panjwani et al. 2016), CD33 (Kenderian et al. 2015). Expression of CAR from introduced mRNA has been shown to transiently redirect T cell specificity to a desired TAA and mediate tumor regression in murine models of mesothelioma and leukemia (Yangbing et al. 2010; Barrett et al. 2013). A new approach of engineering T cells using IVT mRNA to transiently express CAR including both the CD3-ξ and 4-1BB for reducing off-target toxicity is under clinical evaluation. Clinical results indicated CAR-Ts generated from adoptively transferred mRNA to target mesothelin are feasible and safe (Beatty et al. 2014). The lifespan of CART-meso cells was short in the peripheral blood after intravenous injection, and these CAR-T cells effectively migrated to primary and metastatic tumor sites. Bai and colleagues reported an approach that used modified mRNA encoding telomerase reverse transcriptase to transfect CD19 CAR-T cells in order to improve their lifespan and proliferation (Bai et al. 2015). mRNA treatment instantly boosted telomerase activity in the new CD19 CAR-T cells, which

promoted proliferation, delayed replicative senescence, and then, provided long-term antitumor activity in a mouse xenograft model of B-cell leukemia (Bai et al. 2015).

4.5 mRNA Vaccines in Infectious Diseases

mRNA vaccines have been applied for prevention of infectious diseases such as influenza viruses (Richner et al. 2017; Petsch et al. 2012), zika virus (Feldman et al. 2019), rabies virus(Schnee et al. 2016), and Dengue virus (Roth et al. 2019). In 2017, a research group published a study on mRNA vaccines to protect against Zika virus infection (Richner et al. 2017). In these vaccines, the mRNA molecules encoding Zika viral prM/M-E protein antigens contain a proprietary nucleoside modification to minimize indiscriminate activation of innate immunity, although detailed information on the modification was not provided. In addition, a donor methyl group S-adenosylmethionine was added to the methylated capped RNA to enhance translation efficiency. The mRNA molecules were then packaged into lipid nanoparticles before the vaccines were applied to treat mice. The researchers found that a modified mRNA vaccine could prevent Zika disease in animal models. In a follow-up study, the researchers demonstrated protection against Zika virus-induced congenital disease in mice (Richner et al. 2017). With the recent emergence of the corona virus, there is an international effort to develop both prophylactic vaccines and therapeutic vaccines (Steenhuysen and Kelland 2020). Moderna and CureVac announced their involvement in company news release, and Stemirna has applied the LPP platform in vaccine development. Exciting development is anticipated in the coming months. For a more detailed overview of this mRNA application, please refer to the review in the book chapter "Messenger RNA-based vaccines against infectious diseases" reviewed by Mohamad-Gabriel Alameh, Drew Weissman, and Norbert Pardi.

4.6 mRNA in Protein Replacement Therapy

Defective protein translation from DNA genetic information can give rise to various diseases, such as fabry disease, methylmalonic acidemia (MMA), acute intermittent porphyria (AIP), Hemophilia B, and cystic fibrosis (CF) (Kerem et al. 1989; Mehta et al. 2004; Lerner-Ellis et al. 2006; Koeberl et al. 1990). In addition, normal proteins may not be available in certain disease areas due to blood vessel damage, leading to further development of diseases such as heart failure and diabetes foot. Compared with conventional protein drugs for such indications, mRNA therapy provides an effective alternative, since a single mRNA molecule can be translated into a large quantity of protein molecules over the course of hours or days treatment time (Warner et al. 1963). Furthermore, the nascent protein will go through all the

required post-translational modification procedures including phosphorylation, acetylation and glycosylation, a fully functional protein product is guaranteed (Helenius et al. 2013).

4.6.1 Local Injection-Based Protein Replacement Therapy

Myocardial Infarction

Despite advances in curative and preventive medicine, cardiovascular disease still remains one of the leading causes of morbidity and mortality worldwide (Savarese and Lund 2017). To date, a significant clinically feasible and/or verified targeting biologic strategy for treating congestive heart failure is still lacking. Vascular endothelial growth factor A (VEGF-A) has been previously reported to regulate new blood vessel formation, enhance endothelial proliferation from epicardial-derived progenitor cells, and with a pro-survival effect on vascular, endothelial, and cardiac cells (Ferrara et al. 2003; Lui et al. 2013). Same as mRNA molecules for cancer therapy, those used for protein replacement therapy need to overcome the same technical hurdles as immunogenicity, instability, and low expression efficiency, and nucleoside modifications and 5' capping have been applied in mRNA production (Kariko et al. 2008; Mockey et al. 2006). The modified RNA synthesized by Zangi and colleagues showed high production efficiency and dose-dependent expression profile of VEGF-A in a murine myocardial infarction model (Zangi et al. 2013). Direct in vivo comparison of mRNA therapy and DNA therapy showed that the rapid, pulse-like expression profile of mRNA benefited growth of functional vessels, whereas the prolonged VEGF-A expression profile of DNA-induced toxicity due to redundant formation of leaky vessels (Zangi et al. 2013). An effect of expansion and directed differentiation of endogenous heart progenitor cells were shown from the intramyocardial injection of mRNA encoding VEGF-A in the murine myocardial infarction model. Furthermore, Leif and colleagues investigated potential therapeutic application of mRNA in ischemic heart disease in swine (Leif et al. 2018). The purified and optimized (optimization of nucleotide, UTR sequence, capping efficiency, and buffer solution) mRNA showed tissue specific and long-lasting expression of protein without triggering innate immune response. Moreover, the study showed that swine cardiac function was improved after a single intra-cardiac injection of VEGF mRNA one week after myocardial infarction through limiting cardiac infarct expansion and fibrosis, improving systolic function.

Diabetes Foot

Diabetic ischemic ulcer is an intractable and the most devastating diabetic complication. Similar to cardiovascular diseases, angiogenesis is a critical factor for diabetic wound healing (Varu et al. 2010; Harold and Marjana 2007). Application

of VEGF-A and PDGF-β offers a promising approach for treatment of wound and ulceration (Shi et al. 2018). Sun and colleagues analyzed microvascular responses after mice were treated with mRNA therapy (mRNA encoding VEGF-A, AZD8601) or protein therapy (VEGF-A protein) (Sun et al. 2018). Intradermal injection of AZD8601 into mice resulted in dose-dependent vasodilation, upregulation of blood flow and formation of neo-vessel in injection site, results that were not observed in mice treated with the VEGF-A protein or phosphate buffer saline control. In addition, sequential dosing of AZD8601 in diabetic mice resulted in sustained vascularization and tissue oxygenation within wound area. A clinical trial aiming to assess safety and potential therapeutic effects of the mRNA encoding VEGF-A on treating type 2 diabetes mellitus (T2DM) was performed (NCT02935712) (Gan et al. 2019). It was revealed that intradermal VEGF-A mRNA injection was well tolerated and local functional VEGF-A protein was steadily expressed after administration, which led to transient skin blood flow enhancement in patients with T2DM.

4.6.2 Liver Diseases

Liver is the natural target for protein replacement therapy, since most nanoparticles tend to accumulate in this organ via intravenous injection (Fenton et al. 2016; Derosa et al. 2016). Fabry disease is one of these diseases that can be effectively treated with protein replacement therapy. This is a rare inherited disorder of glycosphigolipid metabolism caused by absence or markedly deficient activity in liver α-galactosidase A (α-Gal A), an enzyme that is normally produced by the liver. Patients suffer from progressive decline in renal and cardiac function and develop cardiomyopathy and end-stage renal disease (Hebert et al. 2013; Messalli et al. 2012). mRNA molecules encoding α-Gal A were packaged by two different research groups into LNPs (Zhu et al. 2019) or nanoparticles formulated with lipids and lipid-like materials (DeRosa et al. 2019). A single intravenous injection of α-Gal A mRNA caused not only dose-dependent protein expression and substrate reduction but also long-term (up to 6 weeks) substrate reductions in tissue and plasma in mice. In addition, the product proved to be safe after multiple administrations to non-human primates (Zhu et al. 2019). Hemophilia B is another liver disease that is caused by a deficiency of factor IX (FIX), a serine protease. FIX activation plays a major role in the signaling the coagulation cascade (Jiang et al. 2018). The disease is characterized as sustained, internal bleeding, and easy bruising. The prophylactic treatment is an intravenous application of purified FIX along with blood transfusion, but a heavy administrative burden with continuous treatment over a short period was needed to ensure adequate as-needed dosage given. Ramaswamy and colleagues applied lipid-enabled and unlocked nucleic acid modified RNA (LUNAR) to treat FIX-deficiency and demonstrated its feasibility in mouse model of FIX-deficiency (Ramaswamy et al. 2017). LUNAR is a unique LNP composed of four lipids including a proprietary lipid. Delivery of human FIX mRNA encapsulated in LUNAR resulted in rapid pulse of the FIX protein, and high

protein concentration was maintained for 4–6 days. Therapeutic efficacy from mRNA therapy was comparable to recombinant human FIX protein therapy which is the current standard of care (Ramaswamy et al. 2017). An additional example of protein replaces therapy with mRNA for liver diseases include deficiency of methylmalonyl-CoA mutase (MUT), a vitamin B12-dependent mitochondrial enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA (Chalmers and Lawson 1982; An et al. 2017).

