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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.

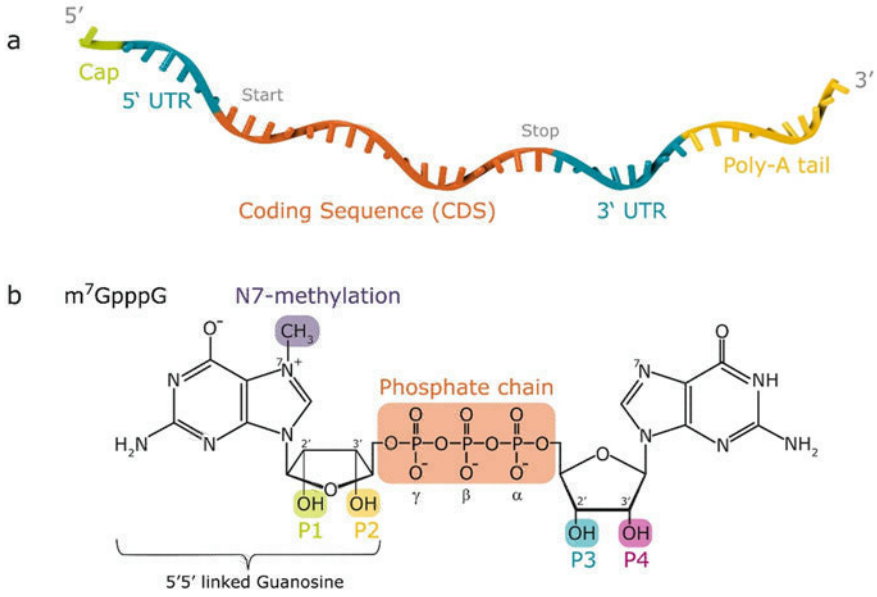

Fig. 1 Schematic structure of the mRNA and cap. **a** The general structure of an mRNA is based on a 5′ Cap, a 5′ UTR, an open reading frame (ORF) coding for the respective antigen, a 3′ UTR and a 3′ end containing a poly(A) stretch. **b** The 5′-cap structure is a N7-methylguanosine (methyl = purple) 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).