RNA Technologies 13

Stefan Jurga
Jan Barciszewski *Editors*

Messenger RNA Therapeutics

Springer

RNA Technologies

Founding Editor

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Volume 13

RNA Technologies provides critical and comprehensive discussions of the most significant areas of RNA research, written by leading international authorities. Each volume in the series reviews both general topics, such as RNA interference, RNA biosynthesis and metabolism, microRNAs and diseases, transcriptome analysis and aptamers, as well as highly topical chapters on very recent findings in the field. Recurring themes of the series include the complexities associated with target recognition, RNA-protein interactions, analysis of genetic networks and the promise of small RNAs as cancer diagnostics and therapeutics in combating cancer.

RNA-based approaches have great potential to revolutionize molecular biology, cell biology, biomedical research and medicine. The nucleic acid-based molecules can be used to regulate the level of gene expression inside the target cells and their potential efficacy against several viruses, including SARS-CoV-2, and other chronic diseases represents a promising research area.

RNA Technologies publishes in both print and electronic format since 2010 and is intended for researchers from academia and industry, as well as graduates that look for a carefully selected collection of high quality review articles on their respective field of expertise.

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Stefan Jurga · Jan Barciszewski
Editors

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Springer

Editors
Stefan Jurga
NanoBioMedical Center
Adam Mickiewicz University
Poznań, Poland

Jan Barciszewski
NanoBioMedical Center
Adam Mickiewicz University
Poznań, Poland

Institute of Bioorganic Chemistry
Polish Academy of Sciences
Poznań, Poland

ISSN 2197-9731 ISSN 2197-9758 (electronic)
RNA Technologies
ISBN 978-3-031-08414-0 ISBN 978-3-031-08415-7 (eBook)
https://doi.org/10.1007/978-3-031-08415-7

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

In memoriam of Professor Stefan Jurga

Introduction: IVT Messenger RNA in the Syringe

The last year marked Diamond Jubilee of four great discoveries: (i) messenger RNA by Sidney Brenner, Francois Jacob, and James Watson; (ii) regulatory mechanisms in the proteins synthesis by Francois Jacob and Jacques Monod; (iii) the genetic code first letter—phenylalanine encoded by poly(U) by Heinrich Matthaei and Marshall Nirenberg; and (iv) the ribosome—the synthetically active, membrane-free particles by Howard M. Dintzis.

The two COVID-19 mRNA vaccines Comirnaty (Tozinameran) and Spikevax (Elastomeren) in clinical trials in 2021 hinged upon those discoveries in the RNA field, leading the way to significant progress for a new RNA technology based on engineering genetic instructions in messenger RNA (mRNA) discovered 60 years ago in 1961. Twenty years later, the RNA World came into reality. The outcome was that RNA tantalized pharmaceutical companies, promising a simple, efficient, and flexible way to deliver nucleic acid drugs and mRNA vaccines (Fig. 1).

These methods are based entirely on various properties of RNA. Francis Crick RNA already in 1959 in the letter to the Tie Club members nicely characterized: *What are properties of genetic RNA? Is he in heaven, is he in hell? That dammed, elusive Pimpernel* (C. B. Johnson, *The Scarlet Pimpernel*, Simson and Schuster 2004, p. 124). Current mRNA vaccines' success again creates an enormous interest and enthusiasm around the different applications of RNA.

The RNA drugs face the challenges of targeting mRNA to specific tissues and giving substantial and lasting benefits without excessive side effects. The potential of mRNA vaccines was already demonstrated in 1990 but without therapy implementation. RNA medicines, especially those that replace beneficial proteins for chronic disease, had a difficult road to the clinic than the other vaccines. For the last 30 years, this approach was not very practical due mainly to an mRNA short half-life and inefficient in vivo delivery.

Recently, messenger RNA went into the clinic is helping patients. Messenger RNAs (mRNAs) are intermediates between the coding genomic DNA and the encoded proteins. mRNAs are blueprints of genes encoded in the genomic DNA synthetic or in vitro synthesized mRNA transcripts of the genes deliver the genetic information to the translational machinery to generate the encoded proteins.

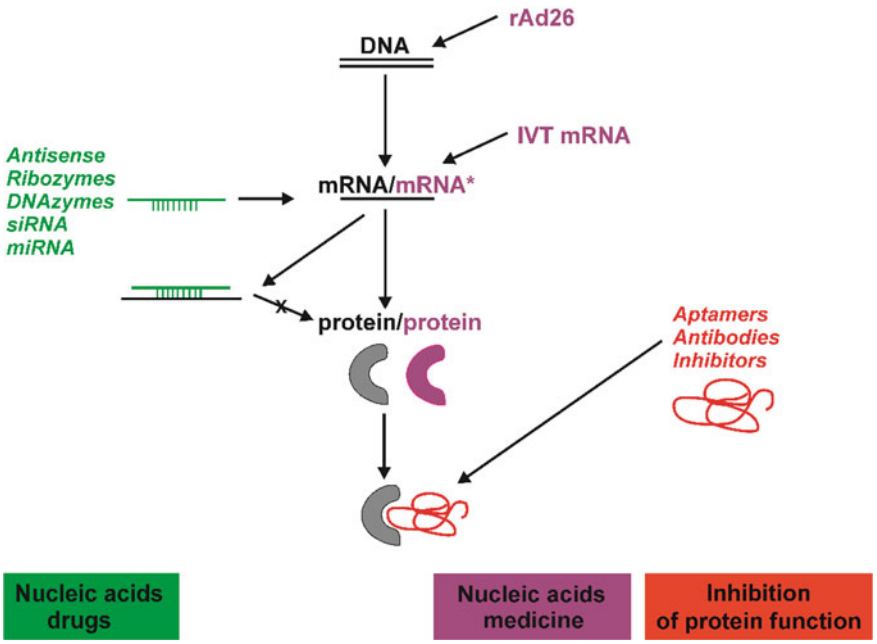

Fig. 1 Regulation of gene expression at the DNA, RNA, and protein levels. In addition to the downregulation of messenger RNA with various RNA technologies, new information can be introduced into the cell using adenovirus or in vitro transcribed (IVT) mRNA. Pathogenic proteins can be blocked with antibodies or inhibitors

Generally, a new mRNA technology development depends on identifying the target antigen and its nucleotide sequence, the preparative scale of mRNA synthesis in GMP conditions, and mRNA encapsulation in lipid nanoparticles. mRNA technology offers advantages for the research, development, and production of various vaccines. It expresses only the antigen(s) of interest. The expression is transient, and there is no risk of genome integration. The same approach can be adapted for various mRNA vaccines with a different coding sequence. The process is cell-free and does not require animal-based materials.

Tailoring mRNA medicine to a disease means tweaking the structures of the mRNA itself and the lipid nanoparticle used to ferry it through the body. Once injected into the cell, mRNA translates into a specific viral protein that trains the immune system to recognize the virus. mRNA vaccines are faster, cheaper, more adaptable, and easier to mass production than traditional vaccines. The mRNA vaccine ultimately trains the body to defeat a given pathogen before it can inflict harm. A local injection into the muscle, under the skin, or into a tumor can also deliver mRNA-based therapies that harness the immune system to fight cancer. Newly produced antigens trigger the body's immune response and the production of antibodies, which ultimately protect the body against future infections from the virus.

Synthetic messenger (IVT) RNA is a common starting material for medical applications. Its transient nature is a benefit, where time-controlled gene expression is required. The immune system will recognize these proteins as antigens or foreign substances that need to be vanquished to protect the body's health.

Messenger RNA production starts with in vitro transcription (IVT) of linear DNA template of target by DNA-dependent bacteriophage (T7, SP6) RNA polymerase. IVT mRNA consists of a single-stranded open reading frame (ORF) flanked by two untranslated regions (UTRs), 5' cap at the 5' end and 3' poly(A) tail important for stability.

Liposome-mediated RNA transfection of mRNA as a therapeutic was conceived in 1989. Next, in 1990, it was shown that after injection into mice is functional in mice. However, the main reasons for a low interest in mRNA as a therapeutic were the lability of RNAs, immunogenicity, low level and transient translatability, and difficulty working with fragile RNA. These difficulties have been overcome after incorporating modified nucleosides, which efficiently reduced immunogenicity and significantly increased its translation. Vaccine's mRNA is stable and no immunogenic when uridine is replaced with pseudouridine, 1-methylpseudouridine, 5-methoxyuridine, or 5-methylcytosine. Deimmunizing IVT mRNA by incorporating modified nucleosides was a fundamental strategy to broaden the potential application.

Improvements in mRNA delivery have significantly impacted the advance of IVT mRNA toward the clinic. Messenger RNA has vast flexibility concerning production and application. Once mRNA encapsulated in lipid nanoparticles (LNP) gets into a body, the mRNA goes into the cells, and their intracellular translation machinery is triggered to produce the antigen protein. LNP is composed of four ingredients. Ionizable lipids with positive charges bind to the negatively charged backbone of mRNA, pegylated lipids that stabilize the particle, phospholipids, and cholesterol contribute to the LNP particle's structure. They also protect mRNA during storage, injection, and transport through the bloodstream.

mRNA therapeutics are efficient because they do not need to cross the nuclear envelope, lack MHC haplotype restriction, and bind to pattern recognition receptors. They are directly translated in the cytoplasm, obviating the need for translocation to the nucleus and resulting in fast protein production. mRNA is capable of activating the immune response and with long-lasting immunity. In addition, mRNA vaccines are self-adjuvanting, which is not the case for peptides—and protein vaccines.

On the other hand, mRNAs have some limitations. The scale is still small. Further process development will likely make mRNA production cost-efficient to manufacture at gram scale. The limited stability of mRNA in vivo and its sensitivity to degradation during handling pose challenges. Also, mRNA may or may not be recognized as foreign nucleic acid by the immune system.

One can find an overview of the composition and performance of mRNA vaccines and fundamental insights into their modes of action. Their rapid development, production, and efficiency demonstrate mRNA therapeutics momentum.

Poznań, Poland

Stefan Jurga
Jan Barciszewski

Contents

Roadmap to the Development of mRNA Therapeutics: From Molecule Design and Delivery Strategies to Manufacturing, Quality Control, and Regulatory Considerations

Randall A. Meyer, Sara Trabulo, Julie A. Douthwaite, and Jose Luis Santos

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Abstract mRNA has been touted as a therapeutic modality since 1990 and has demonstrated potential applications in oncology, protein replacement therapies, and infectious diseases. Since the first demonstration of direct injection of mRNA into muscle showing expression of the encoded protein, tremendous progress has been made to improve mRNA functionality, stability, and safety. Progress has also been made with the development of specialized drug delivery systems that enable delivery of mRNA to virtually any organ, tissue, and cells in the body. The rapid development

R. A. Meyer · J. L. Santos (✉)
Dosage Formulation and Drug Delivery, Biopharmaceutical Development, R&D, AstraZeneca, Gaithersburg, USA

S. Trabulo
Analytical Sciences, BioPharmaceutical Development, R&D, AstraZeneca, Cambridge, UK
e-mail: sara.trabulo@astrazeneca.com

J. A. Douthwaite
In vivo Expressed Biologics, Discovery Sciences, R&D, AstraZeneca, Cambridge, UK
e-mail: julie.douthwaite@astrazeneca.com

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_1

and deployment of mRNA vaccines to address the COVID-19 global pandemic is a true testimony of the significant developments of mRNA technology in the past 30 years. In this book chapter, we will review design strategies used to improve mRNA functionality, stability, and safety and delivery strategies that have been used to deploy mRNA vaccines and provide an overview of manufacturing technologies and regulatory challenges. We will present our own perspective in these areas, gained through building an end-to-end mRNA discovery and development platform to evaluate therapeutic mRNA as a rapid response to pandemic threats and pandemic prevention.

Keywords mRNA · Vaccine · Drug delivery · Nanoparticle · Drug manufacturing · Quality control

1 Introduction

mRNA as a therapeutic modality for protein expression has emerged in recent years as an exciting new frontier in the biotechnology community. Once thought of as an undevelopable modality with insurmountable development-related challenges, mRNA is now the foundation of the two most successful vaccines on the fight of COVID-19 pandemic, developed by Pfizer/BioNTech and Moderna (Buschmann et al. 2021). In addition to the FDA-approved COVID-19 vaccines, multiple mRNA-based products are currently in various stages of clinical development. With respect to infectious disease, novel mRNA vaccines are being developed for influenza, CMV, and Zika virus among others (Hekele et al. 2013; Erasmus et al. 2020; Liang et al. 2017; Kowalski et al. 2019). mRNA is also being explored in immuno-oncology indications with BioNTech's HPV cancer vaccine currently in a Phase 1 clinical trial (Parums 2021). Other applications of mRNA therapeutics include regenerative medicine including AstraZeneca's VEGF mRNA for cardiac regeneration in Phase 2 (Anttila et al. 2020). mRNA encoding for antibodies is also being developed for pandemic prevention as a rapid response passive immunization strategy for first responders and vulnerable populations. As a genetic medicine, mRNA plays an essential role in CRISPR Cas9 applications. These only represent a small fraction of the applications for which mRNA is being developed and the potential applications in other indications are vast. mRNA appears poised to revolutionize the biotechnology industry in the same way monoclonal antibodies did several decades ago.

The success of mRNA therapeutics has only been made possible through substantial developments in mRNA design, formulation, and manufacturing. As recently as two decades ago, mRNA was viewed as too immunogenic to use in humans for any application (Chaudhary et al. 2021). Compounding the challenge, mRNA must be delivered to the intracellular space as opposed to other biologics which typically act extracellularly and that the molecule is inherently unstable in biological fluids (Friedhoff et al. 1994). Significant improvements have been made in the in vitro

synthesis of mRNA enabling the modeling of immunogenicity for different applications and enhanced stability and translatability for longer term protein expression. From a delivery standpoint, multiple novel materials have been developed to stabilize mRNA and enhance delivery to the cytosol of target cells within a tissue of interest for translation into protein (Buschmann et al. 2021). More recently, with the scale-up production of mRNA therapeutics for clinical use, innovative manufacturing approaches have been implemented. These developments, many years in the making, have now successfully coalesced to form mRNA therapeutics and will enable a new generation of medicines to treat human disease.

In this chapter, we will discuss the challenges associated with the development of mRNA therapeutics and the strategies utilized to overcome them. We will review developments in mRNA design/synthesis, mRNA delivery, and mRNA drug product manufacturing/quality control. We will then close with a discussion of existing challenges in the implementation of mRNA therapeutics as well as a perspective on future directions for this novel modality.

2 mRNA Design Strategies

The basic structure of therapeutic mRNA closely resembles a mature eukaryotic mRNA transcript, being composed of the protein-encoding region flanked by 5′ and 3′ untranslated regions (UTRs), a 5′ 7-methyl guanosine cap structure and a 3′ poly(A) tail. Since mRNA is used directly for protein synthesis in the cell cytoplasm, its design is relatively simple compared to other gene delivery modalities such as viral-based therapeutics where delivered DNA must enter the nucleus prior to mRNA transcription and protein production. However, mRNA is inherently unstable and tightly regulated due to its endogenous role as a transient message for protein expression, and cellular entry of RNA from the extracellular compartment is normally only seen during viral infection. The molecular characteristics are key to successful mRNA design to appear self-like and to achieve the desired level of protein synthesis. Close interplay between mRNA half-life, protein expression, and immune recognition also exists. For example, non-self RNA-sensing and an innate immune response leads to protective antiviral mechanisms like RNA degradation and inhibition of protein synthesis. Given these complexities, thorough optimization of all aspects is critical to achieve the most potent mRNA possible, thus reducing the required dose, and improving efficacy and safety. A wealth of knowledge has been built in the last three decades on the design of mRNA therapeutics, mostly based on vaccines. This allows a near "plug and play" approach, as has been perfectly demonstrated by COVID-19 mRNA vaccine development in which mRNA designs were achieved in a matter of days (Xia 2021). Therapeutic mRNA design must consider each element for optimized function for different therapeutic indications, combining suitable immune response, protein expression, and mRNA stability.

2.1 Reducing mRNA Immunogenicity

RNA structures found within pathogens such as viruses contain pathogen-associated molecular patterns (PAMPs) that are recognized by numerous specialized intracellular pattern recognition receptors (PRRs) and elicit innate immune system responses to resolve infection (Olive 2012). Therefore, significant PAMP content in the context of a therapeutic mRNA can lead to loss of the mRNA and low to no translation. In the case of mRNA vaccines where mRNA can act as an adjuvant to the therapeutic goal of an antibody-based adaptive immune response, it is still important to minimize RNA-directed innate inflammation to maintain potency (Linares-Fernández et al. 2020). Strategies for controlling RNA-directed innate immune response revolve around imparting self-like properties to the mRNA to mimic endogenous mRNA and avoid an antiviral response and primarily include choice of the 5′ cap, and use of chemically modified RNA nucleosides to reflect modifications naturally occurring in endogenous RNA nucleotides (Minnaert et al. 2021). The 5′ cap of choice for therapeutic applications is Cap1 which is less immunogenic than Cap0. The most commonly used chemical modification approach is replacement of uridine with N1-methyl-pseudouridine or 5-methoxy-uridine, since unmodified uridine is a significant PAMP; however, other nucleotides can be replaced with chemical modifications such as cytidine with 5-methylcytidine (m5C) or adenosine with N1-methyladenosine (m1A) and N6-methyladenosine (m6A). Sequence optimization can also moderate innate immune recognition, for example minimizing uridine content itself by GC-rich codon choice (Roth et al. 2021). It is important to note that design of the mRNA molecule is only one part of the requirements for avoiding innate immune activation through mRNA, with the manufacturing processes being highly significant in avoiding the generation of or removal of in vitro transcription byproducts that may be highly immunogenic.

2.2 Optimizing Protein Expression

All elements of mRNA from the 5′ to 3′ end play an important role in the regulation of translation and therefore are key to the design of an optimized molecule. Basic prerequisites include a 5′ cap structure for translation initiation through interaction with the poly(A) tail and eukaryotic initiation factors (Roth et al. 2021). Both the 5′ cap and poly(A) tail also regulate mRNA stability, which in turn effects protein expression. However, the two key aspects of mRNA design influencing protein expression outside of immunogenicity and basic function are the 5′ UTR sequence, and the codon choice within the protein coding region. An optimal 5′ UTR structure allows efficient ribosomal scanning in search of the start codon leading to efficient translation initiation which can be rate-limiting in protein expression (Trepotec et al. 2019). Several options to UTR choice exist, from the direct use of known UTR sequences from highly expressed proteins having stable transcripts, for example, those of α-globin

or β-globin (Wang et al. 1999), to a library-based 5′ and 3′ UTR screening activity based on expression and stability of the protein of interest (von Niessen et al. 2019; Cao et al. 2021). Codon choice for the open-reading frame can be based on knowledge of endogenous codon use such as avoiding rare codons, using codon ratios found in highly expressed proteins, and analysis of codon pairs or bi-codons (Diambra 2017). Other aspects less often considered, but potentially significant are codon choice for translation accuracy, for example, selecting the codon with the lowest decoding error rate or following codon use of functionally important as well as highly expressed genes (Xia 2021). Empirical testing of candidate sequences is highly recommended to balance codon optimization for translation efficiency with nucleotide content for reduced PAMP content, although general approaches of guanine and cytidine enrichment or uridine depletion while avoiding rare codons are likely to be successful option.