4.6.3 Lung Diseases

Lung is another organ that offers easy access to drug nanoparticles. Non-invasive aerosol inhalation is a defined method for delivering drugs to the lung, and many technology platforms have been developed for this purpose. For an example, Patel and colleagues synthesized hyperbranched poly (beta amino esters) (hPBAEs) to prepare stable and concentrated mRNA polyplexes for inhalation (Patel et al. 2019). They achieved 24.6% transfection efficiency in lung epithelial cells after a single dose. Cystic fibrosis which is caused by mutations in the CFTR gene is the most widespread life-limiting autosomal-recessive disease in Caucasian (Cutting 2015). Robinson and colleagues applied LNP-packaged mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) to treat cystic fibrosis (Robinson et al. 2018). Mutations in the CFTR gene cause abnormal flux of ions into and out of cells, leading to accumulation of thick airway mucosa and permanent tissue scarring and respiratory failure (Welsh 1990; Lyczak et al. 2002). Nasal application of LNP-CFTR mRNA in CFTR knockout mice recovered CFTR-mediated chloride secretion to conductive airway epithelia for at least 14 days and achieved comparable outcomes with currently approved drug ivacaftor (Robinson et al. 2018).

4.7 Genome Editing

Many genome editing tools have emerged in the past decade including zinc finger nucleases (ZFNs) (Miller et al. 2007), transcription-activator like effector nucleases (TALENs) (Wefers et al. 2013), and the clustered regularly interspersed palindromic repeats (CRISPR)/CRISPR-associated (Cas) enzyme system (Ran et al. 2013). However, off-target effects remain as a major concern. Since the nuclease activity is required for only a short period of time for the action, transient expression of mRNAs encoding ZFNs, LENs, and Cas9 provides a valid option to reduce off-target effects. Recently, Conway and colleagues showed that LNP-packaged mRNA encoding ZFNs to target the TTR and PCSK9 genes achieved over 90% knockout in gene expression in mice (Conway et al. 2019). Finn and colleagues applied LNP to load with Cas9 mRNA and sgRNA delivery system in order to edit the mouse transthyretin (Ttr) gene in the liver and achieved over 97% reduction of the protein level in serum, and the effect lasted for over

12 months after a single administration (Finn et al. 2018). Meanwhile, Miller and colleagues developed zwitterionic amino lipid (ZAL)-based delivery system to co-deliver Cas9 mRNA and sgRNAs and observed permanent DNA editing with 95% decrease in protein expression (Miller et al. 2017). These results suggest that mRNAs packaged in nanoparticles provide an excellent route for genome editing.

5 Conclusions

Application of novel therapies and medical technologies has revolutionized patient care. With the advance in mRNA synthesis and improvements in delivery platforms, mRNA-based therapies will play more and more important roles in the field. As with other types of therapeutic agents, the pharmaceutic industry has taken a very prominent role in advancing mRNA therapies. They have pioneered in mRNA modification in order to minimize innate immune responses. In the meantime, there is a constant need to understand the physical and biological barriers in mRNA delivery and to develop next-generation platforms so as to better overcome the barriers and achieve precision tissue- and cell-targeted delivery, beyond the liver and lung. In addition, approaches to enhance stability of mRNA molecules should continue to be explored. Furthermore, there is a need to standardize production of mRNA molecules to ensure high quality and efficacy of mRNA therapies. Finally, advances in machine learning and bioinformatic analysis will further facilitate sequence optimization of mRNA and medical application of therapeutic mRNA.

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Clinical Development of mRNA Vaccines: Challenges and Opportunities

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Contents

Abstract The emergence of safe and effective mRNA platform-based COVID-19 vaccines from the recent pandemic has changed the face of vaccine development. Compared with conventional technologies used historically, mRNA-based vaccines offer a rapid flexible and robust approach to preventing disease caused by transient viral strains such as SAR2-CoV-2 variants of concern and seasonal influenza. Adaptations in the formulation of the mRNA delivery systems such as with lipid nanoparticle delivery (LNP) used in mRNA-1273 and BNT16b2b have enabled this technology to flourish under the urgent collective response and collaborative regulatory understanding derived from COVID-19 vaccine development.

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The application of mRNA-based therapeutics in other areas holds potential promise including combination vaccines that might deliver protections against multiple infectious diseases. Future studies and further advances in mRNA-based technologies will provide insight into the clinical efficacy and real-world effectiveness of vaccines as well as provisions with respect to the impact of reactogenicity profiles. Overall, the success of mRNA-based COVID-19 vaccines has helped unlock a platform likely to result in many more candidate vaccines entering clinical evaluation to address the unmet medical needs of other diseases including viral respiratory diseases, herpesviruses, and historically challenging vaccine targets such as HIV.

1 Introduction

The promise of mRNA as a new therapeutic modality has been motivated by interest in developing novel prophylactic vaccines to fulfill unmet medical needs and by the recent evidence that demonstrated the effectiveness of the mRNA approach for vaccination against COVID-19 (Polack et al. 2020; Baden et al. 2020). Conventional technologies used historically in vaccine development have included live attenuated virus, killed virus, glycoconjugate, and recombinant protein vaccines and have proven safe and effective (Graham et al. 2018). However, challenges remain in developing vaccine approaches for genetically diverse pathogens, or pathogens for which protective immune responses are difficult to elicit (e.g. HIV) and for which traditional approaches less suitable for rapid development in response to emerging infections and epidemics or for antigen design (Cable et al. 2020).

As a platform, mRNA allows flexibility, unlike production of recombinant proteins or other cell-based biologics. mRNA production efficiencies are achieved through a reduced development time and cost by using a common manufacturing platform and purification methods regardless of the antigen. In addition, the process does not require the handling of potential pathogenic material, increased biosafety measures, or the use of cell lines or reagents that can introduce adventitious agents into the process. As the process is cell-free and is not comprised of any products of human or animal origin, nor does it contain preservatives or adjuvants, it is void of some of the potential safety concerns of older vaccine technologies. Biologically, administration of mRNA provides the production of the antigen in situ, allowing for transmembrane domains to be present, and multimeric complexes to be formed. The capability to express multiple encoded proteins that can fold and assemble into a functional protein is a key advantage of mRNA-based vaccines or therapeutics as demonstrated by the generation of complex antigens such as the cytomegalovirus pentameric complex or mRNA encoded Chikungunya monoclonal antibodies (John et al. 2018; August et al. 2021).

Strategies to optimize pharmacology have enhanced the translation and stability properties of mRNA-based technologies, including mRNA modifications such as the addition of synthetic cap analogs, capping enzyme, regulatory elements, poly

(A) tails and modified nucleosides (Pardi et al. 2018a). Administration of modified mRNA vaccines results in a rapid, transient dose-dependent expression of encoded proteins, typically administered in microgram quantities intramuscularly with adjuvant (Pardi et al. 2018a; Rahman et al. 2021). These mRNA-encoded proteins can mimic a more natural presentation to the immune system, generally resulting in improved T cell responses, and can deliver antigens, which may be otherwise difficult to stabilize in their active biological conformation. Unlike viral vectored vaccines, there are no concerns of anti-vector immunity and as such, responses are more easily boosted, and vaccination with an mRNA vaccine does not preclude vaccination with a different vaccine at a future date.

mRNA technology can enable clinical development programs to address infectious diseases unable to be addressed by conventional vaccine technologies. As mRNA technology has now been established as a key vaccine development platform, several critical aspects applicable to conventional vaccine development need thoughtful consideration on how they may apply to mRNA-based vaccines. Below, we provide an overview of clinical experience with mRNA-based vaccines to date and explore essential issues relevant to vaccine development and propose approaches to integrate this innovative technology into our current toolbox for the clinical development of novel vaccines.

2 Development of Nucleic Acid-Based Vaccines

It was recognized in the late 1980s that available vaccine development technologies, including attenuated viral or bacterial pathogens, inactivated virus, and adjuvanted recombinant proteins, had inherent limitations, and novel approaches were needed. In addition to the manufacturing challenges, the potential safety issues of live attenuated vaccines, the need for an adjuvant for recombinant vaccines, and the limited T-cell response to inactivated viral and recombinant vaccines led to the development of DNA vaccines (Donnelly et al. 2005). The inherent immunostimulatory properties of DNA could circumvent the need for an adjuvant with the encoded antigen generating both neutralizing antibodies as well as CD8 T-cell responses thought to be due to cross-presentation of encoded antigen peptides (Liu et al.1998; Shedlock and Weiner 2000). Early work with DNA vaccines demonstrated robust immunogenicity in rodents but a lack of species translation when the technology was advanced into non-human primates and humans; delivery was a key limiting factor for this lack of translation (Liu and Ulmer 2005; Dupuis et al. 2000). Concurrently, Wolff et al. first demonstrated that mRNA could also result in protein expression in vivo after injection in muscle (Wolff et al. 1990), yet it was not advanced due to the perceived limitations of mRNA instability, degradation, nuclease vulnerability, complex manufacturing processes, and immune system stimulation.

Considerations for the clinical development of mRNA therapeutics and vaccines include an understanding of the biological properties of the different mRNA

technologies and delivery systems. Early mRNA vaccine candidates used canonical RNA bases without modifications (Petsch et al. 2012). However, foreign unmodified mRNA is an important trigger of innate immunity (Diebold et al. 2004). Kariko et al. first demonstrated that modification of mRNA, replacing uridine with pseudouridine or cytosine with methyl cytosine, could reduce innate stimulation (Kariko et al. 2005). Complete replacement of uridine with N1-methyl-pseudouridine results in further decreases in immune activation compared to pseudouridine (Kariko et al. 2005; Andries et al. 2015). In addition to modified and unmodified mRNA, self-amplifying mRNA vaccines have been advanced to clinical testing, which utilize linear, single-stranded, positive-sense viral non-structural proteins, typically an alphavirus RNA polymerase, to increase antigen expression and stimulate immunogenicity (Geall et al. 2012; Deering et al. 2014). Peer-reviewed clinical data on self-amplifying mRNA vaccines have yet to be published; however, preliminary results from Arcturus Therapeutics report showed that the self-amplifying mRNA COVID-19 vaccines induce 47–54-fold increases in neutralizing antibody responses against the Omicron variant, indicating that self-amplifying mRNA vaccines have the potential to combat variants of concern (Arcturus therapeutics reports 2022).

The formulation of mRNA vaccines requires the stabilization of mRNA to facilitate cellular entry and delivery into the cytoplasm of the cell for subsequent translation and antigen production. Unlike traditional vaccine technologies, the aim for an mRNA delivery system, or the antigen itself, is not to over-activate the innate immune system, which otherwise could result in excess interferon induction and down-regulation of antigen expression for mRNA vaccines (Pepini et al. 2017). Although adjuvants have been used to induce potent vaccine responses for herpes zoster and influenza (Cunningham et al. 2016; Izurieta et al. 2020), a delivery system that does not result in additional immune stimulation would be advantageous for mRNA-based vaccines. Three delivery approaches have been or are currently used for mRNA vaccines including an unformulated, protamine-complexed conventional mRNA, cationic lipid-based systems such as cationic nanoemulsions (Brazzoli et al. 2015; Brito et al. 2014), and ionizable lipid-based nanoparticle systems (LNPs). Reviews of mRNA delivery systems have been published elsewhere and are summarized in a previous chapter 'Formulation and Delivery Technologies for mRNA Vaccines' within this textbook, however most mRNA-based vaccine development to date has utilized an ionizable or cationic LNP system (Cullis and Hope 2017; Buschmann et al. 2021).

The strong immunogenic properties of mRNA-based vaccines with LNP delivery have been demonstrated in multiple pre-clinical studies, including the induction of high levels of neutralizing antibodies, CD4 and CD8 T-cells, as well as robust CD4 T follicular helper cell responses (John et al. 2018; Pardi et al. 2018b, 2018c, 2017; Richner et al. 2017; Liang et al. 2017). Importantly, pre-clinical studies of the mechanism of action of modified mRNA/LNP indicate that the primary cells associated with mRNA translation and antigen expression are antigen-presenting cells, including monocytes, dendritic cells, and neutrophils, which subsequently traffic to draining lymph nodes and elicit robust germinal center

reactions and adaptive immunity (Pardi et al. 2018c; Liang et al. 2017). More recently, evaluation of SARS-CoV-2 mRNA vaccines in multiple animal models has demonstrated robust immunogenicity for neutralizing antibodies, CD4 T follicular helper cells, and sterilizing immunity after viral challenge (Corbett et al. 2020a, b).

3 Clinical Experience with mRNA Vaccines

3.1 SARS-CoV-2: mRNA-1273 and BNT162b2

The potential of mRNA-based vaccines for rapid epidemic or pandemic response was previously demonstrated by Hekele et al. who showed that low doses of H7-expressing saRNA could generate potent immune responses in rodents with non-GMP constructs generated within two weeks of the first detection of human-to-human transmission of H7N9 (Hekele et al. 2013). The potential advantages of the mRNA technology were demonstrated with the COVID-19 pandemic, with the first clinical trial of the mRNA-1273 vaccine initiated within two months of the publication of the SARS-CoV-2 sequence and subsequent emergency use authorization (EUA) from the US Food and Drug Administration (FDA) less than one year later after successful phase 3 efficacy trial results (Polack et al. 2020; Jackson et al. 2020; Baden et al. 2021). Likewise, mRNA-based vaccine BNT162b2 received EUA from the US FDA within a similar rapid timeframe followed by subsequent full approval in August 2021 for individuals 16 years of age and over (FDA Approves First COVID-19 Vaccine 2021; Comirnaty and Pfizer-BioNTech COVID-19 Vaccine 2021).

The mRNA-1273 vaccine clinical trial, facilitated by ongoing collaborative research by the US National Institutes of Health and Moderna, was enabled by the identification of a 2-proline substitution (S2P), which stabilizes the SARS-CoV-2 and other betacoronavirus spike proteins in a pre-fusion conformation (Corbett et al. 2020a). The mRNA 1273, and similarly BNT162b2, encode for the S2P stabilized full-length spike (S) protein of SARS-CoV-2 derived from the Wuhan-Hu-1 strain (Wrapp et al. 2020). Both mRNA vaccines have undergone phase 1/2/3 evaluation and given the rapidity of clinical development, those studies are still ongoing to complete safety and immunogenicity follow-up (Jackson et al. 2020; Walsh et al. 2020; Chu et al. 2021). Both vaccines elicited robust neutralizing antibody and Th1-biased CD4 T cell responses including in older adults (Jackson et al. 2020; Walsh et al. 2020; Anderson et al. 2020) and confirmed in clinical studies of recipients of authorized vaccines, peaking after the second dose of vaccine in SARS-CoV-2 naïve subjects (Walsh et al. 2020; Anderson et al. 2020; Painter et al. 2021). Preliminary data also indicate good antibody persistence through 6 months after vaccination with decay rates consistent with those observed after 8 months with natural SAR-Cov-2 infection, to include neutralizing antibodies to

SARS-CoV-2 variants of concern (Widge et al. 2020; Doria-Rose et al. 2021; Pegu et al. 2021). The robust recall responses associated with the second dose of vaccine, including the clinical observation of lymphadenopathy after vaccination (Baden et al. 2020), suggest the induction of a robust germinal center reaction (Turner et al. 2021). Taken together, pre-clinical and clinical data are consistent with a mechanism of action for mRNA/LNP-based vaccines whereby vaccine efficacy is mediated by the uptake and translation of mRNA, primarily in antigen-presenting cells, for presentation in draining lymph nodes, resulting in the generation of robust humoral and cellular immunity (Fig. 1).

Most importantly, validation of the mRNA platform was suggested by the similar high levels of efficacy in pivotal phase 3 trials of mRNA-1273 and BNT162b2, which showed the vaccine efficacy to prevent symptomatic COVID-19 disease to be 94% and 95%, respectively (Polack et al. 2020; Baden et al. 2020); the trials also showed high levels of efficacy against the severe disease that were consistent across age strata and comorbidities, which increase the risk for COVID-19. Emerging data with 6 months of follow-up from these pivotal trials suggest some waning of efficacy for BNT162b2 (Thomas et al. 2021). The effectiveness of these vaccines using real-world evidence has been recently confirmed (Thompson et al. 2021).

Given the widespread implementation of a new vaccine technology for pandemic vaccination, the safety of mRNA vaccines has been a key public health priority monitored closely during the post-authorization period in the US and elsewhere. As of January 2022, over 516 million total doses of mRNA-based COVID-19 vaccines have been administered in the United States (Centers for Disease Control and Prevention 2022). Surveillance data collected from the

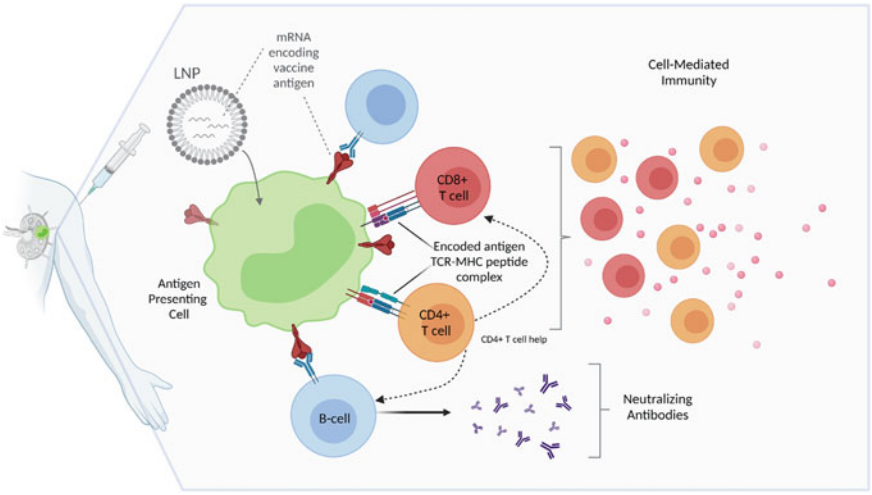

Fig. 1 mRNA-based vaccines induce robust immunogenicity, mediated by cellular uptake of mRNA, and cytosolic translation and production of antigen primarily by antigen-presenting cells in draining lymph nodes for the generation of humoral and cellular immunity
LNP, lipid nanoparticle delivery; *TCR-MHC*, T-cell receptor-Major histocompatibility complex

US CDC VAERS and v-safe systems had not identified a safety concern (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/safety/safety-of-vaccines.html, accessed 22 Jan 2022), while data on the safety of mRNA vaccines in pregnancy indicate similar rates of perinatal and obstetric complications expected in an unvaccinated population, supporting a recommendation for vaccination during pregnancy (Shimabukuro et al. 2021a).

More recently, with the use of authorized mRNA vaccines in adolescents, rare observations of myocarditis and/or pericarditis have been observed primarily in males aged 12–29 temporally associated with vaccine administration, typically within 4 days of the second dose (Montgomery et al. 2021; Marshall et al. 2021). The US Advisory Committee on Immunization Practices recently reviewed available data to date with an analysis of the benefit-risk association. The ACIP recommended continued vaccination in this age group (with information provided about the risk of myo-/pericarditis), as the benefit clearly outweighs the risk at the present time (Gargano et al. 2021). Current data are limited, and further studies are needed to establish causality and pathogenesis (Husby et al. 2021; Witberg et al. 2021; Heymans and Cooper 2022).

Initial concerns about anaphylaxis arose in the early post-authorization period with widespread vaccination, which was not observed in COVID-19 clinical trials (Polack et al. 2020; Baden et al. 2020; Thomas et al. 2021). VAERS data were reviewed by the US CDC between December 14, 2020 and January 18, 2021. Nearly 10 million doses of the BNT162b2 vaccine and over 7.5 million doses of mRNA-1273 were administered in the US, with 66 case reports that met Brighton Collaboration case definition criteria for anaphylaxis (levels 1, 2, or 3): 47 following Pfizer-BioNTech vaccine, for a reporting rate of 4.7 cases/million, and 19 following Moderna vaccine, 2.5 cases/million doses administered (Shimabukuro et al. 2021b), similar to previously reported population rates for anaphylaxis after vaccination (Zent et al. 2002).