2.3 Optimizing mRNA Stability

The polyA tail is a key determinant of mRNA stability where it protects the 3′ end from nuclease degradation and interacts with RNA-binding proteins to modulate stability. For suitable stability (and translation), a poly(A) tail of 80–120 nucleotides is optimal for IVT-mRNAs (Lima et al. 2017). The poly(A) tail is ideally template-encoded and added during IVT, thus leading to a defined product compared to post-IVT enzymatic tailing giving a distribution of heterogenous lengths. mRNA stability is also imparted by the 5′ cap which acts as a protective group against 5′–3′ exonuclease cleavage. UTR elements flanking the coding sequence can influence the stability of mRNA through interactions with RNA-binding proteins and other factors such as microRNA that can recruit or protect from nucleases. For example, microRNA binding sites can be deliberately encoded within the 3′ UTR to initiate mRNA degradation in certain cells where expression is not required, as has been shown for miR-122 effectively functioning as a liver de-targeting mechanism (Jain et al. 2018).

Taken together, the ideal features of an optimized mRNA for therapeutic use can be taken as (i) a 5′ Cap1 incorporated as a cap analogue during IVT, (ii) a short unstructured 5′ UTR from a highly expressed human protein, (iii) a GC-rich open-reading frame optimized for codon choice, (iv) a 3′ UTR from a highly stable mRNA and shown experimentally to be optimal in the mRNA construct under design, and (v) a template-encoded 80–120 long poly(A) tail. Such design principles can allow development of a modular platform with which novel mRNAs can be designed rapidly.

3 Delivery Strategies for mRNA Therapeutics

One of the central challenges of mRNA as a therapeutic modality is the delivery to the intracellular compartment of a target cell. mRNA is unique compared to other biologics in that it leverages its mechanism of action within the cytosol at the ribosome where all other mRNA is translated into protein. mRNA is a very large and highly negatively charged molecule that cannot freely diffuse through the cell membrane in the same way hydrophobic small molecules do. To further complicate this process, there are specifically evolved innate immune defense mechanisms in eukaryotic cells to prevent the invasion of foreign RNA nucleic acids for protection against viral infection. In addition, mRNA is highly unstable in most biologic media due to the ubiquitous presence of RNases. These enzymes, present in nearly all biological fluids, rapidly degrade the mRNA on the order of minutes, long before it can successfully direct the translation of the desired protein (Friedhoff et al. 1994). Although naked mRNA has been successfully used in a few specific scenarios (Gan et al. 2019), these formidable barriers require unique drug delivery solutions to enable mRNA as a viable therapeutic platform for most therapeutic applications.

3.1 Lipid Nanoparticles for mRNA Delivery

Solid lipid nanoparticle (LNP) is the most advanced delivery system for mRNA therapeutics. LNPs are used to deliver the COVID-19 mRNA vaccines developed by Pfizer and Moderna—the only FDA-approved mRNA therapeutics. LNPs are colloidal suspensions of mRNA encapsulated in lipids designed to protect mRNA from extracellular degradation and assist in cellular uptake and endosomal escape of mRNA. LNPs for mRNA delivery typically consist of four component lipids—a cationic lipid, a PEGylated lipid, a helper lipid, and cholesterol. The cationic lipid is generally regarded as the most important lipid for the specific application of mRNA delivery. The positively charged cationic lipid binds to the highly negatively charged mRNA to encapsulate it and protect it from endonucleases. The cationic lipid also assists in endosomal escape through buffering of the endosome, increasing the osmotic pressure of the endosome to facilitate disruption and release of the mRNA into the cytosol. The PEGylated lipid plays an important role in LNP stability during manufacturing, in vivo circulation, and immune stealth. PEGylated lipids are present at the surface of the LNP to prevent individual LNPs from fusing during formulation and during storage. PEG at the LNP/aqueous interface results in a highly hydrophilic surface that can resist opsonization leading to immune elimination. The helper lipid is typically an additional phospholipid that increases the hydrophobicity of the LNP core and also facilitates endosomal escape through interactions with the endosomal membrane. Finally, cholesterol plays an important role in stabilizing the LNP providing a solid structure. Cholesterol is also believed to have a special role as a “targeting” ligand in systemic administration through binding lipoproteins and

driving LDLR-mediated liver uptake of LNPs (Akinc et al. 2010). While a significant number of LNPs have been developed to optimize safety and efficacy in reaching targets in the liver, recent efforts have focused on tuning LNP composition to target cells and tissues beyond the liver, promising a new generation of mRNA therapies.

3.2 Other Materials for mRNA Delivery

Although lipids are the primary material used to formulate mRNA for most therapeutic applications, they are not the only solution to the unique barriers of mRNA delivery. Other biomaterials have shown significant promise through recapitulation of the key functional aspects of the lipids in a typical mRNA-LNP formulation. Although not as mature as lipids in terms of clinical development, polymers have emerged as delivery alternative of interest and shown promise in preclinical studies for mRNA delivery. Polymers offer additional benefits relative to lipids, owing in part to their chemical and physical versatility and additional stability conferred to the formulation. Polymers have been extensively engineered to modulate degradability, stimulus-triggered release, and targeting of specific cells/tissues. Common polymers used in different formulations for mRNA delivery include but are not limited to polyethylenimine, polycarbonates, and poly (β-amino) esters (Kowalski et al. 2019). Block co-polymers have been used to recapitulate the properties of all four LNP lipid subtypes in a single polymer entity. In other instances, various lipids and polymers have been combined in hybrid formulations for mRNA delivery. Short, hydrophobic oligomers termed lipidoids have been developed and used extensively throughout the literature for mRNA delivery. LNP lipids and polymers have also been blended as distinctive entities in an amalgamated particle with mRNA. Additional materials including dendrimers, cell-penetrating peptides, and inorganic metallic nanoparticles have also been successfully used for mRNA delivery in preclinical studies (Kowalski et al. 2019).

3.3 mRNA Drug Product Route of Administration

Once the mRNA is formulated in a LNP, it is ready for clinical use. The route of administration is typically dependent on the therapeutic indication and can have significant impact on the efficacy of the mRNA. For vaccines, including the COVID-19 vaccine, the most common route is intramuscular injection (Hassett et al. 2019). In vaccine applications, the mRNA will typically code for an antigen associated with the pathogen of interest. Once in the intramuscular space, the LNPs can be taken up by resident tissue antigen-presenting cells or drain directly to the lymph nodes, owing in part to their small size. The production of the antigen will elicit an immune response. mRNA vaccines against infectious pathogens have also been

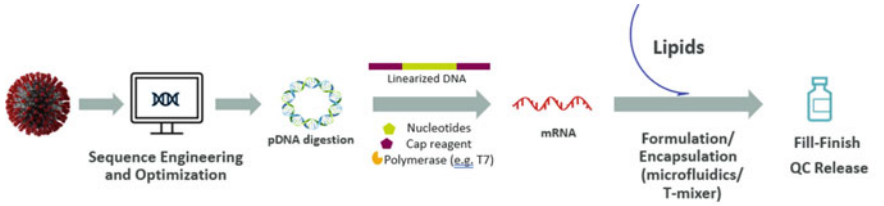
administered subcutaneously, intradermally, and directly to the respiratory system through inhalation (Pardi et al. 2018).

Although the initial clinical success of mRNA therapeutics was for infectious disease vaccine applications, mRNA has significant potential in other therapeutic areas including oncology. mRNA can direct anti-tumor immune responses through either a traditional vaccine/tumor-associated antigen approach or through the production of immunostimulatory ligands and cytokines at the tumor site. mRNA can code directly for the secretion of other anti-tumor proteins such as tumor-related apoptosis-inducing ligand (TRAIL) or sensitizing proteins such as herpes virus kinases to render tumor cells susceptible to antivirals (Tzeng et al. 2016). For most oncology applications, mRNA therapeutics can be administered directly at the tumor site provided it is amenable to a medical procedure.

mRNA therapeutics are also being developed for regenerative medicine and protein replacement therapies for genetic disorders. The route of administration in this therapeutic area is highly application dependent. For regenerative medicine applications, the mRNA is commonly administered directly at site of tissue damage/repair. mRNA encoding for regenerative factors has been administered directly into the cardiac muscle for repair following ischemia (Carlsson et al. 2018) or into the skin for wound healing applications (Gan et al. 2019). For metabolic disorders, the LNP is administered systemically to take advantage of lipoprotein-mediated liver accumulation of mRNA-LNPs. At the liver, the mRNA can direct the production of proteins required for normal metabolism to replace genetically defective ones (Ramaswamy et al. 2017). Liver-targeted mRNA delivery is also of interest for the production of exogenous therapeutic proteins as well. One active area of investigation utilizing this approach is pandemic prevention through use of mRNA encoding for antibodies against the target pathogen (Rybakova et al. 2019). Such a technology could be rapidly produced to induce temporary passive immunity in vulnerable individuals at the onset of a pandemic to prevent rapid spread throughout the population.

4 Manufacturing of mRNA Drug Products

While mRNA has been touted as a therapeutic modality for the past 2 decades, the recent approval of mRNA vaccines to curb the COVID-19 pandemic has shown both the promise and areas for improvement of this therapeutic modality. Notably, the massive demand for mRNA vaccines to meet the global supply has exposed areas in need of higher efficiency: appropriate infrastructure to manufacture the mRNA drug substance (DS) and mRNA-LNP drug product (DP), clinical, safety, and regulatory frameworks. mRNA is typically produced in a cell-free, chemically well-defined, robust, and adaptable manufacturing process that is nearly independent of the encoded protein or antigen. This feature makes mRNA an exceptional tool for rapid response against infectious disease threats. A general representation for the development and manufacturing of mRNA vaccines is provided in Fig. 1.



	Quality Attributes*
Plasmid DNA	Purity, identity, DNA content, process related impurities, endotoxin
mRNA Drug Substance	Purity, identity, content, poly-A length, 5′ capping, residual impurities, endotoxin, sterility
mRNA-LNP Drug Product	Size, polydispersity, encapsulation efficiency, mRNA content, lipid content, potency, residuals, endotoxin, sterility, pH, sub-visible particles

Fig. 1 Schematic representation of mRNA vaccine development starting from pathogen and related sequence identification. mRNA is produced from a pDNA template that is digested using an appropriate enzyme to generate the corresponding linear DNA, which is then combined with nucleotides, capping reagent, and a promoter-specific polymerase to generate the mRNA via in vitro transcription. mRNA is then purified to remove process-related impurities (e.g., dsRNA), formulated in buffer and QC released. mRNA is then combined with lipids to form mRNA encapsulated LNP that is purified, sterile filtered, filled in vials, and released. *General representative list of quality attributes provided for general guidance and is not an exhaustive list

4.1 mRNA Drug Substance Manufacturing

The production of mRNA entails three steps: (i) production of starting material plasmid DNA (pDNA) and corresponding linear DNA template; (ii) in vitro transcription (IVT) reaction, and (iii) mRNA purification and QC release. The starting material is usually a pDNA vector, containing a promoter compatible with a bacteriophage RNA polymerase (e.g., T7), the opening reading frame encoding for the protein or antigen of interest, the 5′ and 3′ untranslatable region sequences, and the encoded poly(A) tail. The pDNA vector is produced using standard *Escherichia Coli* fermentation and ideally enriched in supercoiled isoform. pDNA is then linearized with a restriction enzyme that allows the synthesis of transcripts with a 3′ end poly(A) tail, and the linear DNA template is isolated through purification to remove the enzyme and unwanted process impurities. Following purification, the production of mRNA by IVT takes place through combining the linear DNA template with the appropriate RNA polymerase, nucleoside triphosphates, the chemically synthesized cap analog, and the optimized buffer reaction media. Once the IVT reaction is finalized, the residual DNA is digested using DNase (e.g., DNase I enzyme), and the crude IVT product is subjected to a multi-step purification process to remove enzymes, excess nucleotides, residual contaminants, and in-process–related impurities (e.g., truncated RNA, dsRNA) that can significantly impact the potency and safety of the final drug product. The final mRNA is then formulated into an appropriate storage buffer, sterile filtered, and subsequently filled into compatible storage containers. While the

production of mRNA via IVT is standard, both IVT and downstream purification processes may benefit from optimization depending on the strategies adopted for the nucleotide chemistry substitution, cap analog introduction (co-transcription vs enzymatically added), the polyA tail (encoded in the DNA template vs enzymatically added), and importantly the length of the RNA construct (Gebre et al. 2021).

4.2 mRNA Drug Product Manufacturing

A critical step toward development of RNA-based therapeutics is the manufacture of the final mRNA-LNP drug product that patients receive. mRNA relies on the intracellular machinery to translate the RNA transcript into the therapeutic protein or antigen of interest. Owing to its large size and dense negative charge, mRNA requires a delivery vehicle that confers both protection against enzymatic degradation and delivery to the cell cytoplasm. LNPs have a long history as safe drug delivery systems for nucleic acids and received FDA approval for the delivery of siRNA (ONPATTRO) for the treatment of patients suffering of hereditary transthyretin-mediated amyloidosis (Akinc et al. 2019). Naturally, LNPs were also adopted as state-of-the-art delivery system for mRNA and their use was validated by recent approvals of BioNTech/Pfizer and Moderna mRNA vaccines. Noteworthy, LNPs used for delivery of siRNA or other nucleic acid payloads are not necessarily the best performers when it comes to mRNA delivery. Both the ionizable lipid chemistry and the lipid composition making the LNP need to be optimized through SAR to ensure mRNA can be delivered in a safe and efficacious manner (Hou et al. 2021). In addition, the processes by which mRNA-LNP are produced play a significant role in the quality attributes and potency of the final product. mRNA-LNPs are typically prepared using a rapid mixing process that ensures homogeneous mixing conditions and allows for good control over particle size, distribution, and other key characteristics such as mRNA loading and encapsulation efficiency. Microfluidics, confined impinging jets, multi-inlet vortex mixers, and T-connectors have been among the devices used to prepare mRNA-LNPs at research and clinical grade quality. Lipids are premixed in a water-miscible solvent (typically ethanol) and the mRNA is suspended in acidic buffer before they are brought together to form mRNA-LNPs with the assistance of the aforementioned devices (Samaridou et al. 2020). Process parameters including but not limited to lipids and mRNA concentration input, flow rates, and solvent ratios can be optimized to ensure process robustness and scalability. Once produced, the bulk mRNA-LNP product can be further processed using UF/DF to remove ethanol and formulate mRNA-LNP in the appropriate buffer system. mRNA-LNP is then sterile filtered and fill finished into the appropriate primary container (usually a glass vial) and stored frozen. While before the COVID-19 pandemic the storage temperature for mRNA vaccines was not the focus of major development work, it is now recognized that the storage, transport, and distribution requiring < -70 C constitute a significant logistical challenge (Crommelin et al. 2021). The type of storage (liquid frozen or lyophilized) as well as the buffer components and cryoprotectant used

(e.g., sucrose, trehalose) will affect the long-term stability of the product. Finding solutions that would not require frozen storage will significantly improve the supply chain logistics of mRNA-based products and improve the distribution of vaccines to resource-constrained countries (Schoenmaker et al. 2021).

5 Quality Control of mRNA Therapeutics

mRNA therapeutics are complex dosage forms and require a robust analytical control strategy to ensure product's identity, purity, safety, and potency. Given the novelty of mRNA therapeutics, general guidelines for product characterization and specifications are still being developed by both sponsors and regulatory entities. However, the recent and accelerated development of mRNA vaccines to address the COVID-19 outbreak have resulted in significant experience and knowledge that no doubt serves as a framework for further development of existing quality strategies of this type of product (Mao et al. 2021).

As for other therapeutic products, the establishment of an analytical control strategy is critical and includes the development, qualification, and validation of quality control (QC) tests that ensure the product is safe to be administered in patients. These QC tests have predefined specifications for each test and are performed on each manufactured lot to support both clinical development (including stability and shelf-life determination) or commercialized products. Some of these tests are compendial and are used to monitor the aseptic performance of the process and ensure the general quality and consistency of the process (Poveda et al. 2019). Product attributes tested include the following: Appearance, pH, Osmolality, Subvisible particles, Endotoxin, Bioburden, and Sterility. However, the tests used for Identity, Purity, and Potency are typically product (or platform/process) specific. Due to the complexity of mRNA therapeutics, these require considerable time and resource to be developed. Another particular aspect of this kind of therapeutics is that the DS (mRNA; sometimes more than one molecule per product) and DP (mRNA/LNP) have very different critical quality attributes (CQAs) and therefore require a very different set of QC tests and specifications. It is important to note that the plasmid DNA used for the mRNA production through IVT needs to be produced under cGMP and subjected to appropriate QC testing as well, as it constitutes a key starting material (Knezevic et al. 2021).

From a regulatory perspective, FDA considers all mRNA-based products fall into the category of Gene Therapy Medicinal Products (GTMPs). For EMA, with the exception of mRNA vaccines that follow specific guidelines, all other mRNA-based products are generally considered Advanced Therapy Medicinal Products (ATMPs).