3.2 Other Infectious Disease Vaccines

Prior to the SARS-CoV-2 pandemic, clinical experience with mRNA-based vaccines was limited (Feldman et al. 2019; Feldman et al. 2019), although a number of trials with mRNA vaccines, as well as mRNA therapeutics and cancer vaccines (Cafri et al. 2020) (not addressed in this review) are ongoing. mRNA vaccines for infectious diseases tested to date in the clinic include respiratory viruses (human metapneumovirus, parainfluenza, and influenza), Zika virus, rabies, HIV-1 (intranodal injection) (Leal et al. 2018), and cytomegalovirus (Table 1). Preliminary unpublished results that have been presented publicly are largely consistent with the recently published data for SARS-CoV-2 in terms of safety, reactogenicity, and immunogenicity (Allison August, manuscript in preparation). The first study of an mRNA vaccine published was a protamine-based, unmodified mRNA vaccine encoding rabies virus glycoprotein that was evaluated for safety and

immunogenicity in a Curevac-sponsored phase 1 clinical trial (Alberer et al. 2017a). Study participants received 80–640 μg of mRNA vaccine by intramuscular or intradermal needle injection or administration using a needle-free device intradermally or intramuscularly. More than 90% of participants reported mild to moderate injection site reactions, and 78% experienced a systemic reaction, either by intramuscular or intradermal administration, primarily fatigue, chills, and headache. One case of transient Bell's palsy was considered possibly related to vaccination. Interestingly, only one participant who received a needle-syringe injection had a detectable virus-neutralizing antibody response, while needle-free injections elicited a neutralizing antibody response in the majority of participants (Alberer et al. 2017a). A subsequent study by Curevac that assessed an mRNA/LNP formulation encoding rabies glycoprotein did demonstrate complete neutralizing antibody seroresponse to two doses of either 1 or 2 mcg of mRNA after testing a 5 mcg dose, which had unacceptably high reactogenicity (Aldrich et al. 2021), suggesting that potent immune stimulation by unmodified mRNA may be dose limiting. However, results from the phase 2b/3 HERALD study of the Curevac unmodified mRNA vaccine against SARS-CoV-2 (CVnCoV), which uses the ALC-0315 LNP class, found reactogenicity to be similar to other mRNA vaccine studies with vaccine efficacy against moderate-to-severe COVID-19 to be 70.7% overall and 77.2% in participants aged 18–60 years of age (Kremsner et al. 2021). These studies, although not head-to-head comparisons, will provide important results as to the relative merits of different mRNA technologies and formulations targeting a proof-of-concept antigen with an established serological correlate of protection.

Initial proof-of-concept for a modified mRNA/LNP vaccine was demonstrated for pandemic influenza H7N9 and H10N8. A 50-μg dose of modified mRNA in non-human primates elicited robust hemagglutination inhibition (HAI) responses and in a subsequent dose-ranging, first-in-human clinical trial, H10 mRNA vaccination elicited seroconversion (HAI assay) in all participants dosed with 100 μg of H10 and $\geq$ 90% participants receiving 25 or 50 mcg of H7 mRNA/LNP. In this study, doses up to 400 μg were assessed, to include both intradermal and intramuscular injection. Intradermal injections were discontinued at the 50-μg dose level due to reactogenicity as well as 400 μg administered intramuscularly, due to local and systemic adverse events observed in sentinel participants. Other dose levels up to 100 μg were assessed with no significant safety concerns. Systemic reactogenicity, primarily symptoms of fatigue, headache, and myalgia, was observed most commonly, but usually of short duration. Although the mRNA manufacturing process and LNP assessed in this study differ from the mRNA-1273 COVID-19 vaccine, the reactogenicity data are consistent with the now large safety database for SARS-CoV-2 mRNA-based vaccines, in which the incidence of fever and other systemic symptoms increase with dose level and after administration of the second dose, but are usually of short duration, lasting 1–3 days (Chapin-Bardales et al. 2021).

Table 1 mRNA infectious disease vaccines in clinical trials

Phase 1	Phase 2	Phase 3
Infectious diseases	Infectious diseases	Infectious diseases
HMPV/PIV (NCT03392389)	• CMV (NCT04232280)	• SAR-CoV-2:
RSV (NCT04528719)	• SARS-CoV-2 (Chu et al. 2021)	mRNA-1273 (Baden et al. 2021)
Zika (NCT04064905)		BNT162b2 (Polack et al. 2020)
Chikungunya MAb (NCT03829384)		CVnCoV (NCT04652102) (Kremsner et al. 2021)
SARS-CoV-2 (NCT04283461)		
NextGen SAR-CoV-2 (NCT04813796)		
HSV-2 (NCT04762511)		
Influenza A/B (NCT04956575)		
Influenza A/H3N2 (Centers for Disease Control and Prevention 2021)		
Pandemic Influenza (Feldman et al. 2019)		
Rabies (Sanofi and Translate Bio initiate Phase 1 clinical trial of mRNA influenza vaccine 2021) (NCT04062669)		

4 Key Questions for mRNA Vaccine Clinical Development

With the initial demonstration of safety and efficacy in phase 3 trials, and more recent evidence of effectiveness in the post-Emergency Use Authorization and post-licensing settings with the administration of millions of doses of mRNA vaccines, the establishment of mRNA technology as a vaccine platform marks the beginning of a new era in vaccine development. However, several key questions for mRNA vaccine developers, as well as regulators, need to be considered given the key differences in the mRNA technology and established vaccine technologies.

4.1 Do Correlates of Protection Generated by Conventional Vaccines Apply to mRNA Vaccines?

The licensure of vaccines requires a demonstration that the vaccine provides 'clinical benefit' by showing an effect on a clinical endpoint—a measure of infection or disease. During the course of demonstrating clinical benefit in a clinical trial, sponsors often collect data regarding the immune responses, most often

antibody response, observed in recipients of vaccines and of controls. In this way, a correlate of protection, and a threshold for protection, may be derived. Vaccine correlates of protection are often difficult to identify and establish and may be uniquely applicable to a specific vaccine platform; and establishment of a correlate of protection often requires confirmation in more than one study (Plotkin 2008; Plotkin and Gilbert 2012).

In the case of COVID-19 vaccines, significant resources from the US government, academia, and non-profit organizations have been applied to identifying an immune correlate of protection for COVID-19 vaccines to enable immune bridging studies to establish the effectiveness of new COVID-19 vaccines, primarily focused on levels of neutralizing antibodies, without performing a randomized, controlled efficacy trial (Khoury et al. 2021; Earle et al. 2021). Non-human primate data for mRNA-1273 using doses that allowed breakthrough infections in NHPs indicated that lower antibody levels in the lower airways can mediate protection (lower viral replication), but not the upper airways. Passive transfer of NHP serum in a hamster challenge model confirmed the role of serum antibody levels as a mechanistic correlate (Corbett et al. 2021). Results from the mRNA-1273 Phase 3 trial confirmed the role of antibody as a potential predictor of vaccine efficacy, but a protective level of neutralizing or binding antibodies (potential immune correlate threshold) was not identified, likely due to the high level of initial efficacy observed in the trial and relatively few breakthrough cases at the time of analysis (Gilbert et al. 2021). Additional analyses after longer-term follow-up from clinical trials, coupled with the exploration of additional immunologic biomarkers, may further inform the identification of a correlate of protection given the complexity and interplay among various immunologic measures of vaccine protection (Plotkin 2013).

An obvious candidate for the application of mRNA technology is the development of an improved influenza vaccine. Levels of serum antibody-mediating red blood cell HAI in vitro have been correlated with protection from influenza infection. Regulatory acceptance of this correlate and of the threshold of HAI titers as a surrogate for clinical endpoint efficacy has been employed for accelerated approval of seasonal influenza vaccines. This correlation applies to HAI Ab titers induced by conventional ‘split’ or ‘killed’ influenza virus vaccines. A critical question then follows: does the threshold of protection derived from clinical studies of a killed vaccine apply equally to titers induced by an mRNA vaccine? We would argue that regardless of whether the HAI titers are induced by ‘split’ influenza virus vaccines or by mRNA directly encoding the hemagglutinin of influenza virus, equivalent protection should be assumed. This is a vital step in bringing mRNA vaccines into use. The example of influenza vaccine is an important one: influenza vaccine is in widespread use, but new technologies are needed to address challenges faced with traditional egg-based manufacturing including the coordination of the production of > 100 million pathogen-free embryonated chicken eggs and, additionally that embryonated eggs are not the ideal substrate for all virus strains such as H3N2 strains (Rajaram et al. 2020). An additional consideration is the use of the hemagglutination inhibition assay, which utilizes egg-adapted viruses that may accumulate sequence changes during viral passage similar to those acquired during

the production of egg-based vaccines and, hence, more antigenically similar for HAI assays when compared to an mRNA-based vaccine, which may be better matched to the circulating strain (Katz et al. 2011). Accepting the 'equivalency' of antibody correlates, regardless of the origin of the antigen inducing those responses, may help manufacturers develop the next generation of influenza vaccines and ensure that the vaccine supply remains uninterrupted.

More broadly, in contrast to the established application of correlates of protection, assays such as the single radial immunodiffusion assay (SRID), which measures individual antigen content and has been used to assess the potency of influenza vaccines for decades, are not an appropriate release assay to assess the potency of mRNA-based influenza vaccines (Minor 2015; Weir and Gruber 2016). Regulatory guidance on these and other quality measures are currently under development (World Health Organization 2021). Whether an *in vitro* potency assay can be developed and accepted by regulatory agencies or if an *in vivo* potency study will be required will need to be addressed as mRNA-based vaccines may have advantages over traditional egg-based or recombinant influenza vaccines, such as the incorporation of additional strains (e.g. two or more different H3N2 strains), late strain changes or pandemic application.

4.2 Are Packaged mRNA Vaccines Considered an Adjuvanted Vaccine?

Unlike established vaccines, the effectiveness of mRNA-based vaccines and therapeutics is entirely dependent on the efficient delivery of mRNA to the cell cytosol and, thus, requires a vehicle to both protect mRNA from in vivo degradation and efficiently target its payload. The use of lipid-containing or other moieties to achieve this can induce inflammation at the site of administration, but whether considered an adjuvant or not is an important question to address. In parallel with the rapid refinement of mRNA design and production technology, methods to protect mRNA and assist in intracellular uptake following parenteral injection have been required to include LNPs, which often consist of an ionizable or cationic lipid, cholesterol, PEGylated lipid, and a phospholipid (World Health Organization 2021). Lipid nanoparticles are intended to shuttle the mRNA into the cytosol of cells for translation and expression of the target antigen. As such, the LNP does not accompany the vaccine antigen but rather accompanies the nucleic acid sequence encoding the antigen. This has bearing on consideration of the role LNP plays in mRNA vaccines.

Conventional vaccine formulations, whether an adjuvanted recombinant protein or an attenuated or inactivated virus or bacteria (e.g. BCG), may activate the immune system. Conventional vaccines often are formulated with adjuvants, the most common adjuvant being the aluminum salts (aluminum hydroxide and aluminum phosphate), and more recently the approval of vaccines containing newer

adjuvants such ASO_4 (Cervarix), AS01 (Shingrix), CpG1018 (Heplisav), and MF59 (FLUAD), a water in oil emulsion (Hassett et al. 2019; Burny et al. 2017; Coffman et al. 2010). Recombinant protein antigens often require adjuvants to induce adequate immune responses. Indeed, such formulations meet the common understanding of 'adjuvanted vaccines,' in which the formulation includes antigens as well as an 'additive,' and example being the TLR4 agonist, monophosphoryl lipid A, that is expressly intended to help stimulate an immune response to the companion antigen (Centers for Disease Control and Prevention 2020). Whether the LNP in LNP-formulated mRNA vaccines meets the definition of an adjuvant has important implications to the clinical development of mRNA vaccines. Novel adjuvants require thorough clinical evaluation before vaccines formulated with such adjuvants may be licensed. The administration of antigens in conjunction with potentially immunostimulatory or immunopotentiating compounds raises potential concerns regarding reactogenicity upon injection and even possible autoimmune triggering (Alving et al. 2012). Considering these definitions of vaccine adjuvants, however, it would not appear that LNP-formulated mRNA vaccines meet the definition of adjuvanted vaccines. Importantly, the LNP is not delivered in conjunction with the antigen, and the LNP moiety may not be present at the place or at the time of antigen expression (World Health Organization 2021). Accordingly, we propose that LNP-formulated mRNA vaccines are not 'adjuvanted vaccines' and do not require the unique pre-clinical and clinical evaluations that accompany novel adjuvanted vaccines. Importantly, lipid nanoparticles can induce an inflammatory response at the injection site, which may be detrimental to immunogenicity, perhaps through reduced antigen expression. Selection of a less inflammatory ionizable lipid, therefore, may improve immunogenicity and reduce reactogenicity (World Health Organization 2021). The role of the local inflammatory response to LNP, however, may not be important to the magnitude or quality of the immune response and warrants further study (Liang et al. 2017). In summary, we propose that mRNA-based delivery vehicles such as LNP primarily serve to stabilize and protect the mRNA during cellular uptake following parenteral injection to facilitate intracellular delivery and are not specifically intended to augment or potentiate the immune response to the expressed antigen.

4.3 mRNA Lends Itself to the Development of Combination Vaccines - What are the Implications?

While conventional vaccine technology has proven highly effective and safe, there are some vaccine target pathogens for which mRNA is particularly better suited than conventional technologies. One challenge to conventional vaccine technology is the preparation of multiple antigens to target pathogens with higher serotype diversity. Such diversity has been addressed by recent successful vaccines including the 13-valent pneumococcal vaccine (Prevnar13) and the human papillomavirus

vaccine (Gardasil-9), which includes the major capsid proteins (L1) of nine different HPV serotypes (Silva et al. 2015). These successful examples notwithstanding, the manufacture of such combination vaccines using conventional technology is laborious and lengthy (Joura et al. 2015). In contrast, mRNA production employs a common platform and purification approach that can allow for the synthesis of multiple peptides from a single mRNA or multiple mRNAs. Accordingly, distinct viral serotypes of a given pathogen could be readily and rapidly manufactured as mRNA constructs. Indeed, the opportunity for the platform to generate cocktails of antigen mixtures permits consideration of vaccine development for targets previously considered as too diverse for conventional technology to address (e.g. rhinovirus, norovirus).

The potential for multivalent or combination vaccines that exceed the size of currently available vaccines to date (e.g. 9-, 13-valent, *23-valent pneumococcal vaccines*) presents both an opportunity and a challenge. The opportunity is that mRNA may prove to be a mechanism to finally prevent infection by certain target pathogens by using combinations of antigens to effectively elicit immune responses capable of protecting against the range of pathogen serotypes. As this strategy develops, it will be important to address the challenge of clinical evaluation. Methods for characterizing the manufactured product and demonstrating consistency in proportions between lots and through human immunogenicity studies is one such challenge. In addition, the tolerability profile of novel multivalent or combination vaccines may pose another challenge in terms of increased reactogenicity associated with an increase in total mRNA dose.

The development of mRNA vaccines targeting multiple pathogens are also facilitated by the nature of the manufacturing platform. Design and evaluation of vaccines combining antigens from unrelated pathogens could prove particularly valuable given the crowded pediatric vaccine schedule. The convenience of vaccines targeting like diseases (e.g. a seasonal vaccine targeting viral respiratory infections in addition to influenza), or different populations (e.g. infants, adolescents, older adults, and different combination vaccines) could be valuable. As such combination vaccines are developed, clinical endpoints will need to be established that allow the design of clinical trials that are feasible in terms of size and complexity to support further innovation of combination vaccines.

Beyond such combinations, it is conceivable that mRNA constructs encoding preformed antibodies (i.e. passive immunization) could theoretically be combined with constructs encoding protective antigens (i.e. active immunization). Such scenarios could be imagined for pathogens presenting a defined risk in a known time frame—such as travelers visiting regions with endemic infections on short notice, or post-exposure prophylaxis (e.g. rabies). Such combination passive-active immunizations could provide protection immediately upon arrival in an endemic region and sustained protection following induction of an adaptive immune response to expressed vaccine antigens. Such combination products have not previously been developed and again require consideration of clinical endpoints permitting their development. In addition, different delivery systems with lower immunostimulating

properties, those that generate higher protein expression, and the need for different targeting cells may need to be considered.

4.4 *mRNA Is a Platform Technology: Does Accumulated Safety Data with One mRNA Vaccine Predict Safety to an Unrelated mRNA Vaccine?*

As noted above, mRNA production is a platform in which the translated target sequence is simply exchanged to generate new candidates while other components both within the construct (e.g. untranslated regions) and for the packaging (e.g. LNPs) may remain the same between different mRNA constructs. We propose that, in many cases, the safety profile demonstrated for one mRNA vaccine may apply to the profile of an mRNA vaccine bearing a distinct translated (antigen) sequence. Recent studies of two mRNA vaccines, discussed above, expressing alternate influenza hemagglutinin molecules (of H10N8 and H7N9 strains of influenza) provide insights to this issue (Feldman et al. 2019). Each mRNA expressed the full-length, membrane-bound form of the hemagglutinin glycoprotein from the respective influenza isolates. Each mRNA was vialed at a concentration of 2 mg/mL; each vial contained 40 mg/mL of LNP excipients formulated in isotonic 8.0% sucrose/20 mM buffer. The safety profile observed in the two separate, small-scale Phase 1 trials of each mRNA vaccine was similar. For both vaccine groups, the most common adverse events observed were injection site reactions, occurring at similar frequency; rates of myalgia and headache were also similar between the two groups. While the two antigenic sequences are distinct and do not show cross-reactivity, the tolerability profiles overlapped, with systemic reactogenicity observed more frequently in the H10N8 study.

More recently, data for mRNA-1273 and BNT162b, despite distinct differences in the components of their LNP formulations, appear to show overlapping tolerability profiles, with a general increase in the severity and frequency of solicited adverse events at higher doses and after the second dose in SARS-CoV-2 naïve individuals (Polack et al. 2020; Baden et al. 2020; Jackson et al. 2020; Anderson et al. 2020; Chapin-Bardales et al. 2021). With millions of doses administered, no safety concerns have been identified with the most intense safety monitoring performed in the US to date (Peter et al. 1999). This suggests the possibility that as new mRNA vaccines differing only in the encoded antigen enter clinical development, parameters could be developed to avoid the need to enroll large numbers of subjects, at least during early clinical development, to more efficiently advance vaccine candidates to pivotal licensure studies. Of note, differences introduced with the LNP delivery system (e.g. novel lipids or excipients) for mRNA vaccines entering the clinic may elicit qualitative and quantitative differences in immune response. In this setting, conservative study designs for the assessment of safety and tolerability may be considered.