5.1 Drug Substance (mRNA) CQAs and Quality Control Strategy

mRNA integrity and quality are crucial for the efficient translation of the encoded protein. While hydrolysis of mRNA is thought to be the major contributor to degradation of mRNA, other chemical-related reactions including depurination, deamination of cytosine derivatives, and oxidation of nucleobases or sugar moieties can also contribute to the degradation of mRNA (Pogocki and Schöneich 2000). It has been postulated that even minor degradation events happening anywhere along the mRNA strand have the potential to result in the production of a truncated protein or even preclude translation. Therefore, QC tests that ensure efficient 5′-Capping, a Poly A tail with the right length are required. In addition, methods that confirm mRNA identity (e.g., NGS), as well as methods that assess mRNA integrity and are sensitive to the presence of mRNA fragments or products of its degradation are required for testing and release of mRNA (Poveda et al. 2019). Product-related impurities are commonly mRNA-truncated fragments and dsRNA, the latter bearing the potential to cause immunogenicity and impact translation of mRNA. Methods used to determine mRNA integrity and the presence of process-related impurities are typically based on chromatographic or electrophoretic techniques and could include High Performance Liquid Chromatography (HPLC) and Capillary Gel Electrophoresis (CGE). Although mRNA is typically produced through a cell-free system, some process-related impurities could be carried over from the pDNA starting material, and therefore, assessment of host cell protein, DNA and/or RNA, and residual plasmid DNA could be required to be controlled. The identity of mRNA must be confirmed for each manufactured lot and is particularly important when several different mRNA constructs are produced in the same manufacturing facility. This is typically done through sequencing or RT-PCR methods. The potency of an mRNA product is typically assessed through the quantification of the protein it generates (transgene expression) in vitro and, if required, the biological activity or functionality (e.g., ability to bind to the target protein/receptor or the ability to generate an immune response). Demonstrating that the mRNA can generate the expected product while being functional is of utmost importance to ensure safety and efficacy in patients. However, the complexity of mRNA-based products can present significant challenges to establishing potency assays. FDA regulators acknowledge this complexity and recommend an incremental approach to the implementation of potency assays (i.e., the potency assays will evolve and may change significantly through the clinical development of the product).

5.2 Drug Product (mRNA-LNPs) CQAs and Quality Control Strategy

Lipids forming the LNP encapsulating the mRNA are an integral part of the drug product and play a critical role in the success of mRNA-based products. The chemistry and quality of the lipids used for the formulation play a role on the product's efficiency/potency, biodistribution, and immunogenicity but might also affect its colloidal and chemical stability (Knezevic et al. 2021; Packer et al. 2021). In a recent publication, it was shown that lipid-derived impurities can react with the mRNA molecules and are shown to have a negative impact on the translation efficiency of the mRNA (Packer et al. 2021). The findings of this study strengthen the overall idea that ensuring an appropriate quality of raw materials as well as putting in place an adequate control strategy for the mRNA/LNP drug product is essential to assure consistent quality of the product.

For an mRNA/LNP formulation, the amount of encapsulated versus free mRNA also needs to be measured, is usually presented as encapsulation efficiency and mRNA content, and informs the dosing of the product. It is also important to develop methods that allow the determination of lipid content of each lot (identity and quantification), and the presence of lipid-related impurities if required. In addition, this type of product requires extensive characterization of biophysical attributes, such as particle average size and polydispersity index (PDI), subvisible particles (SVP) counts, particle charge, and morphology. The vesicle size and PDI, which is a measure of the distribution of size populations (size uniformity), are important physical characteristics that have an impact on safety, potency, and stability. They are typically measured by microscopic techniques, diffraction and scattering techniques (e.g., dynamic light scattering, nanoparticle tracking analysis, multi-angle light scattering, and asymmetric field flow fractioning), or hydrodynamic techniques, among others. While microscopic techniques provide additional valuable morphological and structural information, techniques based on light scattering provide more statistically meaningful data by analyzing a large number of particles at a time. Therefore, a more quantitative meaningful method is typically used for batch analysis while microscopy methods, such as cryogenic transmission electron microscopy, are used for additional product understanding. Subvisible particles are quantified and measured using compendial methods such as light obscuration or flow imaging microscopy. The surface charge of mRNA/LNPs is another property that directly affects the pharmacokinetic characteristics and potency of the product and is typically assessed through Zeta potential determination using Laser doppler electrophoresis (also known as Electrophoretic light scattering).

mRNA therapeutics face similar challenges of other novel and complex therapeutic modalities such as viral-based gene therapies and cell therapy, such as the very high cost of materials, scalability, and variability. The implementation of real-time analysis during the manufacturing process, automation, or the overall application of process analytical technologies (PAT) strategies and Next Generation Manufacturing (NGM) could help to reduce batch failures, streamline the manufacturing process,

and potentially improving the speed of the development process, time to market, and ability to support market demand.

6 Conclusion and Future Directions

The rapid development of mRNA vaccines to address the COVID-19 pandemic has demonstrated the potential of mRNA to address infectious diseases. mRNA is also being used in many other disease settings including oncology, cardiovascular, and regenerative medicine. In addition, it is being employed as a tool for the development of genomic medicines. As the mRNA field is rapidly evolving, there are several areas that are in great need of improvement so the mRNA community, and our patients, can realize the full potential that mRNA has to offer. For applications where mRNA may be the platform of choice for more complex proteins such as Cas9, and antibodies, bespoke approaches may be beneficial. For example, a screening or selection campaign for UTR choice, or codon use schemes tested in the context of the whole mRNA molecule and disease indication may prove useful. mRNA design has overcome some challenges of mRNA-based delivery; however, there may be future potential to further evolve mRNA design to address ongoing delivery challenges for mRNA. For example, it has yet to be seen whether the design of an mRNA may improve LNP delivery and endosomal release to improve overall delivery efficiency of mRNA to specific tissues and cells. In addition, it has now been recognized that the mRNA payload and its size can impact delivery and the delivery system composition needs to be optimized based on the mRNA properties and desired target indication. As we learn more from the ongoing clinical trials, the mRNA field will gain access to data that will help to understanding of how the biology of the disease and the state of disease influence on the efficacy and safety of mRNA therapeutics. This in turn could lead to the development of the next generation of mRNA therapeutics where delivery systems such as LNP and others, and the mRNA being delivered are designed to improve safety and efficacy. A great example of the evolution of mRNA design is the development of circular RNA which has been shown to significantly improve expression durability and levels of the target protein. The massive demand for mRNA vaccines to meet the global supply has exposed areas in need of higher efficiency including appropriate infrastructure to manufacture the mRNA and mRNA-LNP DP, clinical, safety, and regulatory frameworks. It has also exposed areas of improvement around the stability of mRNA-LNP to mitigate challenges around supply chain logistics. To expand on the number of therapeutic indications and targets, the drug delivery community has an important role in designing the next generation of delivery systems that are able to deliver mRNA payloads to specific tissues. LNPs have proven to be very successful in delivering mRNA to liver and hepatocytes, and efforts are now focused on further developing LNPs and other delivery platforms to enable and improve delivery outside the liver. Using machine learning and artificial intelligence tools could be very helpful to integrate the development of novel mRNA designs and delivery systems. Finally, the design of the mRNA molecule and the delivery systems

should go hand-in-hand with the design of the manufacturing process to ensure the optimal final product.

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Messenger RNA for Prophylaxis

Nicholas Jackson

Contents

Abstract Remarkable advances in mRNA and ionizable lipid-based carrier innovations have allowed the unprecedented speed of development for these technologies as vaccines to prevent SARS-CoV2 disease. Their validation in the field of prophylaxis now paves the way for other infectious diseases indications and manufacturing advantages over certain traditional vaccine technologies. In this chapter, platform advances and critical quality attributes important for vaccination will be discussed and related to SARS-CoV2 vaccines for which field efficacy data are available.

Keywords mRNA · LNP · Critical quality attributes · Vaccines · Immunogens · Formulations · Manufacturing · Infectious disease · SARS-COV-2 · COVID-19

N. Jackson (✉)
Clover Biopharmaceuticals, Research and Development, London, UK
e-mail: nicholas.jackson@cloverbiopharma.com

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_2

1 Introduction

As the SARS-COV-2 pandemic emerged in early 2020, there were only nine ongoing clinical trials using mRNA technologies as candidate vaccines, seeking indications against seven viral pathogens that did not include SARS-COV-2 (Jackson et al. 2020). In response to this virus, there are two licenced mRNA vaccines which have been administered to over 390 million people in the USA alone (CDC 2021). The two products rapidly achieved Emergency Use Licensure (EUL) after around 300 days since the availability of the SARS-COV-2 sequence and have been distributed globally in 76 (Moderna; mRNA-1273) and 137 (Pfizer-BioNTech; BNT162b2) countries (NYT 2021). This remarkable prophylactic intervention utilizes nucleotide-modified non-amplifying mRNA (NRM), formulated with ionizable lipid-based nanoparticles.

mRNA as a technology is not a universal platform for vaccines, but it does offer distinct advantages for the research, development and production of vaccines compared to other platforms. From a research perspective, the minimal low-cost requirements for small-scale production and the rapid generation of preclinical materials facilitate the proficient evaluation of candidate vaccines for their ability to elicit immunity, or protection, against the target pathogen in animal models. For vaccine development, the very rapid generation of clinical trial materials significantly accelerates the pathway to clinical proof-of-concept, efficacy and licensure. The manufacturing process requires significantly less physical space and is considerably quicker than other vaccines that require large fermentation facilities and many months for production. Moreover, with little or no changes, the same manufacturing process can be adapted for a different mRNA vaccine with a different coding sequence. The process is cell-free and does not require animal-based materials. Scientifically, mRNA is advantageous given that it expresses only the antigen(s) of interest, the expression is transient and there is no risk of genome integration.

In this chapter, scientific advances in mRNA constructs and carrier technologies that led to the historic SARS-COV-2 vaccine achievements in medicine will be reviewed, with emphasis on the biological and molecular parameters important for vaccines. Optimal properties for an mRNA vaccine include a thermally stable formulation, administered through intramuscular or subcutaneous injection, that is effectively taken up intracellularly and subsequently released into the cytoplasm where expression generates the translated immunogen that is efficiency presented to the humoral and cellular components of the immune response. No single chapter can capture the entirety of the past and present mRNA vaccine field. Thus, in order to address definable aspects of RNA and carrier biology that relate to vaccine development, we will concentrate on mRNA vaccines against SARS-COV-2, focussed on BNT162b2, mRNA-1273 and CureVac's CVnCoV vaccine given the availability of field efficacy results and the large clinical safety databases for these vaccines.

2 mRNA Vaccines: Categories and Biological Function

There are currently two categories of mRNA used for vaccines, non-replicating mRNA (NRM) and self-amplifying mRNA (SAM) constructs. Both approaches share common features such as a 5′ cap, 5′ and 3′ untranslated regions (UTRs), a coding sequence for the immunogen(s) of interest and a 3′ poly(A) tail. The SAM construct differs through the expression of a viral RNA-dependent RNA polymerase that directs cytoplasmic amplification of the immunogen coding subgenomic mRNA. As a result, NRM are around 1–5 kbp in size, compared to larger SAM constructs around 9–12 kbp (Blakney et al. 2021).

The production process for clinical trial or licenced products, under compliant Good Manufacturing Practices (cGMPs), commences with the production of mRNA drug substance (DS). This DS process starts with the generation of plasmid-based DNA or PCR-amplified DNA that contains a DNA-dependent RNA polymerase promoter. The DNA is linearized to serve as a template for the *in vitro* transcription (IVT) reaction. IVT at varying reaction volumes requires the DNA-dependent RNA polymerase, nucleotide substrates, polymerase cofactor $MgCl_2$ and an appropriate buffer, from which mRNA is rapidly generated from the DNA template in around two hours. The addition of the 5′ cap and the 3′ poly(A) tail can be achieved either during the IVT (1 step) or enzymatically after transcription (2 steps). Following RNA synthesis, the plasmid DNA is digested using a DNase endonuclease. The crucial process of purification follows using a range of potential methods suitable for large-scale production. The DS is now ready for formulation with a carrier technology, such as lipid nanoparticles (LNP), to create the final formulated vaccine (drug product, DP). Analytical methods are required to assess quality during these processes and to release DP for use (Table 1). For materials used in late-stage vaccine development and for a commercial product, the consistency of manufacturing is essential, as well as validation of the processes and analytical methods (Rosa et al. 2021).

3 Advances in mRNA Constructs

Vaccines possess critical quality attributes (CQAs) that dictate the performance of the product in terms of immunogenicity, efficacy, tolerability, safety and stability. These CQAs must be well characterized, remain consistent and be controlled during and after production. The European Medicines Agency (EMA) Committee for Medicinal Products for Human Use (CHMP) defines a CQA as ‘a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality’ (EMA/ICH 2011). In the case of mRNA, reported CQAs identified to date include purity, stability, integrity, 5′ capping efficiency and 3′ poly(A) tail presence and length. Other important parameters not classified as CQA, but are nevertheless fundamental for the

Table 1 Examples of analytical methods for the release and characterization of mRNA 'drug substance' and mRNA-LNP-formulated 'drug product'

Drug substance (mRNA)	
Assay	Quality attribute or characterizations
Next-generation sequencing/sanger sequencing	Identity
UV spectroscopy absorbance	Quantification/content dose
Extrinsic fluorescent tag	Quantification/content dose
Capillary gel electrophoresis/MALDI-TOF	mRNA integrity/truncation/stability indicator
qPCR	Purity—residual DNA content
Immunoblot/dot blot	Purity—dsRNA content
IP-RP-HPLC–UV-MS/RP-UPLC	mRNA capping efficiency
LC-UV/MS/ddPCR/RP-HPLC	mRNA polyadenylated tail
RP-HPLC	Purity
In vitro translation, cell-free/cell-based	Potency/product biological activity/stability indicator
Residual total protein content assays	Purity/residual host cell proteins
Compendial methods	Visual appearance, pH, endotoxin content, bioburden
Drug product (mRNA-LNP)	
Assay	Quality attributes or characterizations
Next-generation sequencing/sanger sequencing	mRNA identity
Dynamic light scattering/nanoparticle tracking analysis/high-resolution microscopy (cryo-TEM)	Particle size/size distribution polydispersity/zeta potential
Chromatography/MS/UPLC-CAD	Lipid identity/integrity/purity/bound–unbound mRNA
Fluorescence-based RNA-quantitation assay/absorbance dye-binding cryo-TEM	Encapsulation efficiency
In vitro translation, cell-free/cell-based	Potency/product biological activity/stability indicator
Compendial methods	Sterility, bioburden, endotoxin content, pH, osmolality, visual appearance, container closure, extractable volume

Adapted from (Crommelin et al. 2021) and descriptions from (EAR/mRNA1273 2021)
UV ultraviolet–visible, *MALDI-TOF* matrix-assisted laser desorption ionization time-of-flight, *qPCR* quantitative polymerase chain reaction, *IP-RP-HPLC-UV-MS* ion-pairing reversed-phase high-performance liquid chromatography coupled with both ultraviolet and mass spectrometry, *RP-UPLC* reverse-phase ultra-performance liquid chromatography, *LC-UV/MS* liquid chromatography–ultraviolet/mass spectrometry, *ddPCR* droplet digital PCR, *Cryo-TEM* cryogenic electron microscopy, *UPLC-CAD* ultra-performance liquid chromatography charged aerosol detection

performance of mRNA, include the untranslated regions (UTRs) structure and regulatory elements, codon optimization and nucleotide modification. In the case of SAM, the encoded RNA-dependent RNA polymerase complex is an essential component. There are no harmonized criteria or CQAs yet for mRNA vaccines formulated with LNPs; however, regulatory agencies are working towards an aligned set of methods (Table 1). Advances in mRNA biology related to these attributes will be addressed in the following section.

3.1 5' Capping

A 5′-cap structure bound to the first nucleotide, common to all eukaryotic mRNA, is based on a 7-methylguanosine (m^7G) that exists in three configurations (Fig. 1): cap 0 (m^7GpppN) with no further methylation, cap 1 ($m^7GpppNm$) methylated 2′hydroxyl group on the first adjacent nucleotide and cap 2 ($m^7GpppNmNm$) methylated 2′hydroxyl groups on the first two nucleotides (Furuichi 2015; Ramanathan et al. 2016). Capped mRNA has several functions relevant to its utility as a vaccine. Firstly, the cap structure plays a crucial role in the efficiency of protein synthesis through cap-dependent initiation of translation (Borden and Volpon 2020). The 5′ cap also serves to stabilize mRNA by preventing 5′ to 3′ exonuclease degradation (Schoenberg 2011). Essential for the ability of the mRNA to express the coding sequence, the cap structure influences innate immune response recognition that can abrogate expression. Retinoic Acid Inducible Gene-I (RIG-I) has a primary role in innate responses through the detection of viral RNAs and the 2′O-methylation of the 5′-end

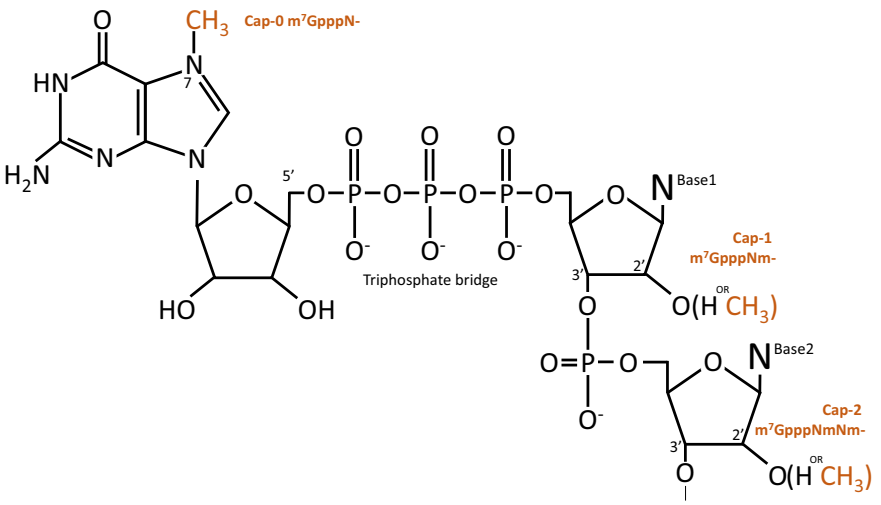

Fig. 1 Structures of cap 0, cap 1 and cap 2 mRNAs. The 5′ cap 0 structure ($m^7GpppN-$) is further methylated at the 2′ hydroxyl group positions to generate the cap 1 or cap 2 structures

nucleotide of the cap 1 structure avoids RIG-I recognition (Devarkar et al. 2016). Selecting a cap structure is therefore a careful consideration for a vaccine developer. Both BNT162b2 and mRNA-1273 use a m^7GpppNm cap 1 structure (Sahin et al. 2020; VRBPAC/Moderna 2020).

For mRNA vaccine production purposes, 5′ capping can be performed co-transcriptionally or by post-transcriptional modification (Grudzien-Nogalska et al. 2007; Martin and Moss 1975). Enzymatically, guanylyl transferase transfers the Gp molecule onto the 5′-end of the nascent mRNA to create GpppNp-RNA and subsequent methylation of the guanosine performed by guanine-N7-methyltransferase to create a cap 0 structure (m^7GpppN). Additional methylations of the ribose at the 2′O position by (nucleotide-2′-*O*)-methyltransferase produce cap 1 or 2 structures (Decroly et al. 2011). Given the crucial need for a very high percentage of capping during manufacturing to ensure stability and translation efficiency, CleanCap™ technologies are widely used in the field, for example to generate Pfizer's BNT162b2 vaccine (Supply-Agreement 2020). Capping is performed co-transcriptionally by Pfizer-BioNTech (Sahin et al. 2020). Alternatively, Moderna's process performs capping post-transcriptional, using capping enzymes, and this requires a purification and filtration steps between the IVT reaction and enzymatic capping (EAR/mRNA1273 2021).