5 Summary

The recent success in rapid deployment of mRNA vaccines to address the world's most devastating pandemic in 100 years has confirmed the promise of mRNA as a vaccine platform for the prevention of infectious diseases. Differences in modified, unmodified, and self-amplifying mRNA technologies are beginning to be elucidated by clinical data emanating from the COVID-19 pandemic. It is possible that these different mRNA technologies may be similarly effective for some pathogens as vaccines; however, due to differences in the immunostimulatory properties of modified vs. unmodified mRNA, the former is better suited for different applications such as for the expression of secreted monoclonal antibodies. Although this potential for broader therapeutic applications has yet to be realized, advances in mRNA manufacturing, formulation, and delivery will likely achieve similar clinical success. In the near term, the success of mRNA vaccines for COVID-19 is likely to result in many more candidate vaccines entering clinical evaluation to address unmet medical needs in viral respiratory diseases, herpesvirus, and challenging vaccine targets such as HIV, and will improve existing licensed vaccine and remain a key countermeasure for emerging infectious diseases with pandemic potential.

Statement and Declarations

All authors are employees of Moderna Inc. and may hold stock/stock options.

The data summarized in this review are from published articles and are publicly available.

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Regulatory Considerations on the Development of mRNA Vaccines

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Abstract Developing traditional viral vaccines for infectious diseases usually takes years, as these are usually produced either by chemical inactivation of the virus or attenuation of the pathogen, processes that can take considerable time to validate and also require the live pathogen. With the advent of nucleic-acid vaccines (DNA and mRNA), the time to vaccine design and production is considerably shortened, since once the platform has been established, all that is required is the sequence of the antigen gene, its synthesis and insertion into an appropriate expression vector; importantly, no infectious virus is required. mRNA vaccines have some advantages over DNA vaccines, such as expression in non-dividing cells and the absence of the perceived risk of integration into host genome. Also, generally lower doses are required to induce the immune response. Based on experience in recent clinical trials, mRNA-based vaccines are a promising novel platform that might be useful for the development of vaccines against emerging pandemic infectious diseases. This chapter discusses some of the specific issues that mRNA vaccines raise with respect to production, quality, safety and efficacy, and how they have been addressed so as to allow their evaluation in clinical trials.

1 Introduction

Most of the licensed prophylactic viral vaccines for infectious diseases have been generated by conventional methods, such as by chemical inactivation of the pathogenic virus or by developing live-attenuated versions of the pathogen (Plotkin et al. 2017). Developing these types of vaccines and demonstrating their safety and effectiveness can take many years. In some recent cases, vaccine antigens have been produced using recombinant DNA methodologies, which can reduce the development time. However, if there is a need to generate a vaccine rapidly in response to a bioterrorism attack or an infectious disease emergency, a different strategy is required to develop a vaccine that induces both cellular and humoral immunity. Over the last decade or so, nucleic acid-based vaccines have been explored to fill this gap.

Although it had been known for several decades that both DNA and RNA transfected into cells in culture can produce proteins, it was not until the pioneering work of Wolf and colleagues in 1990 showing that both DNA and RNA could produce proteins following inoculation into mouse muscle that the rationale for nucleic acid vaccines was demonstrated (Wolff et al. 1990). RNA has some advantages over DNA, such as being able to enter non-dividing cells and to produce proteins in the cytosol once inside the cell. In contrast, DNA requires both uptake

into cells and entry into the nucleus for expression—a process that necessitates the breakdown of the nuclear membrane during cell division. However, mRNA vaccines were not pursued until recently because of the difficulties in producing the quantities of mRNA required to produce a vaccine and the lability of mRNA in vivo. Because of its stability in vivo and its ease of production, plasmid DNA was the first nucleic acid to be explored as a vaccine. DNA vaccines were initially evaluated in clinical trials in the 1990s, but for various reasons, none was successful in human trials. However, recent approaches to enhance immunogenicity of DNA vaccines have shown promise (Kutzler and Weiner 2008; Abdulhaqq and Weiner 2008; Manickan et al. 2017; Porter and Raviprakash 2017; Tregoning and Kinnear 2014; Lee et al. 2018; Hobernik and Bros 2018; Li et al. 2012; Saade and Petrovsky 2012; Chi et al. 2017; Modjarrad et al. 2019; Al-Amri et al. 2017; Muthumani et al. 2016; Muthumani et al. 2015; Zakhartchouk et al. 2007; Huang et al. 2006; Ahn et al. 2017; Morrow et al. 2018; Cheng et al. 2018; Williams 2014), and a number of DNA vaccines are in clinical trials.

Although candidate RNA vaccines have been recently developed and entered into clinical studies, some of the general manufacturing and pre-clinical issues relevant to development of these vaccines are addressed in the *Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications* (https://www.fda.gov/media/73667/download).

Finally, it is important to note that both types of nucleic acid-based vaccines have the significant advantage over conventional vaccines in that they can be designed and produced rapidly and thus could be ready for evaluation in the clinic in the short timeframes needed to combat a new infectious disease threat. Also, there is no need to handle the infectious virus, which may be highly pathogenic and require high-containment laboratories for its growth. All that is required for both types of nucleic acid vaccines is the availability of the sequence of the antigen gene(s) so that an optimized DNA sequence can be synthesized and cloned into appropriate expression vectors. Furthermore, with experience gained from evaluating the safety of both types of nucleic acid vaccines, it is possible that certain preclinical safety studies may not be necessary, which would further shorten development times and cost. This chapter will discuss only mRNA vaccines and leave DNA vaccines to another venue. The goal is not to provide detailed checklists of regulatory requirements for mRNA vaccines, since different regulatory authorities may have different requirements and requirements may change as experience is gained. Rather, it is to discuss some of the specific issues that mRNA vaccines raise with respect to safety and efficacy and how they have been addressed so as to allow their evaluation in clinical trials.

2 RNA Vaccine Development

Early attempts to stabilize RNA by encapsulation in liposomes showed that encapsulated mRNA delivered to a mouse was able to express proteins that induced an immune response (Martinon et al. 1993). Despite this result, RNA vaccines were not developed until the last decade, when several technical advances coalesced to render the field of mRNA vaccines practical. Several aspects of mRNA vaccines have been touted as being advantageous over DNA vaccines. Not only can RNA be expressed in non-dividing cells (as mentioned above), but perceived safety risks associated with DNA (Myhr 2017; Klinman et al. 1997, 2000; Medjitna et al. 2006) were not raised with RNA. For example, there was a concern that DNA would integrate into the host genome and induce mutagenic and possibly oncogenic events. It should be noted, however, that experimental evidence has shown that the frequency with which plasmid DNA integrates into the host chromosome is low (Ledwith et al. 2000; Manam et al. 2000), thus lessening the concern for integration. However, integration was not considered relevant with RNA, since, in the absence of specific enzymatic activities such as those provided by retroviruses or retrotransposons, there was no known mechanism by which integration of RNA could occur. Another potential concern with DNA was that administration of large quantities of plasmid DNA could induce anti-DNA antibodies in the host and, if that occurred, auto-immune disease could result. Experience has shown that this concern was also unfounded (Gurunathan et al. 2000; Langer et al. 2013).

The work of Wolf and colleagues (Wolff et al. 1990) and Martinon and colleagues (Martinon et al. 1993) provided the rationale for mRNA vaccines. Subsequent studies have shown that both RNA and DNA vaccines are able to elicit both humoral and cellular immune responses (Conry et al. 1995; Hoerr et al. 2000; Zhou et al. 1999), a not-surprising result as antigens expressed from mRNA are processed inside the cell and displayed by MHC class I and class II molecules (Bryant et al. 2002; Rock et al. 2002, 2016). Thus, mRNA vaccines have the potential to elicit antibodies against infectious viruses and to target the infected cells through cellular immunity.

RNA vaccines fall into two basic types: Those that are introduced as mRNA and do not replicate, and those that contain an amplicon and thus can increase the copy number of the RNA, thereby enhancing antigen expression levels (for reviews, see references Ulmer et al. 2012; Brito et al. 2015; Uematsu et al. 2012; Tews and Meyers 2017). Although both types of RNA vaccines are produced using similar methods, we will discuss some specific issues associated with self-amplifying RNA vaccines separately. We will not cover self-amplifying RNA vaccines that are introduced by viral vectors.

There were several factors, both technical and scientific, that needed to be addressed before mRNA vaccines could become practical. Some of these are: (1) the ability to produce sufficient quantities of mRNA; (2) the reactogenicity of the RNA through its interaction with the innate immune system; (3) the stability of RNA; (4) the potential toxicity of the components used to encapsulate the RNAs;

and (5) the possibility that the encapsulated RNA will distribute to unexpected and undesired tissues and have negative consequences. We will discuss these in the following sections.

3 Production Limitations

One of the questions with RNA-based vaccines was whether sufficient quantities of an RNA of defined sequence, integrity and purity could be manufactured to supply sufficient doses for use in the target population. The problem with production was solved by using an enzymatic method that transcribes RNA from a DNA template, the feasibility of which was first shown by Krieg and Melton (1984, 1987), who determined that functional mRNA could be transcribed from a DNA template using the bacteriophage SP6 DNA-dependent RNA polymerase. As reported (Pascolo 2004), because the DNA template is used multiple times for transcription, the efficiency of RNA production is high. For example, up to 30 μg of capped mRNA can be obtained from about 1 μg of DNA (Pascolo 2008). By scaling up the in vitro transcription (IVT) reaction, yields of mRNA up to 10 g have been reported by some groups. And, since the doses of a mRNA vaccine are generally lower than for DNA vaccines (50–100 μg for mRNA compared with 1–5 mg for a DNA vaccine), generation of up to 100,000 vaccine doses should be possible from 10 g of mRNA. Further scale up is likely in the future. The various methods now used are the subject of many reviews (Weissman and Kariko 2015; Pardi et al. 2013, 2018; Pardi and Weissman 2017; Weissman 2015; Sergeeva et al. 2016; Maruggi et al. 2019; Zhang et al. 2019; Schlake et al. 2012, 2019; Kallen and Thess 2014) and are covered in other chapters of this volume.