3.2 Untranslated Regions (UTRs)

5′-end UTRs function to stabilize mRNA and regulate expression, and 3′ UTRs are also known to influence translational efficiency (Fig. 2) (Leppek et al. 2018; Jackson 1993). Regulatory elements in both UTRs are known to influence expression, and studies with viral RNA have shown how elements such as Internal Ribosome Entry Site (IRES) regions can allow cap-independent translation initiation (Francisco-Velilla et al. 2015). Cellular RNA may also contain 5′ UTR IRESs that permit ribosome initiation of translation distal from the cap structure, under special conditions where cap-dependent protein synthesis is significantly diminished (Stoneley and Willis 2004). At the 3′-end, data have demonstrated that the length of the UTR can affect poly(A) tail-mediated stimulation of translation and its length impacts stability (Tanguay and Gallie 1996).

In totality, the selection and optimization of UTRs are important considerations for an mRNA vaccine construct. Systematic work, in the therapeutic setting, screening a large library of different 5′ and 3′ UTRs in various combinations, demonstrated several superior 5′ UTR sequences for protein translation and the dominance of the 5′ UTR (Asrani et al. 2018). The UTRs of BNT162b2 are known (WHO/INNP 2020). The 5'-end is derived from human α-globin mRNA with an optimized Kozak sequence that promotes translation (Babendure et al. 2006; Waggoner and Liebhaber 2003). The 3′-end is derived from two sequences: the amino-terminal enhancer of split (AES) mRNA and mitochondrial encoded 12S rRNA that both stabilize mRNA and were the best protein expressors from amongst a panel of different 3′ UTRs (Orlandini

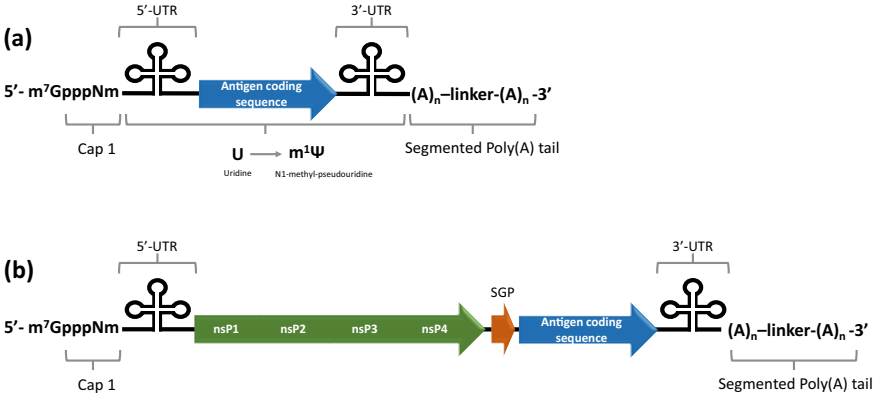

Fig. 2 **a** Schematic representation of a generic modified non-replicating mRNA vaccine construct with a 5′ UTR, antigen coding sequence, 3′ UTR and a bi-segmented poly A tail. Uridine replaced with N1-methyl-pseudouridine. Typically 1–5kb in size. **b** Schematic representation of a generic self-amplifying mRNA vaccine construct with a 5′ cap 1 structure, followed by a 5′ UTR, the replicon apparatus nsP1/nsP2/nsP3/nsP4, a bi-segmented poly A tail. In total, around 9–12kb in size. Adapted from Blakney et al. 2021

von Niessen et al. 2019). The design of the mRNA-1273 UTRs is putatively known; a 5′ V1-UTR and 110-nucleotides of the 3′-UTR of human α-globin gene (HBA1) (Xia 2021).

3.3 Poly(A)-Tail

Poly(A)-tails, composed of repeating adenosines at the 3′-end of the majority of eukaryotic mRNA are multifunctional: translation, stability and binding Poly(A) Binding Protein (PABP) (Nicholson and Pasquinelli 2019). Despite conflicting evidence for certain roles, the binding of PABP to the poly(A)-tail promotes stability by abrogating deadenylation and the depletion of PABP from cell extracts significantly reduces translation (Wang et al. 1999; Kahvejian et al. 2005). The binding of PABP to the poly(A)-tail has been hypothesized to form a complex structure with the initiating ribosome complex (Kahvejian et al. 2005). The length of the poly(A)-tail can impact translation (Holtkamp et al. 2006; Park et al. 2016).

Essential for manufacturing vaccine considerations, the addition of a poly(A)-tail to an mRNA construct can be achieved either enzymatically after IVT of the DNA plasmid template, or through a co-transcriptional approach by direct inclusion of a poly(A) coding sequence into the DNA template. The former process is problematic to control, requires an additional enzymatic step and results in varying poly(A) tail lengths (Holtkamp et al. 2006). It is therefore not favoured by vaccine developers as a production process. The latter is a far more consistent method resulting in a homogenous poly(A)-tail length that is desirable from a CQA perspective. However,

DNA plasmids containing poly(A)-tail sequences can recombine in *E. coli*. This led to the innovative use of segmented poly(A)-tails that reduce recombination without any detrimental impact on the mRNA constructs' stability or translation (Trepotec et al. 2019). The segmented design can be enhanced by reducing the spacer length that improves expression versus a homogeneous poly(A)-tail, and on a particular note, a single G spacer demonstrated no measurable recombination (Trepotec et al. 2019). Pfizer-BioNTech have taken advantage of this approach with a bi-segmented poly(A)-tail (Nance and Meier 2021).

3.4 Nucleotide Modification and Codon Optimization

A central premise for the use of mRNA for prophylaxis is finding the right balance between a construct that minimizes intracellular innate sensing recognition to promote optimal expression of the immunogen(s) and, the actual local/systemic stimulation of the innate response to potentiate the quality and quantity of the adaptive immune response. Host innate immunity is the first line of defence against pathogen infection, and as referenced above, the cap structure can act like a virus-derived pathogen-associated molecular patterns (PAMPs) and be subsequently recognized by host pattern recognition receptors (PRRs) (Lee et al. 2019). The activated PRRs drive a cascade of antiviral signalling pathways that result in the production of inflammatory cytokines.

To circumvent detection, modified nucleotides have played an important role in the use of mRNA to express immunogens. A prominent example is pseudouridine (Ψ), an isomer of uridine, that is highly abundant in cellular rRNAs, tRNAs and snRNAs. Certain cell lines are not activated when exposed to Ψ-modified RNA (Kariko et al. 2005). Murine studies have demonstrated significantly higher expression levels using Ψ-modified mRNA compared to unmodified mRNA (Kariko et al. 2008). These observations were corroborated through the measurement of proinflammatory cytokines; high IFN-α levels were only detected in animals that received unmodified mRNA. RNA-dependent protein kinase regulates translation and amongst other factors can be activated by a variety of RNA structures. These translational differences appear related, in part, to a lower activation of PKR by Ψ-modified mRNA (Anderson et al. 2010). Modified RNA can also promote the stability of the construct intracellularly. The interferon-induced enzyme 2′–5′-oligoadenylate synthetase (OAS) is activated upon binding to RNA and subsequently activates RNase L which in turn cleaves ssRNA. Compared to unmodified RNA, Ψ-modified mRNA causes less OAS activation, confers greater resistance to RNase L cleavage and results in longer translation and an extended half-life (Anderson et al. 2011).

Given that there are around 170 RNA modifications identified in coding and non-coding RNAs, there is significant potential in the field to identify other base modifications that improve the performance of mRNA (Boo and Kim 2020). Constructs containing the N1-methyl-Ψ ($m^1\Psi$) or 5-methylcytidine (m^5C) modified bases result

in significantly higher expression in vitro and in vivo compared to Ψ. $m^5C/m^1Ψ$-modified compared to m5C/Ψ-modified mRNA elicits reduced innate responses (Andries et al. 2015). Biophysical studies have shown distinctly different stabilizing structural properties with $m^1Ψ$ compared to unmodified U-based constructs and the spatial position of these secondary structures correlate with expression (Mauger et al. 2019).

In totality, our understanding of nucleotide modifications has allowed the generation of mRNA constructs better able circumvent intracellular innate immune responses that would otherwise curtail protein expression and such modifications have found their way into the design of vaccines (Boo and Kim 2020). $m^1Ψ$ has been used instead of uridine during IVT production of the Pfizer and Moderna COVID-19 vaccines (Table 2). However, CureVac used unmodified mRNA for their COVID-19 vaccine.

Related to further adaptation of the coding sequence, codon optimization has played a prominent role in the design of mRNA vaccines. The field of optimization strives to enhance codon selection that results in superior protein expression using computational programs based on scoring a selection of functions (Gustafsson et al. 2004; Quax et al. 2015). A common function pursues an increased GC content given a correlation between an increased content and increased transcription (Bauer et al. 2010; Kudla et al. 2006). Bias in codon usage is another common function widely thought to influence expression (Plotkin and Kudla 2011). Codon optimization has been utilized by Pfizer, Moderna and CureVac for their SARS-COV-2 vaccines (Nance and Meier 2021; Corbett et al. 2020; Rauch et al. 2020). The specifics of their algorithms have not been disclosed but attempts have been made to decipher the optimization approaches taken (Xia 2021).

3.5 Purity

It is now widely acknowledged that the purity of mRNA in the final vaccine formulation is essential for an optimal immunogenicity and reactogenicity profile. A particularly important source of impurity created during IVT production results from imperfect DNA-dependent RNA polymerase activity that generates varying short length oligoribonucleotides because of abortive initiation events (Milligan et al. 1987). Additionally, dsRNA by-products from the IVT reaction can be formed in one of two possible ways: firstly, self-complementary 3' extension of run-off products, and secondly, hybridization of an antisense RNA molecule to the run-off transcript (Triana-Alonso et al. 1995; Mu et al. 2018). Cellular innate sensing has evolved a particular propensity to detect dsRNA as exemplified through multiple PRRs able to recognize the molecule, and the downstream impact can potentiate inflammatory responses that in turn diminish expression and promote undesirable reactogenicity (Tatematsu et al. 2018).

Depending on the manufacturing process, there are other potential sources of impurities that should be removed to the extent feasible and release specifications

Table 2 NRM features of Pfizer-BioNTech, Moderna and CureVac's mRNA vaccine constructs

mRNA	DNA template	Cap	5′ UTRs	3′ UTRs	Codon optimization	Antigen	Modifications	Poly-A-tail
Pfizer-BioNTech	Linearized plasmid DNA	Cap 1 structure	Human α-globin mRNA with an optimized Kozak sequence	Amino-terminal enhancer of split mRNA and mitochondrial-encoded 12S rRNA	Yes, liberal strategy[b]	Trans-membrane anchored, full-length S protein, prefusion stabilized[a]	N1-methyl-pseudouridine	Two segmented (30 adenosines, 10-nucleotide linker, 70 adenosines)
Moderna	Linearized plasmid DNA	Cap 1 structure [c]	V1-UTR	110-nt 3'-UTR of human α-globin gene (HBA1)	Yes, fundamentalist strategy[b]	Full-length S protein, prefusion stabilized[a]	N1-methyl-pseudouridine	Not known
CureVac	Not known	Cap 1 structure	Not known	Parts of human α-globin gene (HBA1)	GC-enriched	Full-length S protein, prefusion stabilized[a]	Unmodified nucleotides	Poly-A (64) stretch

NB The sequence of Pfizer/BioNTech's BNT162b2 is publicly available (WHO/mRNA 2020; Nance and Meier, 2021), and Moderna's mRNA-1273 has been sequenced and related to patents; therefore, certain details should be considered putative (Xia, 2021). CureVac's mRNA are derived from (Gebre et al., 2021).
[a]Proline-substitution (K968P and V969P) stabilize the S protein in a prefusion state that allows optimal recognition of the viral protein by the immune system.
[b]For details of codon optimization evaluation, see (Xia, 2021).
[c](VRBPAC/Moderna 2020).

defined for the acceptable limits of inclusion: residual DNA template, unutilized caps, unincorporated nucleotides, enzymes and any other process-related materials used. Manufacturers of mRNA vaccines therefore go to great lengths to generate constructs, batch-to-batch, of the highest purity. In general terms, a series of filtration and chromatography-based techniques are utilized to purify the mRNA drug substance. These can include amongst other methods, tangential flow ultrafiltration and diafiltration combined with an array of potential chromatography procedures: ion exchange chromatography, reverse-phase chromatography, cellulose-based chromatography, oligo dT affinity chromatography and anion exchange chromatography.

There are little specific details known about our SARS-COV-2 mRNA vaccine examples, which is not unusual because vaccine manufacturers rarely disclose proprietary processes. Pfizer-BioNTech use the broadly acting proteinase K to digest proteins in their IVT preparations, and ultrafiltration and diafiltration steps to derive their BNT162b2 drug substance and finally sterile filtration for mRNA-LNP drug product (EAR/Comirnaty 2021). Moderna's process for mRNA-1273 drug substance includes purification and filtration steps after the IVT reaction, further purification and filtration after enzymatic capping and final aseptic 0.2 μm sterilizing filtration of the formulated mRNA-LNP drug product (EAR/mRNA1273 2021). Even less is known related to CureVac's CVnCoV purification process except for the potential application of a protein denaturing agent after IVT production followed by tangential flow filtration (Patent 2016). In all three vaccines, release specifications for purity are unknown but one can assume that very high levels of purity were required.

3.6 Self-Amplifying Specific Features

Although this chapter focusses on NRM SARS-COV-2 vaccines given the availability of clinical phase 3 efficacy data for those technologies, it is important to briefly discuss features specific for self-amplifying mRNA (SAM) as the technology is being used for candidate SARS-COV-2 vaccines in late clinical development. The fundamental difference between SAM and NRM is the presence of replicons machinery (RNA-dependent RNA polymerase [RDRP] and a subgenomic promoter for the coding sequence; Fig. 2) derived from self-replicating RNA viruses that aim to replicate multiple copies of the mRNA template upon release in the cytoplasm (Tews and Meyers 2017). The coding sequence is downstream of the replicon genes, and in totality SAM constructs are much larger (around 12 kb) than NRM which has important implications for the vaccine formulation discussed later. Following release of the SAM into the cytoplasm, the translated RDRP subsequently amplifies the construct and the subgenomic promoter drives expression of the coding sequence. The process is self-limiting. An underappreciated important intracellular feature required for optimal amplification is the association of the RDRP with the plasma membrane. The induction of membrane invaginations, called spherules, forms a shield against the innate sensing of dsRNA intermediates (Pietila et al. 2017). The

efficiency of this process remains unknown and may have important consequences on the success of SAM technologies.

In principle, the net result of self-amplified mRNA should allow a significantly lower dose of SAM vaccine to achieve the same or superior expression levels as NRM (Vogel et al. 2018). A generally comparable IVT reaction and manufacturing process can be used to generate SAM constructs as described above for NRM and CQA's are equally important. However, SAM-specific improvements in production may apply given the larger size of the construct compared to NRM. To date, replicon machinery has been derived largely from the replicase genes of alphaviruses (Bloom et al. 2021).

4 mRNA Carrier Technologies

As important as the mRNA construct per se, the carrier technology and formulation are critical for the performance of the vaccine. Carrier approaches need to ensure that (i) the mRNA payload is delivered to the surface of a cell (ideally an antigen presenting cell) and avoid nuclease digestion en route, (ii) efficient uptake by the cell into the endosomal pathway and (iii) proficient release from the endosome into the cytoplasm. Moreover, the approach should ideally result in a thermally stable vaccine formulation and not induce undesirable toxicity, reactogenicity or safety issues. This tall order held the mRNA vaccine field back until the advent of ionizable lipid-based nanoparticles described below.

Pfizer-BioNTech, Moderna and CureVac all use the same four basic components to create the LNP used in their SARS-COV-2 vaccines: an ionizable lipid, phospholipid, cholesterol and a PEGylated lipid (Table 3). Although certain properties are not definitively proven, one can summarize the contribution of these components towards the overall function of the LNP in the following manner: (i) *Ionizable lipids* have a central role in facilitating endosomal release through their transition from a neutral charge at pH ~ 7.4 to a protonated state at pH ~ 6.5. They complex with mRNA to form a core structure and reside in the outer bilayer (Eygeris et al. 2020; Buschmann et al. 2021). (ii) *Phospholipids* help drive the structure of the LNP, mainly located at the surface, forming part of the envelope bilayer around the lipid-mRNA complex (Yanez Arteta et al. 2018). (iii) *PEGylated lipids* serve many functions, including the formation of a steric surface barrier due to the hydrophilic polyethylene glycols (PEGs), prevent inter-LNP fusion, influence particle size, help prevent phagocyte uptake thereby promoting systemic circulation and dictate uptake efficiency (Semple et al. 2001; Kulkarni et al. 2019; Immordino et al. 2006; Mui et al. 2013). (iv) *Cholesterol* plays a multipurpose role, including the provision of structural integrity, stability and surface localization that may aid endosome release (Cheng and Lee 2016; Rodrigueza et al. 1995; Pozzi et al. 2012).

Table 3 LNP composition, mRNA content, formulation and storage conditions for Pfizer, Moderna and CureVac's SARS-COV-2 vaccines

	Pfizer-BioNTech[a]	• Moderna[b]	CureVac[c]
Status	• EUA and full licensure	• EUA	• Phase 3 completed
LNP contents per dose	• 0.43 mg lipid (4-hydroxybutyl) (azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) • 0.05 mg 2-(polyethylene glycol 2000)-N,N-ditetradecylacetamide • 0.09 mg 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) • 0.2 mg cholesterol	• 1.93 mg total lipid content • SM-102 (9-Heptadecanyl 8-{(2-hydroxyethyl) [6-oxo-6-(undecyloxy)hexyl]amino}octanoate) • Polyethylene glycol [PEG] 2000 dimyristoyl glycerol [DMG] • 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) • Cholesterol	• Cationic lipid • PEGylated lipid • 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) • Cholesterol
mRNA content per dose	30 μg	100 μg	12 μg
Formulation/state	Phosphate buffer/liquid	Tris buffer/liquid	Not known/liquid
Storage	• Frozen, −60 ^{0}C to -90 ^{0}C ≤ 6mo • Frozen, −15 ^{0}C to -25 ^{0}C ≤ 2wks • Thawed, 2 ^{0}C to 8 ^{0}C ≤ 1mo • Thawed, RT ≤ 2 h • Thawed (diluted), 2 ^{0}C to 25 ^{0}C ≤ 6 h	• Frozen, −15–−50^{0}C until expiry [US label]; −15$^-$−25^{0}C ≤ 7mo [EU label] • Thawed, 2 ^{0}C–8 ^{0}C ≤ 30d • Thawed, 8 ^{0}C to 25 ^{0}C ≤ 24 h	• < -60 ^{0}C ≤ 3mo • + 5 ^{0}C ≤ 3mo • RT ≤ 24 h

mg milligrams, *mcg* micrograms, *mo* month, *wks* weeks, *hrs* hours, *EUL* emergency use licensure, *RT* room temperature, *EUA* emergency use authorization, *EU* European, *US* United States

[a](US/Prescribing/Information 2021; Pfizer-BioNTech/Factsheet 2021)

[b](Moderna/Protocol 2020; FDA/Factsheet 2021; EAR/mRNA1273 2021); ; , ,

[c](Curevac/Protocol 2021; Curevac/Press-release 2020)

4.1 Tropism and Uptake Efficiency

Vaccines are typically administered by intramuscular (IM) or subcutaneous (SC) injection. It is important to note that many studies investigating the biodistribution of mRNA-LNPs following intravenous administration cannot be translated to a vaccination setting that uses SC or IM routes. At the bolus of injection, the dispersal of the vaccine begins, ideally presented to the innate and adaptive arms of the immune system. It is particularly important for the antigen(s) to be detected by antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, in the presence of innate 'danger' signals. APCs are present in the skin and muscle. In addition, drainage from the site of injection to lymph nodes (LNs) exposes the immunogen to high concentrations of APCs, incoming naïve T/B cells and resident memory cells (Jiang et al. 2017). These spatial events following mRNA IM administration in non-human primates (NHP) have been investigated using non-invasive whole-body imaging technologies combined with necropsy sampling. In one study, 4 h post IM vaccination, labelled mRNA (complexed with the aminoglycoside lipidic derivative CholK) was detected in draining LNs (Lindsay et al. 2019). Over a 28 h period, the signal increased in the draining LNs and decreased in the muscle injection site. mRNA expression was seen in the muscle associated with recruitment of APCs to the injection site, and in the LNs, APCs were the main cell type containing mRNA (Lindsay et al. 2019). In another NHP study, mRNA-LNP IM administration was associated with germinal centre formation in draining LNs and an increase in circulating specific T follicular helper cells, both important indicators of a maturing immune response (Lindgren et al. 2017).

Formal Good Laboratory Practice (GLP) preclinical biodistribution studies have provided further detailed insight into the fate of an mRNA-LNP vaccine following IM administration. An mRNA candidate vaccine formulated with an LNP was given to mice via the IM route. The highest concentrations of mRNA were detected in the muscle and proximal LNs with a half-life of 19 and 25 h, respectively (Bahl et al. 2017). Much less (around 10–30-fold) was found in distal LNs and further smaller quantities detected in the spleen and liver. Trace amounts at further reduced levels (100–1000-fold) were seen in numerous other tissues.

Similar biodistribution studies for the Pfizer-BioNTech, Moderna and CureVac SARS-COV-2 vaccines have been reported, but not all numerical data are disclosed. Pfizer-BioNTech performed a time-course biodistribution study in mice measuring IM delivered mRNA expression of a bioluminescence marker (formulated with an identical LNP composition to that of BNT162b2) (EAR/Comirnaty 2021). Highest expression was detected at the injection site (likely including local LNs) and peaked 6 h after IM injection with a signal around 10,000 times higher than background. The signal declined gradually over the first 72 h and down to very low levels after 6–9 days (18- and 7-times background, respectively). Detection in the liver also peaked at 6 h post immunization and decreased to background levels 48 h after injection. An additional IM route biodistribution study in rats was performed using radiolabelled LNP for greater sensitivity of detection over a 48-h period (EAR/Comirnaty 2021).

At the early first time point (0.25 h), the injection site and the liver were the major sites of distribution. Distribution from the injection site to most tissues occurred at low levels with the greatest levels in plasma observed 1–4 h post-dose. Over 48 h, distribution was mainly observed to liver, adrenal glands, spleen and ovaries. In terms of total quantitative recovery, as a percentage of the injected dose, there was up to 21.5% in the liver and considerably less in the spleen ($\leq$ 1.1%), adrenal glands ($\leq$ 0.1%) and ovaries ($\leq$ 0.1%) (EAR/Comirnaty 2021).

Moderna has reported a time-course biodistribution study in rats after IM administration of mRNA-1647 (a candidate CMV vaccine) using the same SM-102–containing LNP composition as that used for its SARS-COV-2 vaccine (EAR/mRNA1273 2021). Quantifiable mRNA was recovered in the majority of tissues examined at the first time point collected (2 h post-dose) and peak concentrations were reached between 2 and 24 h post-dose in tissues with exposures above that of plasma. Quantifiable mRNA was distributed throughout the body (including brain, heart, lung, eye, testis) and was rapidly cleared from plasma during the first 24 h, with a half-life estimation of around 3–4 h. The mRNA concentration was highest at the injection site and following plasma clearance, LNs (proximal and distal) and the spleen were the major distant organs. Like other studies, distribution to the liver was also detected.

In summary, mRNA-LNP are not atypical compared to other vaccines in their biodistribution. Following IM injection, there is a local accumulation in the muscle tissue leading to drainage into the local lymph nodes and systemic distribution through vasculature. Size plays an important role in the fate of nanoparticles following injection, and murine studies have shown that larger polystyrene fluorescent nanoparticles (500–2000 nm) were largely associated with injection site DCs and smaller particles (20–200 nm) also associated with LN-resident DC and macrophages, suggesting drainage to the LNs (Manolova et al. 2008). LNPs are typically around 100 nm in size. Other murine studies have also shown that more negatively charged LNPs result in greater off-target expression in the liver following IM administration and therefore the potential importance of charge-related biodistribution (Carrasco et al. 2021).

4.2 The Art of Endosomal Escape

Several investigations have determined the central role of ionizable lipids in the disruption of the endosome, after the extracellular uptake of an LNP, that crucially facilitates the release of the mRNA payload into the cytoplasm. Such studies using the ionizable amino-lipid dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA, or MC3) as part of a LNP demonstrated the very low inherent efficiency of release (1–2%) (Gilleron et al. 2013). As such, activities have focussed on optimizing release related to protonation by adjusting the acid dissociation constant (pKa) through different amino head molecules (Buschmann et al. 2021). The early to late endosomal lumen maintains an acidic environment with a pH range of around 6.5 and

5.5, respectively (Hu et al. 2015). This drives protonation of the headgroups of the ionizable lipids resulting in their positive charge, and this in turn promotes association with the negatively charged membrane of the endosome. Using a large panel of amino lipids with varying head group adaptations, in a controlled setting, the relationship between the pKa was assessed against preclinical activity. A distinct association between the preclinical activity and the acid dissociation constant of the headgroup demonstrated an optimal pKa of 6.2–6.5 (Jayaraman et al. 2012). However, this activity was based on siRNA and hepatocyte targeting following intravenous administration. Thus, a more relevant assessment for vaccines has been performed in murine studies and shown that lipid pKa is a strong determinant of humoral immunogenicity with a higher optimal range of 6.6–6.9 (Hassett et al. 2019).

At the juncture of association between the LNP (with a positively charged ionizable lipid content) and the negatively charged endosome membrane, it is postulated that the cone structure of the lipid is important for membrane disruption, driven by the molecular geometry of the lipid tails (Hajj et al. 2019). On a molecule-to-molecule basis, ion pairs form between the positively charged lipid and the anionic endosome lipid and the pair prefers an inverted cone position (termed a hexagonal structure) associated with non-bilayers that disrupt the endosome membrane as the LNP dissembles (Jayaraman et al. 2012; Schlich et al. 2021). Compared to MC3 with two hydrophobic tails, ionizable Lipid 5 with an extra tail (but comparable pKa) results in significantly higher endosomal escape efficiency, supporting the hypothesis of structural importance (Sabnis et al. 2018).

These and other principal studies have significantly advanced our understanding, but we remain potentially far from knowing all the adaptable factors that could further enhance release and new findings continue to open other avenues of investigation. For example, the comparison of several LNPs, including MOD5 (an analogue of the SM-102 lipid used in Moderna's mRNA-1273 SARS-CoV-2 vaccine), has been tracked using high-resolution methods to assess endosomal distribution in several cell lines (Paramasivam et al. 2021). Observations suggest that different LNPs have distinctly different trafficking in varying endosomal compartments, and the best delivery was associated with endosomes that have a high proportion of recycling tubules. Thus, a greater propensity for LNPs to reach these recycling endosomes warrants further investigation. Other observations suggest that defective endosomal acidification may explain putative cytotoxic effects of LNP (Paramasivam et al. 2021). In all the above-mentioned studies, there are currently no head-to-head comparisons of the LNP used by Pfizer-BioNTech, Moderna and CureVac in their SARS-COV-2 vaccines. We do know however, related to the cone-shape hypothesis, that Pfizer-BioNTech ALC-0315 licenced from Acuitas and Moderna's SM-102 are ionizable lipids with increased branching: four and three alkyl tails, respectively (Pfizer-BioNTech/EUA/Letter 2020; Moderna/Protocol 2020).

4.3 Formulation

The final vaccine formulation is achieved through the principle of rapid mixing of an ethanolic mixture of the lipid components with an aqueous suspension of mRNA at a low pH, in which the ionizable lipid is initially positively charged and begins to form complexes with RNA and excess ionizable lipid begins to seed vesicular bilayer structures that grow in size and encapsulate the complexed RNA as the pH is raised to neutral (i.e. the lipids becoming less soluble) by dialysis (Jeffs et al. 2005; Kulkarni et al. 2018; Leung et al. 2015). During this formation, PEG lipid applies its size-limiting role on the forming LNPs (Kulkarni et al. 2019). Encapsulation efficiency of the mRNA is crucial, and larger-sized payloads can influence the proportion of loaded LNPs (Kulkarni et al. 2019). As a result, control and measurement of the encapsulation efficiency is a requirement from vaccine regulators, amongst other parameters (WHO/Draft/Guidelines 2021).

In terms of the equipment used for formulation mixing, methods and hardware need to ensure consistency between batch production and be suitable for large-scale manufacturing to have vaccines available at sufficient volumes for population needs. Current approaches include microfluidics mixers, T-junction mixers, impingement jet mixers or pressurized stainless-steel tanks. As one example, T-junction mixing involves two incoming pressurized fluid streams (one an ethanolic lipid mixture and the other aqueous mRNA) that collide at a junction, creating sufficient turbulence in the outflowing stem to mix the components and through dilution result in a pH change (Evers et al. 2018). The process allows controlled rapid mixing, superior encapsulation efficiency, uniform size and compatibility with a broad range of solvents. Pfizer uses a comparable approach—impingement jet mixers—two pressurized jets from opposite directions impinge directly in a mixing chamber (Sealy 2021). Following this procedure, the developer would typically proceed with buffer exchange and concentration by TFF, followed by dilution for the desired dosing and ultimately conduct a sterile filtration step before filling and storing the DP; followed by analytical release testing and the initiation of stability testing programs.

4.4 Stability

Despite the successes of the Pfizer-BioNTech and Moderna mRNA-LNP SARS-COV-2 vaccines demonstrating protection and helping to end the pandemic, their thermal instability and required storage conditions have been a limitation to their distribution and use, even in high-income countries. The products have short shelf-lives at room temperature or at refrigerated temperatures, requiring frozen and/or ultra-cold frozen conditions for shipment and longer storage (Table 3). This necessitates a complex cold-chain distribution network from the point of manufacturing, onto national storage facilities, regional facilities, and healthcare centres or other points of vaccination; all tightly controlled and monitored to avoid and detect

any temperate deviations. The effectiveness of this cold-chain distribution requires extensive training programmes, significant financial resources, clearly documented instructions, reliable and secure storage equipment and diligent management at all stages (CDC/Vaccine/Storage 2021). CureVac claim to have superior thermal stability and the possibility of refrigeration for 3 months, but this is not a licenced product, and the basis for this improved thermal stability is unknown (Table 3).

Assuming the absence of RNases, RNA as a pure molecule is thermally stable (Pascolo 2021). However, hydrolysis and resultant cleavage of the RNA backbone phosphodiester bond is a major degradation pathway initiated by deprotonation of the 2′-hydroxyl group of the ribose; dictated by pH, temperature and ionic concentration (Li and Breaker 1999; Fabre et al. 2014). There is evidence that the rate of hydrolysis is influenced by secondary structures (Mikkola et al. 2001). Oxidation is another source of degradation although it is considered less of an issue compared to hydrolysis (Pogocki and Schöneich 2000).

The licenced siRNA product, Onpattro, has a 36-month shelf life at 2 °C and 8 °C and is based on an M3-based LNP comparable in composition to our three SARS-COV-2 mRNA-LNP vaccine examples (EMA/SPC 2018). Another siRNA-LNP liquid formulation has shown stability over 1.5 years and maintained high encapsulation and no change in particle size (Suzuki et al. 2015). In totality, all these data suggest the limitation is rather mRNA instability than LNP instability. Nevertheless, potential oxidation and the physical degradation of LNPs need to be considered by a vaccine developer (Schoenmaker et al. 2021). For the latter, particle fusion or aggregation can be an issue, but this has been largely addressed using PEG lipids that help prevent this. Evidence for the interior of an mRNA-LNP suggests the presence of water with the mRNA-ionizable lipid complexes and therefore the mRNA is prone to hydrolysis in a liquid state (Yanez Arteta et al. 2018; Schoenmaker et al. 2021).

Cryo-preservatives/cryo-protectants have been investigated, the addition of sucrose or trehalose has been shown to promote mRNA-LNP stability when stored in liquid nitrogen, and it should be noted that both Pfizer-BioNTech and Moderna's SARS-COV-2 vaccines include sucrose as an excipient (Table 3) (Zhao et al. 2020). Clearly, pH needs to be controlled given its influence on hydrolysis, and the pH of Moderna and Pfizer-BioNTech vaccines are between 7 and 8 (Li and Breaker 1999; EPAR/Comirnaty/SPC 2021; EPAR/Spikevax/SPC 2021). Freeze drying has successfully improved the thermal stability of mRNA in the presence of trehalose, and there is at least one mRNA-LNP candidate vaccine in development that has been lyophilized for storage at 5 °C storage $\geq$ 18 months shelf life (although no details are available for the formulation or process) (Jones et al. 2007; Moderna/CMV 2021). This indicates that other lyophilized vaccine formulations are likely to emerge although it should be noted that the process of lyophilization can be a significant bottleneck in the manufacturing process for even large-scale developers. Overall, there is much needed work on mRNA-LNP vaccine formulations and the factors that dictate the overall stability.

5 Conclusions

The two categories of mRNA used in the vaccine field, NRM and SAM, have significantly advanced in the last decade based on fundamental advances in mRNA biology and the ability to deliver the construct to the cytoplasm of cells following parenteral administration. In particular, ionizable-lipid-based nanoparticles (LNP) are currently the most commonly used class of delivery technology. Both NRM and SAM have been used as candidate vaccines against several viral targets, but only NRM has been tested in completed SARS-COV-2 field efficacy trials to date. The manufacturing processes for mRNA and LNP are sufficiently scaled to support mass COVID-19 vaccination campaigns, but there are still numerous areas of improvement needed in productivity and thermal stability. Critical quality attributes and other important features that dictate expression efficiency and stability, expression regulation and intracellular innate immune response sensing include 5-capping efficiency and structure, 5′ and 3′ UTRs, codon optimization and nucleotide modification and the poly(A) tail. The unique components of LNPs and subtle differences in their composition drive successful evasion of mRNA degradation, tissue targeting, cellular uptake and endosomal release of their mRNA payload. The final vaccine formulation, and in particular its purity, needs to be consistent and well characterized analytically during production and release.

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Messenger RNA Therapeutics: Start of a New Era in Medicine

Saloni Jain, Abhilash J. George, Vasu Sharma, Gagandeep Singh, and Vandana Gupta

Contents

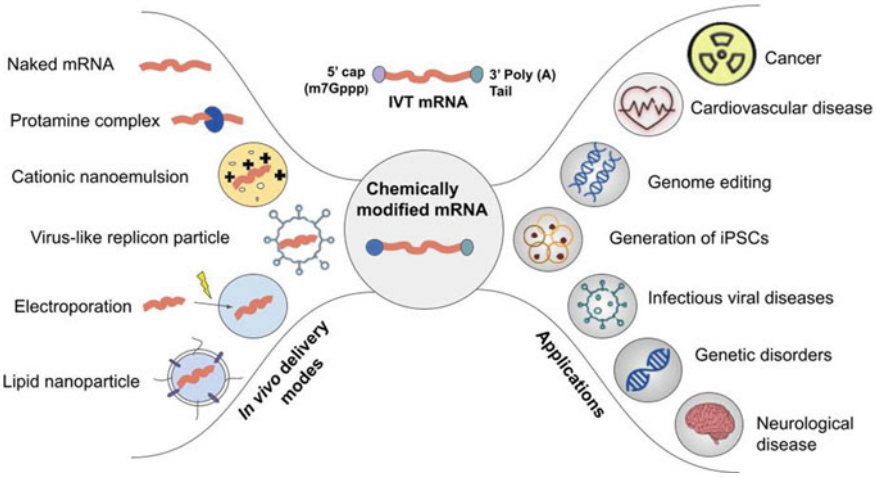
S. Jain · A. J. George · V. Sharma · V. Gupta (✉)
Department of Microbiology, Ram Lal Anand College, University of Delhi, Benito Juarez Road, New Delhi 110021, India
e-mail: vandanagupta@rla.du.ac.in

G. Singh
Section of Microbiology, Central Ayurveda Research Institute, Jhansi, Uttar Pradesh 284003, India

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_3

Abstract Last decade has witnessed tremendous growth in the new promising treatment options based on mRNA, RNAi, antisense RNA, and RNA aptamers, the four classes of RNA-based therapeutics. Among these, mRNA-based therapy is centered on producing proteins within the cells to supplant deficient or abnormal proteins and in vaccination to a target pathogen. The potential of mRNA therapeutics is evident from the two major mRNA vaccines approved for COVID-19: developed by *Moderna* and by *Pfizer*. Nonetheless, mRNA therapeutic potential extends far beyond this, such as in treating genetic diseases, cancers, and other infectious diseases. Given the potential of mRNA therapeutics, this chapter is written to provide the reader an insight into the features of several synthetic mRNA platforms, production, purification; strategies to increase the stability and reduce the immunogenicity of therapeutic mRNA molecules; delivery methods of these mRNAs in vivo; and their applications, safety, and efficacy.