4 Product Quality Issues

4.1 Chemistry, Manufacture and Controls (CMC) of Drug Substance

The overall steps in the production of a mRNA drug substance (DS) are similar whether for a conventional mRNA vaccine or a self-replicating mRNA vaccine. The starting material is a plasmid DNA into which the desired open reading frame (ORF) for the vaccine antigen has been inserted. The ORF is frequently codon optimized for expression in human cells, whereby codons used infrequently are substituted with codons used more frequently. This has the aim of increasing the rate of translation elongation and improving the yield of the expressed vaccine antigen. However, replacing rare codons with those used more frequently does not always produce the desired effects (Mauro 2018; Mauro and Chappell 2014). For

example, if translation is too rapid, the protein might not fold correctly into a functional antigen, and this can have the undesired consequences of reducing vaccine efficacy (Mauro and Chappell 2018). Because of these possibilities, several mRNA versions of the antigen gene may need to be evaluated in preclinical toxicology and immunogenicity studies.

The ORF is flanked by a 5′ untranslated region (5′-UTR) and a 3′-UTR followed by a stretch of 100–200 adenylate residues as the poly(A) region. The 5′- and 3′-UTRs can have significant effects on expression levels (Wilkie et al. 2003; Jia et al. 2013), and different sequences may need to be evaluated. The promoter for the ORF is usually from a bacteriophage such as SP6, T7, or T3, the sequences of which are known, and the bacteriophage DNA-dependent RNA polymerases are commercially available. The plasmid DNA is propagated in an appropriate bacterial strain, usually derived from *Escherichia coli,* and prepared under standard conditions using defined reagents. The plasmid DNA is linearized using a restriction enzyme whose site is downstream of the poly(A) site, and the resultant linear DNA is purified and used for IVT.

The IVT reaction uses a promoter-specific bacteriophage DNA-dependent, RNA polymerase (SP6, T7, or T3) and nucleoside triphosphates (NTPs) from commercial sources. Because these reagents (and other materials) are used to produce the DS, their quality must be documented. As described below, the NTPs often contain modified nucleosides to reduce the innate immune responses to RNA.

Following the IVT step, the DNA template is removed by DNase digestion, and the RNA is purified by different methods, such as alcohol precipitation, LiCl precipitation, HPLC, or FPLC. For translation and stability, the RNA needs to be capped and polyadenylated. The capping can either be done enzymatically after RNA synthesis using a capping enzyme, such as from vaccinia virus, or it can be done during IVT with the incorporation of different versions of a cap such as the anti-reverse cap analogue (ARCA) (Jemielity et al. 2003; Thran et al. 2017). There are advantages and disadvantages of each approach. The enzymatic capping method requires a separate step, although the yields are often higher than with the reverse-capping method.

The other modification required for production of a stable mRNA is the addition of the poly(A) tail. The length of the poly(A) tail can affect the translation efficiency, and thus, the expression level of the protein, and therefore, needs to be considered (Jalkanen et al. 2014). There are two approaches to add these adenylate residues. First, a sequence of A:T base pairs can be a component of the DNA template and is thus transcribed into poly(A) during the IVT step. It has been noted that long stretches of adenylate residues are not always stably maintained in plasmids and thus might be a problem for the propagation of some plasmids. An alternative approach is to add the poly(A) sequence to the RNA after transcription using poly(A) polymerase. Additional adenylate residues are sometimes added by poly(A) polymerase if the poly(A) sequence generated during IVT is too short.

Following capping and polyadenylation, the mRNA is purified, and the percentage of the mRNA that is capped and polyadenylated has to be determined as

one way to assess how well the manufacturing process has been controlled. Before the DS can be used for the mRNA vaccine drug product (DP), it will need to meet several assigned quality specifications, some of which are discussed below.

4.2 Identity

The identity of a nucleic acid vaccine is provided by its sequence. For mRNA vaccines, the sequence of the starting DNA plasmid used as template is generally sufficient, as the DNA is transcribed directly into RNA; sequencing the mRNA either by direct sequencing or by converting the mRNA to a cDNA and the sequencing of that DNA is not considered to provide additional information. Although the error rates of RNA polymerases are higher than those with DNA polymerases, the reported rates of 1 error in 10^4–10^5 nucleotides would not be readily detected in the RNA, since the errors are introduced randomly. Based on existing data that mRNA vaccines generate protective immunity in animal models, this error rate does not appear to affect the generation of an immunogenic antigen.

4.3 Consistency of Production

As with the production of any vaccine, consistency of manufacture is important and necessary to document. In the case of mRNA vaccines, which are synthesized enzymatically, there are several steps in the production of the mRNA that need to be monitored. Although the RNA polymerases are highly processive (Pardi et al. 2013; Borkotoky and Murali 2018; Zhu et al. 2014; Arnaud-Barbe et al. 1998), allowing the synthesis of long transcripts (Pardi et al. 2013), it is important to monitor the size of the transcript and to have appropriate assays in place to measure the size. Gel electrophoresis with size markers is one method to assess RNA integrity. As mentioned above, the percentage of the RNA that is capped and polyadenylated needs to be documented for each product.

4.4 Purity of the DS

The assays used to measure RNA purity and the acceptability criteria for a determination of purity must be developed and refined during vaccine development. Product- and process-related residuals in the DS include the components used in the IVT reaction, such as enzymes, nucleotides, nuclease, incomplete transcripts, and DNA fragments. These potential residuals can be removed by various methods such as precipitations and chromatography (Pascolo 2004). As discussed below, the presence of double-stranded RNA (dsRNA) in the mRNA DS—an inevitable

by-product of the IVT reaction—can cause stimulation of the innate immune system. As reported by Weissman and colleagues (Weissman et al. 2013), HPLC is effective in removing these types of by-products.

4.5 Stability of the DS

The DS can be stored, usually at ≤ -70 °C, and will be assessed for stability according to a protocol. This protocol should include periodic testing of the DS to ensure that the RNA retains critical attributes, such as percent full-length transcript and total RNA amount. It is well known that RNA is chemically more labile than DNA in high pH solutions, as the vicinal glycols in the ribose sugar are susceptible to base-catalyzed hydrolysis (Nielsen 2011). Such hydrolysis occurs more readily in single-stranded RNA than in double-stranded RNA or in regions of secondary structure due to base pairing. However, at neutral pH, RNA is more chemically stable than is commonly recognized (Pascolo 2004). The perceived lability of RNA is predominantly due to the presence of ribonucleases (RNases), the enzymes that cleave RNA. These RNases are abundant in nature and are themselves quite stable; hence, the concern with the use of naked RNA as a biological product and the need to provide it with some form of protection as the DP.

We point out here that the term DS is mainly historical and originates from the production of other types of vaccines. While we recognize that manufacturing processes that move directly from the mRNA transcript to the production of an encapsulated DP without a formal and stored DS are being developed, the term DS is retained as not all manufacturers are applying continuous-manufacturing methods and they do characterize and store a DS.

Additional quality attributes of the DS that should be evaluated include appearance, endotoxin level, pH, bioburden, and sterility.

4.6 CMC of Drug Product (DP)

The mRNA vaccine DP is usually produced by mixing the DS with various chemicals under defined conditions. One commonly used approach is to encapsulate the RNA in lipid-based particles (Kaczmarek et al. 2017; Sedic et al. 2018; Kulkarni et al. 2018; Hassett et al. 2019) and another is to complex it with positively charged molecules such as protamine (Kallen et al. 2013; Fotin-Mleczek et al. 2011, 2012; Weide et al. 2009). The aim of encapsulating the DS is both to protect the mRNA from degradation in vivo and to facilitate its entry into cells. The various approaches and methods to protect the mRNA DP are covered in other chapters and are the subject of several reviews (Pardi et al. 2018; Kulkarni et al. 2018; Zhou et al. 2013; Kowalski et al. 2019; Midoux and Pichon 2015).

However, there is a balance between making the particle so compact and inaccessible to cleavage by RNases such that the RNA is not able to be released from the particle upon entry into the cell *versus* making a particle that is not compact or protected enough. Lipid nanoparticles (LNPs) are commonly used to protect and enhance the delivery of mRNAs, as LNPs are of a size that may be taken up by cells in vivo and they usually contain ionizable cationic lipids, which facilitate release of the mRNA payload once inside the cell (Hassett et al. 2019; Midoux and Pichon 2015; Whitehead et al. 2009). If the LNPs contain a lipid component that is not a natural chemical (such as cholesterol) but instead is a novel compound that has not been a part of another drug used in humans, it may need to be tested for toxicity, particularly as some cationic lipids have been found to be toxic in vivo (Lv et al. 2006). Whether toxicology studies will need to be done on the individual components of the mRNA DP or on the final DP should be determined in consultation with the National Regulatory Authority (NRA).

Assembly of the mRNA vaccine DP from the DS and the various components is done by a number of methods. The important issue is whether the DP can be produced consistently. If the DP is a particle, then the particle-size distribution is an important indicator of consistency of manufacture. Dynamic light scattering is a common method used for determining the particle size.

Among the release specifications of the DP are the composition and the proportion of each component of the DP, the amount of mRNA encapsulated in the particles, and the integrity of the RNA in the particles. Determination of RNA purity and integrity with multi-component mRNA vaccines might require development of assays to measure and quantify each component specifically.

4.7 Potency Determination

Initially, it is advisable that a biological assay be used to assess expression of the vaccine antigens by the mRNA-based vaccine. While in vivo assays were originally requested for all vaccines, because of the variability of the immune responses in indicator animals, the cost and time involved, and the goal of reducing animal use in general, cell-culture-based assays have frequently replaced in vivo assays to quantify the expression of the vaccine antigen. For some vaccines, such as viral vaccines, potency can be determined by physicochemical methods once a correlation between biological activity and attributes of the physical particle has been documented. In the case of DNA vaccines, potency is generally provided by a measured amount of nucleic acid. It is anticipated that this will also become accepted with mRNA vaccines once regulatory authorities gather experience with this vaccine platform and a correlation between RNA amount and biological activity is demonstrated. As of now, the expression of the protein antigen following transfection of cells in culture with the encapsulated mRNA has been used as a measure of vaccine activity, although the dose of the mRNA vaccine is usually provided by the amount of RNA as determined by a quantitative assay such as

RT-qPCR. In the future, other types of assays might be developed to measure potency, and their appropriateness should be discussed with the NRA.