Graphical abstract



Keywords Chemically modified mRNA · In vitro transcription · mRNA therapeutics · mRNA vaccines · Immunogenicity · Immunostimulatory · In vivo delivery · Cell-mediated immunity

Abbreviations

ACE2	Angiotensin-converting enzyme 2
ADAR	Adenosine deaminases acting on RNA
AKP	Alkaline phosphatase
APC	Antigen presenting cell
ARCA	Anti-reverse cap analog
BVDV	Bovine viral diarrhea virus
CFTR	Cystic fibrosis transmembrane conductance regulator
CLRs	C-type lectin receptors
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
CNE	Cationic nanoemulsion
CNS	Central nervous system
COVID	Coronavirus disease
CPE	Cytopathic effect
CPP	Cationic cell-penetrating peptides
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR Associated Protein
DDA	Dimethyldioctadecylammonium
DOTAP	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DPPC	Dipalmitoylphosphatidylcholine
ESC	Embryonic stem cells
HA-antigen	Hemagglutinin-antigen of Influenza Virus
HCE	Human carcinoembryonic
HFF	Human foreskin fibroblast
HPLC	High-performance liquid chromatography
HSP	Heat shock protein
iDCs	Immature Dendritic Cells
IFN	Interferon
IgG	Immunoglobulin gamma
IL-2	Interleukin-2
iPSC	Induced pluripotent stem cell
IRE	Iron-responsive element
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
IVT	*In vitro* Transcribed
KUNV	Kunjin virus
LDNP	Lipidoid nanoparticle

LIONs	Lipid inorganic nanoparticles
LNP	Lipid Nanoparticle
m Ψ	N-methyl pseudouridine
m^5C	5-Methylcytidine
m^5U	5-Methyluridine
mo^5U	5- Methoxyuridine
m^6A	N6-methyladenosine
m^7G	N7-methylated guanosine
MHC	Major histocompatibility complex
MYD88	Myeloid differentiation primary response 88
NAb	Neutralizing antibody
N-Antigen	Neuraminidase antigen
NF-κB	Nuclear factor-κB
NLR	NOD-like receptor
NSP	Non-structural protein
OAS	2,5-Oligoadenylate synthetase
ORF	Open reading frame
PABPs	Poly(A) binding proteins
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PEG-PAsp	Polyethylene glycol polyaspartamide
PKR	Protein kinase
PLA	Polylactic acid
PRR	Pattern recognition receptor
PSA	Polyethyleneimine-stearic acid
Ψ	Pseudouridine
RBD	Receptor-binding region
RE	Restriction enzyme
RIG – 1	Retinoic acid-inducible gene I
RNAP	RNA polymerase
RNTP	Ribonucleoside triphosphate
RLR	Retinoic acid-inducible gene I like receptor
RSV	Respiratory syncytial virus
RVG	Rabies viral glycoproteins
SEAP	Secreted embryonic alkaline phosphatase
sgRNA	Single-guide RNA
SINV	Sindbis virus
S protein	Spike protein
S^2U	2-Thiouridine
TAA	Tumor-associated antigen
TALEN	Transcription activator-like effector nuclease
T_H	T helper
TLR	Toll-like receptor
TNF-α	Tumor-necrosis factor-alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β

UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VEGF-A	Vascular endothelial growth factor-A
VRP	Virus-like replicon particle
YTH	YT521-B homology
ZAL	Zwitterionic amino lipid
ZFN	Zinc finger nuclease

1 Introduction

Ever since its discovery in 1961, the mRNA molecule has been under research and discussion. With the advancement in technology, in vitro transcribed (IVT) mRNA was investigated for its properties, assorted uses, and functions. Of the various applications, the use of IVT mRNA in the replacement of protein and vaccinations for cancers and several infectious diseases have been investigated. Some characteristics that make IVT mRNA a better candidate for therapeutics as compared to DNA are as follows:

(i) IVT mRNA can be translated into desired protein immediately upon its arrival into the cytoplasm without any prerequisite to enter the nucleus.
(ii) It will not lead to insertional mutations as it doesn't get incorporated into the host genome and hence, it is fast-acting and safe (Sahin et al. 2014; Pardi et al. 2018).
(iii) It is active for a short duration and can easily be degraded, which makes it suitable in various pharmaceutical applications.
(iv) It can be produced cost-effectively with ease within the specified time (Sahin et al. 2014; Zhong et al. 2018).
(v) It can also be used to produce pluripotent stem cells (Sergeeva et al. 2016).

Additionally, its in vivo efficacy is well documented. The transfected liver cells might attain 100% efficacy (Sergeeva et al. 2016). Besides, mRNA can also encode zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and even CRISPR-Cas9 and can further be employed in genome editing (Sergeeva et al. 2016). IVT mRNA-based vaccines are a promising alternative to conventional vaccines as they can induce immune modulation with an increase in humoral as well as cell-mediated immunity (CMI) due to their self-adjuvanting nature (Sahin et al. 2014). mRNA vaccines are a safe platform and proved to be very effective as in the case of COVID-19 vaccines. They elicited elevated B-cell responses, $CD4^+$ Type 1 T helper cell responses, and strong interferon-gamma (IFN-γ), and interleukin-2 (IL-2) producing $CD8^+$ cytotoxic T-cell (T_C) responses (Walsh et al. 2020; Bettini and Locci 2021).

Formulation of mRNA into carrier molecules can lead to efficient in vivo delivery. Moreover, mRNA vaccines can be administered multiple times, as there is no anti-vector immunity because mRNA is the smallest genetic vector (Pardi et al. 2018). The mRNA is usually manufactured either from a linearized plasmid DNA (pDNA) template or from a PCR product in a cell-free system by in vitro transcription with the help of T7 or SP6 RNA polymerase and is capped enzymatically. IVT mRNA undergoes translation process in vivo and forms the protein which in turn undergoes post-translational modification achieving its bioactive configuration. The pharmacokinetics of mRNA-based therapeutics largely depends on the half-life of IVT mRNA as well as of the encoded protein, and the pharmacodynamics depends on the different processing pathways encountered by the encoded protein. Several other factors of the encoded protein like the biological functions, mode of action, among others are responsible for determining the total amount of IVT mRNA dose required for a particular therapeutic regimen (Sahin et al. 2014). IVT mRNA-based therapeutics have undergone leaps and bounds due to immense potential to deliver personalized intervention permitting patients to synthesize therapeutic proteins within themselves, eliminating the need for purification, glycosylation, and other solubility barriers linked with the conventional recombinant protein therapies.

Three major hurdles allied with mRNA are its short half-life, unpropitious immunogenicity, and in vivo delivery (Sahin et al. 2014). Structural modifications of IVT mRNA can enhance stability and encapsulating mRNA with suitable carrier molecules will ease their delivery and lead to rapid uptake. Codon optimization and nucleoside modifications can alleviate the immunogenicity to varying degrees and also affect the secondary structure of mRNA, kinetics, and accuracy of translation and proper folding (Sergeeva et al. 2016).

2 Production of IVT mRNA

For therapeutic uses, mRNA is produced synthetically by in vitro transcription of either a linear pDNA or a PCR template containing a promoter of bacteriophage origin. IVT requires modified nucleotides and an RNA polymerase (RNAP) enzyme usually derived from a bacteriophage (T7, SP6, or T3) which recognizes the bacteriophage promoter present on the DNA template to be transcribed. Capping and tailing of the synthetically produced mRNA are carried out as the translation of mRNA in the eukaryotic cell requires the presence of a 5′ cap and a 3′ poly(A) tail. A mature IVT mRNA contains a 5′ cap, a 5′ untranslated region (UTR), an open-reading frame (ORF), a 3′ UTR, and a 3′ poly(A)/polyadenylation tail. This kind of production process leads to the formation of certain by-products resulting in increased immunogenicity, which must be removed through the purification steps (see Sect. 5) (Sergeeva et al. 2016).

Recently, a novel, simple, and scalable method has been developed to synthesize functional mRNA with reduced immunogenicity in vivo. This method makes use of high temperatures, a thermostable T7 RNAP, and template-encoded poly(A) tail. All

these features together prevented the formation of dsRNA—an unwanted by-product (Wu et al. 2020).

3 Immunogenicity of IVT mRNA

The immune response elicited depends on the size and type of the carrier of IVT mRNA. Several components like RNA sensors in cells and structural parts of mRNA involved in the activation of the immune system have been identified. When the IVT mRNA is recognized by pattern recognition receptors (PRRs) like toll-like receptors (TLRs), protein kinases (PKRs), or the retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), an immune response is generated. TLR3 and PKR identify dsRNA, whereas TLR7 and TLR8 identify ssRNA and upon activation, both the sets induce IFNs secretion (Sahin et al. 2014; Sergeeva et al. 2016). The most effective inducer of IFN is Poly(U) which acts through binding with TLR7 (Sahin et al. 2014).

Several applications like protein-replacement therapies do not require activation of the innate immune system which rather is a drawback (Sahin et al. 2014). A unique approach to curb the innate immune response makes use of innate immune inhibitors which either avert the identification of synthetic mRNA by PRRs or block several downstream mediators in the TLR-3, TLR-7, RIG-1 pathways. Some inhibitors for released IFNs and cytokines have been recognized. Chloroquine, an anti-malarial drug, was the first candidate to reduce tumor-necrosis factor-alpha (TNF-α), ILs, and IFNs type-I. Although it was anticipated that TLRs present in the endosomes do not get activated as chloroquine is capable of averting endosomal acidification, it was reported to be ineffective in the human foreskin fibroblast (HFF) cell line. Other inhibitors like trichostatin A, Pepinh-TRIF, and Pepinh-MYD are also partially effective. Trichostatin A blocks the nuclear translocation of IRF7 (Interferon Regulatory Factor 7), Pepinh-TRIF blocks the interaction of TRIF (TIR-domain-containing adapter-inducing interferon-β) with TLR3 and Pepinh-MYD blocks the interaction of MYD88 (Myeloid differentiation primary response 88) with TLR7/8. Two appealing small inhibitors—BAY11, an anti-inflammatory compound, and BX975, an aminopyrimidine compound inhibits Nuclear Factor (NF-κB) and IRF3/7 activation respectively, thereby curbing the immunogenicity of synthetic mRNA (Zhong et al. 2018).

4 Strategies to Increase the Stability and Reduce the Immunogenicity of IVT mRNA

The stability, translational efficiency, and immunogenicity of mRNA can be regulated by modifying its structural elements as per the requirement (Sahin et al. 2014).

4.1 Capping (m7GpppN or m7Gp3N)

The addition of a 5′ cap (m7GpppN, also called cap-0) through a 5′-5′ triphosphate bond is important as it stabilizes the mRNA by abolishing the free phosphate groups and preventing its degradation by the nucleases. It is also important for the initiation of translation, helps avoid the recognition of the synthetically produced mRNA as a foreign entity by innate immune sensors and activities like splicing, transport, and translation of mRNA. Cap-0 is later on methylated in the cytoplasm on the first and second nucleotides (nucleoside-2′-*O*) of mRNA to generate cap-1 and cap-2 structures. 2′-O-methylated cap is required for efficient translation of mRNA (Sergeeva et al. 2016; Zhong et al. 2018; Xu et al. 2020). The cap is added either during in vitro transcription called co-transcriptional capping or post-transcription. Several editions of 5′ cap such as anti-reverse cap analogs (ARCAs) or synthetic caps can be added with the help of vaccinia virus capping enzymes (Pardi et al. 2018). ARCA is a cap analog that is modified with the replacement of the 3′ OH group with the $-OCH_3$ group. The initiation of transcription with the remaining hydroxyl group forces ARCA incorporation only in the forward orientation. Therefore, ARCA results in 100% of the transcripts produced with the capping at the 5′ end, and all such transcripts are translatable in the cell.

In 2018, a co-transcriptional CleanCap® capping method was developed that made use of an initiating capped trimer to generate cap-1 on the 5′ end of IVT mRNA. This resulted in the production of naturally occurring 5′ cap with increased capping efficiency reaching up to 90–99% (Xu et al. 2020). 100% efficacy is not possible when capping is done in vitro. Hence, 5′ triphosphates of mRNA transcripts must be removed with the help of phosphatase, thereby decreasing the immunogenicity as well as degradation of IVT mRNA (Sergeeva et al. 2016). Remaining uncapped or incompletely capped mRNA molecules can lead to the production of unnatural ends or anomalous RNAs such as dsRNAs produced by self-complementarity and extension resulting from self-priming (Schlake et al. 2019). In the cell cytosol, mRNA is decapped by the decapping enzymes of the cellular machinery, leading to instability. To reduce the pace of the decapping, reduce immunogenicity, and increase stability, chemically modified analogs of 5′ cap can be used (Grudzien-Nogalska et al. 2007).

(i) Methylation/removal of the 3′ OH group which wedges N7-methylated guanosine (m^7G) residue elongation (Sergeeva et al. 2016).

(ii) Addition of a phosphorothioate group results in a twofold reduction in retinoic acid-inducible gene 1 (RIG-1) activation by ssRNA and adjusts the binding between the cap-region and mRNA degrader protein Dcp2 (Sergeeva et al. 2016). Phosphorothioate modification of ARCA further enhances stability and translational efficiency (Xu et al. 2020).

(iii) Addition of 2-thiouridine (S^2U), N6-methyladenosine (m^6A), 5-methyluridine (m^5U), 5-methoxyuridine (mo^5U), 5-methylcytidine (m^5C), pseudouridine (Ψ), and N-methyl pseudouridine (m Ψ) could bring down the activation of TLRs, PKRs, and RIG-1 receptors and downregulate type-1 IFN signaling. As a consequence of m^6A addition, there is an interaction

of YT521-B homology (YTH) family proteins that reduces the half-life of mRNA by inciting its degradation. So, this modification should be avoided. The addition of Ψ enhances the translational rate, stabilizes secondary structure, and does not lead to any kind of toxicity since they are found naturally (Sergeeva et al. 2016).

(iv) Capping with m^7Gmpp_SpG (β-S-ARCA) increased the stability and translational efficiency appreciably in the immature dendritic cells (iDCs) (Xu et al. 2020).

(v) 2S analogs which are derived from the combination of 1,2—dithiophosphate modification, ARCA, and an elongated polyphosphate chain outshines any other capping methodology (Xu et al. 2020).

4.2 Tailing

There are many different ways to achieve polyadenylation of the synthetic mRNA, namely post-transcriptional tailing by the enzyme poly(A)polymerase or by introducing a poly(T) sequence into the DNA template that encodes for poly(A) tail. Also, while using the PCR template for synthetic mRNA production, poly(T) primers which bind at the 3′ end of the insert have been successfully used. Poly(A) tail shows a cooperative effect with other elements like the 5′ cap, the internal ribosomal entry site (IRES), poly(A)-binding proteins (PABPs), translation initiation factor eIF4G, etc., to regulate the stability and translatability of mRNA (Sahin et al. 2014). The cap and the tail circularize the mRNA and form an association with PABPs and eIF4G. This leads to a development in binding to the ribosome and further prevents the destruction of mRNA. To reduce the deadenylation through the activity of poly(A)-specific nucleases, modified nucleotides must be integrated into the poly(A) tail (Sahin et al. 2014; Sergeeva et al. 2016). But some of the modifications were a disappointment, e.g., cordycepin (3'-deoxyadenosine) could not increase the half-life of mRNA probably due to its failure to fully incorporate at the 3′ end as it is a chain terminator (Sahin et al. 2014). Nonetheless, cordycepin can be readily converted to cordycepin 5′ triphosphate which can further be used for 3′ end labeling. The estimated length of the tail capable of plummeting mRNA immunogenicity is between 120 and 150 nucleotides. But in many cases, short tails work better. The length of the poly(A) tail should be appropriate for the binding of PABPs, both at the poly(A) tail and the cap, leading to mRNA circularization. Therefore, one must be very careful about the choice made on the tail length (Sahin et al. 2014; Sergeeva et al. 2016).

4.3 Untranslated Regions (UTRs)

IVT mRNA has UTRs at 5′ as well as 3′ ends flanking the coding region. 5′ UTR has a direct influence on the translational process of the downstream sequence of mRNA.

Certain sequences like CC-(A/G)-CCAUGG can be incorporated to augment the stability and translational efficiency of mRNA. Some studies revealed that short and loose 5′ UTR is much more favorable for ribosomal binding compared to that of over-stabilized secondary structures (Xu et al. 2020).

The 3′ UTRs of many IVT mRNAs are derived from α- and β-globin mRNAs having certain sequence elements like iron-responsive elements (IRE; bounded by iron regulatory proteins—IRPs) that enhance the stability and translation of mRNA. When two human β-globin 3′ UTRs are set in head-to-tail orientation, the stability is amplified to a greater extent. Likewise, human heat shock proteins (Hsps) and several viral UTRs have also been employed to augment the expression of the protein. UTRs have some unstable regions (AU-rich or GU-rich regions) which must be substituted by stable structured sequences. This boosts the half-life of the mRNA (Sahin et al. 2014; Sergeeva et al. 2016; Xu et al. 2020).

A novel 3′ UTR motif has been identified with the help of cell culture-based systematic selection process. As compared to the 3′ UTR of the human β-globin, this motif could synthesize ~ threefold higher protein. These sequences are obtained from viral or eukaryotic genes and further can be altered through a method of systemic enrichment of RNA sequences that exist naturally. This increases the efficiency of mRNA and also its half-life (von Niessen et al. 2019).

4.4 Coding Region

The coding region in IVT mRNA may or may not be codon-optimized. Optimization of rare codons with frequent similar/synonymous codons is believed to enhance the translation efficiency. They do not alter the amino acid sequence, but can still increase the stability of mRNA and in some cases affect its secondary structure as well (Wang et al. 2021). The expression is increased by ~ 1.6 fold in a human T lymphocyte cell line with codon optimization of gag protein of HIV-1 (Ngumbela et al. 2008). Codon-optimized mRNA-encoding angiotensin-converting enzyme 2 (ACE2) exhibiting enhanced expression were transfected into A549 and HepG2 cells (Schrom et al. 2017; Schlake et al. 2019). Nonetheless, it should not be ignored that some proteins rely on slow translation for proper folding which can be guaranteed by the rare codons only. Some of the IVT mRNA vaccines work better with original ORF (Kimchi-Sarfaty et al. 2007).

5 Purification of Synthetic mRNA

The IVT mRNA has to be purified to remove DNA template, RNA polymerase, and unincorporated RNTPs. After the capping of the purified mRNA, the capping machinery also needs to be removed. Synthetic IVT mRNA may also be contaminated

with short abortive RNA fragments, RNA-RNA hybrids, etc., which are immunostimulatory in nature and reduce the efficiency of translation. Hence, all these elements ought to be removed to reduce the magnitude of innate immunity responses. During the commercial production of IVT mRNA, initial purification is done by commercial purification kits succeeded by precipitation methods. Several methods are being used for the purification process which includes $LiCl_2$ precipitation, silica membrane columns, etc., among others (Zhong et al. 2018). However, these methods fail to remove dsRNAs and other short abortive RNA fragments completely, and therefore another more efficient method such as reverse-phase high-performance liquid chromatography (HPLC) is used to reduce the immunogenicity of mRNA (Sahin et al. 2014; Sergeeva et al. 2016; Schlake et al. 2019). But this method is efficient for small length mRNAs only. Hence, other techniques such as size-exclusion chromatography, cross-flow filtration have emerged as better options for the purification of IVT mRNAs (Edelmann et al. 2014). Chromatographic techniques such as anion-exchange chromatography have also been successfully used for the purification of smaller synthetic mRNA (less than 500 nucleotides) (Zhong et al. 2018). Different techniques in combination may also be used to enhance the purity of synthetic mRNA. One such example is the use of hydroxyapatite chromatography (a common method used for separating nucleic acids and proteins) in conjunction with cellulose chromatography (Urayama et al. 2015).

6 Synthetic mRNA Platforms and their Features

There are various platforms for synthetic mRNA which are discussed below along with their advantages and disadvantages.

6.1 Unmodified mRNA

Unmodified mRNA are the mRNA molecules that do not have any modifications before in vivo delivery. These are recognized by the PRRs, and innate immune responses are induced by activating several such receptors (see Sect. 3). The unmodified mRNAs are also recognized by the nod-like receptors (NLRs), which can lead to pyroptosis mediated by caspase-1. By the activation of PRRs such as TLR, expression of type-I interferons such as IFN-α, IFN-β, and pro-inflammatory cytokines such as IL-12 are induced. These, unmodified mRNA molecules used as vaccines can act as brilliant self-adjuvants due to their ability to induce robust cellular and humoral immune response. However, these innate immune responses may be so severe that it may inhibit translation and lead to mRNA degradation by means of molecules such as dsRNA-dependent PKR, 2, 5-oligoadenylate synthetase (OAS) and adenosine deaminases (ADARs). Therefore, there should be a perfect balance between the

mRNA expression and the innate immune responses. Unnecessary immune stimulation by the synthetic mRNA is a side effect of several protein-replacement therapies, reducing the efficacy of the treatment. Although some approaches are being used to reduce the immunogenicity of the unmodified mRNA (see Sect. 3) so that its full potential may be exploited for therapeutic usage, yet more exploration is required in this area (Zhong et al. 2018).

6.2 Modified mRNA

Addition of $m^5C/S^2U/\Psi/m^6A/m^5U$, etc., to the 5′ cap decreases the immune stimulation. Despite reduced immune responses against the modified mRNA, the level of protein expression may vary from cell to cell. Modified mRNA resulted in escalated protein expression in RAW 264.7 cells (Uchida et al. 2013), but lesser protein expression in HuH7 and MEFs cells which may be attributed to the differences in PRR activity in these cells. Another reason could be that modified mRNAs have lesser ability to be translated than their unmodified counterparts, due to the alteration of their secondary structure, leading to a decrease in the protein binding affinity to regulatory sequence elements located within the UTRs involved in the modulation of translation and stability of the synthetic mRNA molecule (Zhong et al. 2018).

The advantage of having reduced immunostimulatory action is the escalation in the production of the encoded protein. But, it is important to highlight that the modification of the mRNA does not always guarantee an increase in the expression levels as it may also depend on the nature of the target cell and the delivery system.

Apart from modified nucleotides, mRNAs may be modified by the use of specific regulatory sequences in the 5′ and 3′ UTRs. For example, the 5′ and 3′ UTRs of 17-β-hydroxysteroid dehydrogenase 4 can be incorporated within the synthetic mRNA to increase its efficacy. Some other UTRs used for modification of mRNA include UTRs of α- and β-globin, viral UTRs of Venezuelan equine encephalitis virus (VEEV) and Sindbis virus (SINV), etc. The addition of a histone stem-loop after the poly (A) tail has also been reported to increase the efficacy of synthetic mRNA (Zhong et al. 2018).

6.3 Sequence-Optimized Unmodified mRNA

Some studies have indicated that sequence-optimized unmodified mRNA is non-immunostimulatory with excellent levels of expression in vivo. Sequence-optimized unmodified mRNA coding for the protein erythropoietin was non-immunogenic and exhibited protein expression several times better than the ψ-modified mRNA (Zhong et al. 2018). Another approach for producing sequence-optimized unmodified mRNA can be the replacement of codons poor in GC content by similar codons which are rich in GC content. This leads to a decrease in the AU-rich codons resulting in lesser

immune responses as AU-rich codons act as decay signals and are recognized by the TLR-3 and 7. Also, since uridine is the most commonly modified nucleoside in eukaryotic mRNA, a decrease in the uridine content also means reduced chances of PRRs recognizing the synthetic mRNA as a foreign entity (Meng and Limbach 2006; Zhong et al. 2018).

6.4 Replicon RNA

As the name suggests, replicon RNAs are self-amplifying RNAs containing the gene of interest, the sequence coding for viral replicase, and promoters (both genomic and subgenomic). These replicon RNAs can be used for the production of multiple proteins at a time. The most commonly used replicon RNAs are derived from viruses belonging to *Alphaviruses* such as SINV, VEEV, Kunjin virus (KUNV) (Zhong et al. 2018).

These replicons act through some basic steps such as translation of the gene-encoding viral replicase complex upon delivery into the cell cytoplasm, transcription of replicon RNA by replicase complex into a (−) sense complementary RNA strand, further used by replicase complex to generate a large number of (+) sense RNA, encoding the protein of interest. As observed with unmodified mRNA, replicon RNAs are also immunostimulatory (Akhrymuk et al. 2016). pDNA vectors can also be used for launching replicon RNA. These pDNA vectors have the gene coding for replicon RNA and hence helps achieve additional amplification as a result of the production of several copies of replicon for each pDNA molecule. These pDNA-launched replicon RNA are reported to be more immunostimulatory than replicon RNAs (Johansson et al. 2012; Zhong et al. 2018). Also, during the amplification process, a large number of single-stranded DNA and double-stranded RNA species are produced, eliciting immunity (Akhrymuk et al. 2016; Zhong et al. 2018). Hence, replicons may be beneficial for creating RNA vaccines. Several studies have suggested that non-structural proteins (NSPs) forming the replicase complex of alphaviruses cause severe cytopathic effect (CPE), resulting in cell death (Petrakova et al. 2005). This might raise the replicon RNA vaccine's efficacy due to the production of certain immunostimulatory factors as well as encoded intracellular antigens in the extracellular environment. These are then taken up by the antigen presenting cells (APCs) and presented to the respective major histocompatibility complex (MHC)—molecules (Leitner et al. 2004). Certainly, when replicon RNAs are employed for protein therapies, such untimely death of target cells is not at all required as this would cause an obstruction in the overall protein production. To prevent the CPE and untimely death of the target cells, amino acid substitution is frequently done in the NSP2 protein.

7 In Vivo Delivery Strategies of Exogenous mRNA

As mRNA molecules are not very stable, it requires the assistance of a suitable carrier for its delivery in vivo. Approaches to the in vivo delivery of synthetic mRNA include direct uptake of naked mRNA, cationic-liposome-mediated RNA transfection or cationic nanoemulsion, peptide-based delivery, electroporation and nucleoporation, gene gun-mediated delivery of mRNA, use of polymer nanomaterials, virus-like replicon particle-based delivery of mRNA, and some other lesser-known techniques.

7.1 Delivery of Naked mRNA

This approach was first described already in 1990, which demonstrated temporal transgene expression after injection of naked mRNA into the skeletal muscle of mice (Rhoads 2016). Reversal of condition in diabetic rats with mRNA-encoding vasopressin, decrease in viral load in the lungs after delivery of naked mRNA expressing antigens against influenza A virus, respiratory syncytial virus (RSV) and louping ill virus are some examples of the efficacy of naked mRNA (Jirikowski et al. 1992; Fleeton et al. 2001). Delivery of naked mRNAs involves the direct injection of the mRNA in solution (commonly used mRNA solutions include Ringer's solution and lactated Ringer's solution) (Wang et al. 2021). Although it is assumed that naked mRNA are not able to freely cross the cellular membranes, a number of studies have proposed some hypotheses pertaining to its uptake mechanism. One of such studies hypothesizes that the uptake of naked mRNA molecule by the DCs in the central nervous system (CNS), after an intranodal injection, involves macropinocytosis, which allows the expression of the antigen-encoding mRNA along with the activation of T cells/DC (Wang et al. 2021). Intradermal injection of synthetic mRNA-encoding several antigens such as telomerase, survivin, human EGF2, in 30 patients resulted in benefits to a few patients (Rittig et al. 2011). Intradermal injection of naked mRNA-encoding influenza virus hemagglutinin (HA) antigen in animals such as mouse, pigs, resulted in these animals gaining immunity to influenza A infections (Petsch et al. 2012).

7.2 Cationic Liposome-Mediated or Cationic Nanoemulsion (CNE)-based RNA Transfection

The use of complexing agents for the delivery of mRNA has an advantage over naked mRNA, as it helps mRNA resist the degrading action of nucleases. Also, by binding to the self-amplifying RNAs, it potentiates the mRNA vaccine. The most important component for achieving delivery via this method is the cationic lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOTAP). In the very first attempt,

incorporation of IVT mRNA-encoding luciferase enzyme into cationic liposome exhibited a considerable response in transfected mouse NIH 3T3 cells, where the luciferase expression was demonstrated to be linearly correlated to dose (Rhoads 2016). Furthermore, a liposome-protected mRNA vaccine-encoding human carcinoembryonic (HCE) tumor antigen has been developed (Conry et al. 1995). Intramuscular injection of this vaccine into mice produced a good immune response against the HCE antigen. Injection of a liposome-protected mRNA vaccine-encoding melanoma antigen glycoprotein 100 (gp100) into the spleen led to delayed tumor growth (Zhou et al. 1999). Also, immunization by injecting mRNA into the skin has been done. They isolated mRNA from spindle cell tumors (S1509a) and encapsulated it with the cationic lipid dioleoyl-3-trimethylammonium propane (DOTAP), and injected it into mice epidermal cells. Upon challenge with alive S1509a cells, these mice exhibited a greatly reduced rate of tumor formation (Granstein et al. 2000). Nucleoside-modified mRNA, encapsulated within cationic lipid, Lipofectamine 2000, was used for the expression of therapeutically significant proteins (Kormann et al. 2011). Based on all these studies, it can be concluded that CNE has the potential to be evaluated and subjected to human clinical trials.

7.3 Peptide-based Delivery

The mRNA, being negatively charged, can be easily delivered by adsorption onto the surface of cationic peptides, as a result of electrostatic interactions between them. The positive charge is due to the presence of basic amino acids such as lysine and arginine. The amount of mRNA which gets adsorbed onto the cationic peptide is dependent upon the negative:positive charge ratio (Wang et al. 2021).

7.3.1 Protamine-Complexed mRNA

Protamine is one of the positively charged peptides which has been evaluated for the delivery of mRNA. Two important properties make this peptide an important delivery agent. First, protamines have the ability to protect mRNA from degradation by host nucleases. Second, protamine is an adjuvant and helps achieve heightens immune response (Wang et al. 2021).

The β-galactosidase-encoding mRNA condensed with protamine into a negatively charged nanoparticle complex, and further encapsulated in a cationic lipid liposome was demonstrated to be effective in mice. This complex resists the mRNA degradation inside the cell and could express the protein in vivo followed by T-cell response and production of anti-β-galactosidase IgG antibodies. Protected mRNA persists longer than naked mRNA and hence remains immunogenic for a longer period. Protamine-complex mRNA encoding for proteins such as tyrosinase, gp100, melan-A was tested in clinical trials in seven metastatic melanoma patients, out of which, one patient showed a promising response (Rhoads 2016).

7.3.2 Cationic Cell-Penetrating Peptides (CPP)

These are small peptides, which are positively charged and are composed of 8–30 amino acid residues. They act as an important delivery agent for the synthetic mRNA due to two major reasons. Firstly, these peptides possess low charge densities. Secondly, they are able to disrupt the membranes for an endosomal escape. This is an important aspect with respect to synthesis of proteins. In a recent experiment, three CPP-mRNA platforms, viz. RALA (WEARLARALARALARHLARALARALRACEA), LAH4 (KKALLALALHHLAHLALHLALALKKA), and LAH4-L1 (KKALLAHALHLLALLALHLAHALKKA) were compared. All these three cationic peptides complexed with the mRNA were introduced into the dendritic cells, and elicited both innate as well as adaptive immune responses. But, the LAH4-L1 complex with mRNA exhibited the best protein expression. It is noteworthy that the uptake and other intracellular activities for such CPP-mRNA complex involve processes such as clathrin-mediated endocytosis and phagocytosis (Coolen et al. 2019). In a different set of experiments, attempts were made to combine the cationic characteristics of protamine and cell-penetrating characteristic by formulating a fused protamine-CPP protein, which was then used to deliver mRNA to human cell lines (Wang et al. 2021).

7.4 Electroporation and Nucleoporation

To increase the uptake of naked mRNA by cell, the electroporation approach has been widely used. A significant increase in both the cell-mediated and humoral immune response was recorded when SFV vector RNA-encoding β-galactosidase was electroporated intradermally into mice (Zhong et al. 2018). Electroporation technique aided in enhancing the expression levels as well as the immune response to intramuscularly injected VEEV chimeric replicon RNA coding for two different proteins, viz. secreted embryonic alkaline phosphatase (SEAP) and HIV envelope protein (Cu et al. 2013). Gain and loss of function studies in live animals may be performed by in vivo electroporation of synthetic mRNA in brain tissue (Bugeon et al. 2017).

Nucleoporation is similar to electroporation, but it is based on the use of proprietary nucleofection reagents. The synthetic mRNA is introduced into both the nucleus and cytosol (Lenz et al. 2003). It is a milder process than electroporation, and cells are found to recover faster than electroporation.

7.5 Gene Gun-Mediated Delivery of mRNA

Also known as the biolistic method, it helps in the cellular uptake of larger molecules as a result of membrane damage. Gene gun mediated in vivo delivery led to an

increase in uptake efficacy and delivery of synthetic mRNA encoding for human α-1 antitrypsin and produced a significant immune response against the expressed antigen in rodents (Qiu et al. 1996). Gold microcarrier particles coated with the infectious Flavivirus RNA delivered through the gene gun method exhibited good efficiency of infection in mice (Mandl et al. 1998). In another significant experiment, synthetically produced Bovine viral diarrhea virus (BVDV) mRNA was introduced in cows and sheep using gene gun, which led to a robust immune response against BVDV, at levels similar to natural exposure to the virus (Vassilev et al. 2001).

7.6 Use of Polymer Nanomaterials

Polymer Nanomaterials are usually made up of synthetic polymeric materials such as polylactic acid (PLA), chitosan, gelatin, polycaprolactone, etc. These materials are very much stable in nature, and they show the ability to encapsulate both hydrophilic as well as hydrophobic compounds, apart from proteins and other biomolecules. They are also instrumental in the tuned delivery of therapeutic compounds to the target site (Damase et al. 2021). These polymeric substances can be utilized for the purpose of producing nanoparticles that can be delivered via injections. The injections can be done via intravenous, intramuscular, intraperitoneal, or subcutaneous routes (Molina et al. 2015). For instance, cationic polyethyleneimine-stearic acid (PSA) copolymer has been developed for the delivery of mRNA coding for HIV-1 gag, into the dendritic cells and a 6-8 week-old female BALB/c mice (Zhao et al., 2016). This modified mRNA (encapsulated within PSA) was used to immunize the mice against the HIV-1 gag antigen and to detect the titers of antibodies specific to the HIV-1 gag (anti-HIV-1 gag antibodies) along with the levels of $CD8^+$ and $CD4^+$ T-cell responses.

7.7 Virus-like Replicon Particle (VRP)-based Delivery of mRNA

Virus-like particles have the ability to encapsulate mRNA and deliver it into the target cell, in a manner similar to that of a virus infection. The synthesis of viral structural proteins takes place in vitro followed by the encapsulation of the antigen-encoding mRNA (Li et al. 2017). In a study, Venezuelan equine encephalitis VRP encapsulated mRNA, coding for different kinds of Dengue virus E-antigen, viz. subviral particles [prME] and soluble E dimers [E85] were used for immunization, which displayed induction of protection against the encoded antigens. Moreover, E85 antigen-VRP gained importance in terms of both speed and magnitude of immunity (White et al. 2013). In another study, HIV-derived mRNA that encoded clade C trimeric envelope glycoprotein was used as a VRP source. The complex triggered cellular immune responses in rhesus macaques (Bogers et al. 2015).

Recently, it has been shown that lipid inorganic nanoparticles (LIONs) encapsulated alphavirus-derived replicon RNA coding for SARS-CoV-2 spike (S) protein when administered intramuscularly into mice and primates, increased the titers of anti SARS-CoV-2 S protein IgG antibodies (Erasmus and Khandar 2020).

7.8 Other Lesser-Known Methods

Several other methods can be used for the delivery of mRNA into the cells but are not as common as the above-mentioned ones. The use of lipid nanoparticles for the delivery of mRNA into the cells is being exploited as a technique for delivering self-amplifying RNA vaccines (Rhoads 2016). Lipid nanoparticles (LNP) are excellent delivery platforms. They are negatively charged in nature and can be categorized as cholesterol, ionizable amino lipids, phospholipids, and polyethylene glycol. The interaction between the essential ionizable amino lipids and ionizable amino lipids as well as the endosomal membrane helps mRNA escape from the endosome. The advantages of using LNP-based systems for the delivery of mRNA are twofold. First, LNPs shield the mRNA from being degraded by the enzymes present within the endosome, which ensure high efficiency of encapsulation. The second major advantage that LNPs confer is their decent biocompatibility, by means of a series of bioprocesses for the delivery of mRNAs to be expressed (Wang et al. 2021).