One important question is whether vaccine potency for each mRNA is reduced if the vaccine contains multiple mRNA components. Therefore, while it will be critical to characterize the quality of each component as described above, it will be necessary to evaluate the expression of each component individually and in the vaccine to confirm that there is no negative effect on antigen expression by the individual mRNA components when administered in combination or after being mixed together as a composite vaccine.

4.8 Stability of the DP

As for all vaccines, the stability of the mRNA vaccine needs to be documented. For conventional vaccines, stability is often evaluated under different storage conditions as well as under stressed conditions, such as at elevated temperatures. This also needs to be done for mRNA vaccines, where maintenance of the structure of the vaccine particle needs to be determined as well as the integrity of the mRNA molecule. As stated above, for multi-component mRNA vaccines, methods need to be developed to be able to assess the integrity of each mRNA component.

5 Potential Safety Issues

5.1 Immunogenicity of RNA

One of the potential safety issues with RNA vaccines was thought to be the fact that RNA itself can be immunostimulatory. In fact, there are many receptors that recognize RNA, and this subject has been reviewed thoroughly (Wu and Chen 2014; Cerboni et al. 2013; Crampton and Bolland 2013; Kindler and Thiel 2014; Freund et al. 2019; Sioud 2006; Chen et al. 2017; Fukui and Miyake 2012). The consequences of stimulating the innate immune system could be beneficial in that the RNA could have an adjuvant effect and increase the immune response to the antigen, or it could be detrimental by reducing the expression of the antigen (Weissman 2015). There are several approaches to reducing the immunogenicity of RNA. A major finding was that incorporating naturally occurring modified nucleosides into the IVT reaction reduced the immunogenicity of mRNA (Kariko et al. 2005). For example, the immunostimulatory activity of the IVT mRNA was greatly reduced if all the uridines were replaced by pseudouridine (Kariko et al. 2008). Other modifications of mRNAs have been reported to be at least as effective (Svitkin et al. 2017).

5.2 Reactogenicity of RNA

By-products from the DS manufacturing process that may be present in the mRNA DP and could cause adverse immune responses in vaccine recipients may include dsRNA, which is normally found in cells following infection with RNA viruses or DNA viruses (Matsumoto et al. 2011). These dsRNAs are detected by Toll-like receptor (TLR) 3 and may lead to a safety signal. While dsRNA might be difficult to eliminate if it is intrinsic to the mRNA structure, dsRNA as by-products of the IVT reaction can be reduced by such methods as HPLC or FPLC (Pardi and Weissman 2017; Pardi et al. 2018; Kariko et al. 2011).

5.3 Self-Amplifying mRNA (SAM) Vaccines

While SAM vaccines are produced in similar ways to conventional mRNA vaccines, there might be some differences in how they are evaluated for safety. Although the immunostimulatory activity of the SAM vaccines can be reduced by incorporating modified nucleosides, as done for conventional mRNA vaccines, because the RNA is amplified by the encoded viral RNA polymerase once inside the cell, the modified nucleosides are not available to be incorporated into the amplified RNA, and thus, the in vivo synthesized RNA is likely to be more immunogenic than the initial mRNA. And, because this increased reactogenicity may not be detected in preclinical studies in rodents and only become manifested during clinical trials, vaccinated individuals may need to be monitored closely, and dose-escalation studies may need to be carefully designed.

A second issue with SAM vaccines is the possibility that the vaccine could be transmitted from cell to cell if the vaccine antigen is itself a viral envelope protein that could form particles with the mRNA vaccine. Such particles have been described and are variously referred to as virus-like vesicles (Reynolds et al. 2015) or propagating replicons (Rose et al. 2008), and these particles have been shown to be "infectious." However, it should be noted that these infectious particles were produced in vitro following transfection of cells in culture with DNA expression vectors. Therefore, such particles are unlikely to be generated in vivo after inoculation of the self-amplifying RNA itself. Nevertheless, because such "infectious" particles if generated could distribute to distant tissues and be retained in the body longer, cell-culture studies and preclinical biodistribution studies might be requested to explore this possibility.

6 Safety Evaluation

6.1 Toxicity

While it is generally true that typical toxicology studies have not revealed safety signals with nucleic acid-based vaccines, because the mRNA vaccine components may be novel and not previously administered to humans, toxicology studies for this new vaccine platform might be required. However, whether the whole formulated DP or the individual components are assessed for toxicity is a decision for individual NRAs.

A toxicology study of a mRNA vaccine would generally include an assessment of the reactogenicity of the vaccine in a relevant animal model. However, because the mouse and human immune systems are not always concordant (Mestas and Hughes 2004), and the two species can respond differently to TLR agonists (Fukui and Miyake 2012; Gorden et al. 2006a, b; Jurk et al. 2002; Lee et al. 2003; Hemmi et al. 2002), the assessment of potential reactogenicity of the RNA vaccine can be complicated. Considerations for selection of the animal species for nonclinical evaluation of mRNA vaccines should be discussed with the NRA.

6.2 Genotoxicity

Because mRNA has no means of integration into the host chromosome and does not alter the host genetic sequence, mRNA vaccines against infectious diseases are not considered as gene-therapy products (Hinz et al. 2017), and thus, genotoxicity studies are not considered to be informative (Pascolo 2008). Nevertheless, manufacturers should consult their NRA for guidance.

6.3 Biodistribution and Retention of the DP

Since most vaccines are introduced parenterally either by the intramuscular, subcutaneous, or intradermal routes, they have the potential to be distributed throughout the body by the blood and lymphatic systems. Biodistribution and retention studies are designed to determine whether the vaccine migrates after inoculation, to which tissues, and for how long the vaccine remains in those tissues. These studies are generally done in rabbits or rats, but other small animals can be substituted.

Various nucleic acid detection methodologies, such as standard or quantitative PCR assays and branched DNA assays, have been used to detect vaccine-specific RNA. Historically, for conventional vaccines, biodistribution studies have not been particularly informative, with the vaccines generally being localized to the site of

inoculation and the draining lymph nodes and sometimes the spleen. This may not be the case for viruses that are known to be neurotropic and is the reason why biodistribution studies continue to be requested for viruses with this capacity. For mRNA vaccines that do not have components that target the vaccine to neurological tissues, it is unlikely that biodistribution studies will be informative. In addition, data have shown that retention times for the mRNA vaccines tested to date are short, usually within a week or so, and these studies have not raised safety concerns. This fairly rapid clearance of the vaccine is likely facilitated by the labile nature of the components of the mRNA vaccine (Maier et al. 2013).

The caveat with biodistribution studies is that the ability to detect the vaccine nucleic acid at sites distant from the site of inoculation is limited by the sensitivity of the assay used and the dilution of the vaccine in the body. Likely because the number of genome copies is generally low for conventional vaccines [e.g., under 10^{11} copies per dose for replication-defective adenovirus vaccines (Barry 2018)], it has not been possible to detect the vaccine at distant sites. Even with DNA vaccines, which have been administered at doses of 4 mg or more, the distribution has been difficult to detect at sites other than the site of injection and the draining lymph nodes, and the DNA signal at distant sites, if detected, rapidly deceases (Sheets et al. 2006a, b). For a DNA vaccine with a genome size of 10,000 base pairs, the genome copy number for a 4-mg dose is about 4×10^{14}. Similar calculations for RNA copy numbers can be made for mRNA vaccines, although the behavior of these vaccines in biodistribution and retention studies would likely be different from DNA vaccines because a lipid-based particle encapsulating the mRNA might facilitate passage to distant organs and result in longer retention times. For this reason, biodistribution and retention studies have been requested for mRNA vaccines.

6.4 Adjuvant Versus Delivery Vehicle

The WHO guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines published in 2013 refer to vaccine delivery systems as a type of vaccine adjuvant https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf. However, whether the lipid components of the mRNA vaccine DP should be considered as adjuvants or as protectants/drug delivery vehicles needs to be decided case by case, and the manufacturer should consult CBER.

7 Concluding Remarks

It is well recognized that having a common platform for vaccine production that can be used for the rapid introduction of effective vaccines against various infectious diseases will be critical should threats from emerging agents arise, as well as against

agents of bioterrorism. Because traditional vaccines usually take years to develop, having several proven platforms would be advantageous. It is unlikely that a single "platform" will be appropriate to develop safe and effective vaccines for all infectious diseases, and therefore, vaccines should be developed using several "platforms." Nucleic acid vaccines have shown promise, and in several cases, products have reached the clinic within months rather than years, which has generally been the case for conventional vaccines. In addition, it is possible and even likely that once experience with the different platforms is obtained, regulatory agencies will be able to leverage existing preclinical data and potentially reduce preclinical safety testing. For example, carrying out biodistribution and retention studies in vivo for mRNA vaccines using the same delivery system might not be informative once data for several vaccines have been obtained and no safety signals have been identified. In addition, while IVT from a DNA template appears to work well, other methods of RNA production may become available in the future, e.g., RNA amplification by RNA synthetase may be possible. Also, as we have seen with the DNA synthesis, purely chemical synthesis of RNA may become practical in the future. Because of these and other technological advances, combined with results from the several ongoing human clinical trials, mRNA-based vaccines are a promising novel platform that might be useful for the development of vaccines against emerging pandemic infectious diseases.

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