Cationic oil-in-water nanoemulsions for the effective delivery of mRNA into the cells have also been documented (Brito et al. 2014). The use of polymeric mRNA nanomicelles, for the delivery of mRNA in vivo, was a significant breakthrough in the field of molecular therapy. In that method, polyethylene glycol polyaspartamide (PEG-PAsp) polymer was used to form small 50–100 nm in diameter nanomicelles with mRNA (Kataoka et al., 2012). The use of PEG-polyamino acid polymers has demonstrated continuous expression of the protein in cerebrospinal fluid for a week when mRNA-encoding luciferase with modified nucleosides was administered into mice CNS intrathecally (Uchida et al. 2013).

8 Applications of mRNA Therapeutics

Synthetic mRNAs find several applications in the field of medicine and genetic engineering such as protein-replacement therapies, vaccines against several diseases and genome editing.

8.1 mRNA as a Therapeutic Agent for Replacement of Defective Protein within the Cell

Several genetic diseases such as hemophilia B, cystic fibrosis are characterized by the presence of a defective protein or defective translation of mRNA encoding that particular protein (Huang et al. 2020). Such conditions can be treated by the delivery of a functional protein-encoding mRNA into the cell, known as protein-replacement therapy. But, unlike mRNA vaccines, delivery of mRNA intended for such therapy should not be immunostimulatory as this may lead to mRNA degradation (Rhoads 2016). This method is more effective when compared to conventional protein therapy, as a single mRNA molecule can be used to produce a considerable amount of protein produced in the cell during the course of treatment (Warren et al. 2010). Several studies have been conducted which supplement the application of mRNA for protein-replacement therapy. As early as 1992, naked IVT mRNA coding for the hormone vasopressin was demonstrated to be effective in diabetes insipidus in Brattleboro rats. The synthetic mRNA injection showed promising results within 5 h of its injection into the hypothalamus, expressing vasopressin in magnocellular neurons (Jirikowski et al. 1992). Modified synthetic mRNA coding for surfactant protein B has been demonstrated to be effective in mouse models suffering from congenital lung disease (Kormann et al. 2011). Lipid nanoparticle-packaged mRNA coding for cystic fibrosis transmembrane conductance regulator (CFTR), for the treatment of cystic fibrosis, which is an inherited genetic disorder resulting due to a mutation in the chloride channel, the CFTR. The disruption of CFTR leads to the accumulation of a thick mucous layer in various organs such as the pancreas and the lungs. The LNP-packaged mRNA has been tested in knockout mice with intranasal delivery and recorded an efficacy equivalent to the presently used drug ivacaftor (Robinson et al. 2018). Apart from this, MRT5005 (Translate Bio) is being developed for the treatment of cystic fibrosis, which codes for a functional CFTR protein, and delivered into the lung epithelia via nebulization (TranslateBio 2019).

An mRNA drug has been formulated for treating citrin deficiency, caused by a mutation occurring in the SLC25A13 gene-encoding citrin. Citrin is a mitochondrial membrane transport protein which has a role in the urea cycle. The deficiency of citrin leads to hyperammonemia and neuropsychiatric disturbances. Upon administration of an mRNA coding for human citrin in SLC25A13 knockout mice, it was observed that there was a drastic reduction in the hepatic citrulline and blood ammonia levels succeeding an oral sucrose challenge and reduced aversion of sucrose, which is a hallmark of citrin deficiency (Cao et al. 2019). Also, promising data were obtained from the pre-clinical studies of an mRNA therapy for the fabry disease, caused as a result of mutations in the GLA gene coding for enzyme α-galactosidase, which is very essential for the utilization of the glycolipids. In the Fabry disease, glycolipid derivatives such as globotriaosylceramide, globotriaosylsphingosine tend to accumulate within a number of tissues with time leading to a wide range of clinical manifestations. Administration of a single dose of GLA-encoding mRNA in GLA-deficient mice models leads to a drastic reduction in the accumulation of globotriaosylsphingosine

in plasma and tissues. It is important to note here that this beneficial effect of mRNA administration was observed for up to 6 weeks after the dose (Zhu et al. 2019).

8.2 mRNA as Vaccines Against Cancer

As discussed earlier, mRNA-based vaccines against cancer have significantly more advantages over DNA vaccines and conventional peptide-based or protein-based vaccines. Also, the adaptability of these mRNA molecules to both dividing and resting cells makes them more favorable for therapeutic purposes (Huang et al. 2020). Proteasome, a protein complex, degrades the mRNA-encoded proteins and presents the resulting peptides to the MHC-I molecules which in turn activates the $CD8^+$ T_C cells. Generally, the MHC-II processing pathway is out of reach of intracellular proteins. So, T_H2 cell responses are not efficiently stimulated. But with the help of certain secretion signals engineered into mRNA, the protein can be directed to the extracellular secretion pathways activating the effective T_H2 responses (Sahin et al. 2014). Upon delivery, these tumor antigen-encoding mRNAs are expressed into the APCs and are presented majorly with MHC class I molecules, activating T-cell mediated response required for effective tumor clearance (Fiedler et al. 2016). Tumor-associated antigens (TAAs) are expressed abundantly in cancerous cells; hence, mRNAs coding for these antigens have been exploited as anti-cancer vaccines. Some studies by CureVac demonstrated that upon intradermal injection of cationic-liposome-protected mRNA, a sufficiently good amount of CMI and antibody-mediated immune response was induced (Hoerr et al. 2000). Also, results from some other clinical trials showed that the injection of mRNA complexed with protamine intradermally produced strong T-cell and B-cell responses and are highly safe and effective (Rittig et al. 2011). Since mRNA encoding a single antigen may not always be sufficient for inducing a robust immune response, mRNA vaccines based on multiple antigens or "antigen-cocktail" have been validated for enhancing the immunogenicity of vaccines (Vansteenkiste et al. 2016). Some of the therapeutics developed by *Moderna*, intended for the treatment of cancer include mRNA2416, mRNA-2752, and MEDI1191. These therapeutics are *immunomodulatory* in nature. mRNA2416 codes for OX40 ligand (OX40L), a membrane-bound co-stimulatory protein involved in the enhancement of expansion, function and survival of T-lymphocytes for mounting a robust immune response against the cancer cells. After delivery of this molecule into the tumor (by means of an intratumor injection), the tumor cells express it on their surface, which attracts a stronger T-cell response against these tumor cells. mRNA2416 is also being investigated by *Moderna* for abscopal effect in metastatic cancer, i.e., whether a localized injection into the target tumor cell would elicit secondary immune response and also show an effective result in the surrounding metastases or not (Moderna 2020). Another therapeutic ligand, mRNA2752, is based on delivery of OX40L into the tumors, in addition to the

immunostimulatory cytokines IL-23- and IL-36γ-encoding mRNA for promoting T-cell-mediated cellular cytotoxicity. The therapeutic drug MEDI1191 can be administered for the solid tumors also, and it encodes IL-12, one of the most potent cytokines which is involved in mediating antitumor activity (Tugues et al., 2015).

8.3 Dendritic Cell (DC) Vaccines

Dendritic cells are an excellent vaccine target. The primary reason for this is that, as professional APCs, they tend to take up the antigen, subject it to processing and present it to the cells of the immune system, which leads to generation of a strong adaptive immune response (Beck et al. 2021).

Loading of DC-based mRNA vaccines can be achieved both in situ and ex vivo. Dendritic cells are first obtained from the peripheral blood of the patient. After the DCs undergo maturation, they are subjected to loading with mRNA encoding the desired antigen and the loaded DCs are returned back to the patients. There are multiple ways in which the loading of antigen-encoding mRNAs into mature DCs can be performed including electroporation, lipofection, nucleofection, and sonoporation. Among these, the most frequently utilized technique is electroporation (Ahmed et al. 2020; Wang et al. 2021). For achieving the delivery of mRNA into DCs in situ, the antigen-encoding mRNAs, in a complex with TriMix, can be directly injected into the lymph nodes. For instance, the first clinical trial of a TriMix-DC complex vaccine was NTC01066390 in patients suffering from advanced melanoma (Wang et al. 2021). As part of a phase 1b clinical trial in melanoma patients, it was noted that the administration of DCs electroporated with mRNA coding for the tumor antigen and TriMix showed extended progression-free survival time (Wilgenhof et al. 2013).

Targeting tumor antigen-encoding mRNAs into the monocyte-derived DCs ex vivo, and then re-injecting them in the animal stimulates effective immune responses against the tumor cells. It was shown that cationic lipid mRNA coding for ovalbumin pulsed into autologous DCs and re-injected in mice, protected them against the cancer cells expressing ovalbumin (Boczkowski et al. 1996). Injection of FOXP3-encoding mRNA into the dendritic cells led to an effective T-cell-mediated immune response against breast cancer cells (Nair et al. 2013). One of the most famous DC vaccines for viral diseases is the HIV-1 vaccine. In this, the patients suffering from HIV-1 infection are administered with DCs having mRNA vaccines coding for multiple antigens of HIV-1. The delivery of the mRNA is done by electroporation due to its high delivery efficacy. Evaluation of the elicited cellular immune responses displayed antigen-specific T cells action with not much clinical benefits (Wang et al. 2021).

8.4 mRNA Vaccines in Prevention of Diseases

mRNA vaccines can be administered for a number of infectious as well as non-infectious diseases. Such vaccines have been tested for infectious diseases such as Influenza virus infection, Rabies, Zika virus, COVID-19 infection. Apart from infectious diseases, a number of non-infectious diseases such as cardiovascular diseases, neurological disorders have the potential to be corrected by the use of mRNA vaccines. With a greater number of developments expected on the mRNA modification technology and delivery strategies, it is expected that the applications of such vaccines would gain more popularity and its use would become more widespread (Li et al. 2021).

8.4.1 mRNA Vaccines Against Infectious Diseases

mRNA vaccines have been tested for the prevention of infectious diseases caused by different pathogens such as Influenza viruses (Petsch et al. 2012), Zika virus (Feldman et al. 2019), Rabies virus (Schnee et al. 2016), Dengue virus (Roth et al. 2019), and SARS-CoV-2 infection (Kaur and Gupta 2020a, b). Influenza A was the first viral infection against which an mRNA vaccine was investigated (Borch and Svane 2016). Subcutaneous injection of liposome-encapsulated mRNA-encoding Neuraminidase (N)—antigen of Influenza A in mice elicited immune response similar to the response to natural infection in terms of specificity and strength of immune response (Martinon et al. 1993). An unmodified IVT mRNA Influenza A vaccine-encoding Hemagglutinin (HA) and N antigens led to a robust antibody-mediated as well as cell-mediated immune response upon intradermal injection into animal models such as mice, ferrets, and pigs that were similar to the immune response induced by a licensed vaccine against Influenza A virus in pigs (Petsch et al. 2012). A self-amplifying mRNA, in a complex with LNP-encoding HA antigen of Influenza A virus, was delivered into mice models intramuscularly. After two weeks of a second dose, mice developed satisfactory immunity, which was thought to be sufficient to impart protection (Hekele et al. 2013).

COVID-19 is the first viral disease for which mRNA-based vaccines gained approvals for use in human beings. Currently, two such vaccines are in use to impart effective immunity against COVID-19, mRNA-1273 by *Moderna* and BNT162b1 by *Pfizer*. The mRNA-1273 vaccine is an LNP-capsulated mRNA encoding for the S protein of SARS-CoV-2 and elicits a strong antiviral response against the S antigen. In phase-I clinical trials, it was found that the participants who received 25 μg dose of viral S mRNA had neutralizing Ab (nAb) levels which were comparable to the convalescent sera, whereas the participants who received 100 μg dose of mRNA had an Ab level surpassing the levels in convalescent sera. The vaccine was well tolerated by the patients who received both 25 μg and 100 μg doses, but grade 3 systemic symptoms were shown by three volunteers who received 250 μg doses.

The BNT162b1 vaccine is a codon-optimized mRNA vaccine coding for the SARS-CoV-2 receptor-binding region (RBD) of the S protein, encapsulated in ionizable cationic LNPs. In the vaccinated individuals, RBD-specific IgG levels were found to be higher than the levels in convalescent serum. The levels of SARS-CoV-2 neutralizing antibodies (nAbs) were also found to be significantly higher than the levels found in convalescent serum (Kaur and Gupta 2020a, 2020b).

The efficacy of mRNA vaccines is not only limited to respiratory pathogens, such as influenza and coronaviruses but also rabies in rodents and pigs. It has been shown that the vaccine (CV7202)-encoding rabies viral glycoproteins (RVG) can elicit adaptive immune response ($CD4^+$ T cells) in the body which is comparatively more than the vaccines already in use. Throughout the observation period of one year, there was no change in the nAb titer value in mice. CV7202 is currently being studied in a phase 1 trial of CureVac for its safety, reactivity, immune response, and immunogenicity (Schnee et al. 2016). Also in another study, the amplified mRNA vaccine encoding the RVG formulated with LNPs showed a promising effect against rabies virus. They made a comparison among frequently used cationic lipids and on the basis of that chose the most efficient cationic lipids-DOTAP or dimethyldioctadecylammonium (DDA). These two were capable of inducing efficient antigen expression for animal evaluation of LNPs designed (Lou et al. 2020).

The idea of mRNA vaccines for protection against the Zika virus was published in 2017 (Richner et al. 2017). Synthetic modified mRNA-encoding prM/M-E antigens of the virus were produced. Apart from a modified nucleoside, a molecule of S-adenosylmethionine was added to the capped end of the mRNA to increase the translatability of the mRNA and coated with LNPs before administration in the mice models. Results suggested that the modified synthetic mRNA vaccine provided sufficient immunity against the virus in animal models and protected the mice against Zika-virus-mediated congenital disease (Richner et al. 2017; Huang et al. 2020). A cytomegalovirus (CMV) vaccine has also been developed for the prevention of CMV infection in pregnant women and in patients who have undergone transplantation. The constituents of the vaccine were six modified mRNA which coded for CMV glycoprotein and pentameric complexes. Delivery of the modified mRNA vaccine (encapsulated within LNPs) was done by means of an intramuscular injection. It was noted that a single dose of the vaccine was able to induce strong immune responses in mice and non-human primates. As a result, the vaccine underwent clinical trials, sponsored by *Moderna* (mRNA-1647) (John et al. 2018).

8.4.2 mRNA-based Therapeutics Against Non-Infectious Diseases

A number of recent studies have shown that many of the previously "undruggable" pathways which are involved in the progression and development of cardiovascular diseases could be targeted by mRNA. AZD-8601 developed by *Moderna* coding for vascular endothelial growth factor-A (VEGF-A) was intended to be used during coronary artery bypass surgery, delivered via an epicardial injection. It was thought to reduce myocardial ischemia, along with improvement in left ventricular systolic

function in people suffering from ischemic heart disease, by means of enhancement of local angiogenesis (Carlsson et al. 2018). A phase II clinical trial conducted by *AstraZeneca* is currently evaluating the efficacy of this drug. This trial is randomized, double-blind, placebo-controlled, as well as multicenter. The trial is being conducted in patients who have moderate contractile dysfunction and are undergoing coronary artery bypass surgery. With the aid of epicardial injections, patients are randomly assigned doses of 0, 3, or 30 mg of mRNA-encoding VEGF-A in a citrate buffer. If this study shows effective results, it would suggest that there is an improvement in the blood flow and function when mRNA is injected directly into an ischemic tissue (Anttila et al. 2020).

With respect to neurological disorders, administration of mRNA proves to be an effective approach because they can offer native proteins and peptides to the target site perpetually, allowing a synchrony between the dynamics of signal receptor expression and the availability of the bioactive factor. However, direct delivery of mRNA to neural tissues in vivo has proven to be difficult because of the instability and high immunogenicity of introduced mRNA, limiting the studies and attempts to target neural tissue.

Recently, an mRNA formulated with cationic liposomes has been developed for the treatment of chronic disorders. This was a novel approach in which nucleic acids were employed for the treatment of neurological disorders. The cationic liposome used for the delivery of the therapeutic mRNA was composed of DOTAP, dipalmitoylphosphatidylcholine (DPPC) and cholesterol. The potential for the delivery of such mRNA to the brain via the nasal passage has been evaluated in mice models, and the results suggested that the delivery of non-mRNA into the brain via the intranasal route is feasible for the treatment of neurological disorders (Dhaliwal et al. 2020).

8.5 mRNA-Mediated Genome Editing

Several tools are available for editing the genome, including ZFNs, TALENs, and CRISPR/Cas system (Sergeeva et al. 2016). But a point of major concern is their off-target effects. To reduce the off-target effects, mRNAs coding for ZFNs, TALENs, and CRISPR/Cas systems can be produced to achieve a transient expression of these nucleases, only requiring a short period for their action. A delivery system based on zwitterionic amino lipid (ZAL) to co-deliver mRNA coding for Cas9 and single-guide RNA (sgRNA) has been developed. As a result, a 95% reduction in the protein expression was observed along with permanent editing of DNA (Miller et al. 2017). LNP-coated mRNA coding for Cas9, along with sgRNA for editing transerythrin gene found in mouse liver reduced transerythrin protein levels in the serum, for at least 12 months after the administration of the synthetic mRNA (Finn et al. 2018). Targeting TTR and PCSK9 genes by an LNP-coated mRNA coding for ZFNs in mice significantly reduced the gene expression (Conway et al. 2019). All these experiments suggest that LNP-based mRNA delivery systems for genome editing are very promising and should be explored further.

8.6 Generation of Induced Pluripotent Stem Cells (iPSCs) using mRNA

Somatic cells can be successfully converted into stem cells by the introduction of DNA or mRNA coding for transcription factors into the cell. Shinya Yamanaka and co-workers demonstrated that "mature cells can be re-programmed to become pluripotent" (Takahashi et al. 2007). Although their work was based on DNA vectors, the same can be achieved by using mRNA (Rhoads 2016). The re-programmed cells are now being widely explored for the treatment of various diseases such as diabetes, muscular dystrophies (Okano et al. 2013; Fox et al. 2014).

A nucleoside-modified cationic lipid-mRNA capped with ARCA encoding four transcription factors, viz. KLF4, c-MYC, OCT4, and SOX2, has been developed. It was introduced into different cells such as human epidermal keratinocytes, and reprogramming of cells took place to produce iPSCs, proving that the use of mRNA for the generation of iPSCs was highly efficient (Warren et al. 2010). The most interesting fact is that these induced stem cells are quite similar to the embryonic stem cells (ESC) with respect to their ability to self-renewal and to differentiate into all three germ layers of the body (endoderm, mesoderm, and ectoderm). Therefore, it can be ascertained that these human iPSCs can be used as an alternative option for human ESCs, which also helps nullify the associated ethical concerns. This discovery provided a transformation for the field of regenerative medicine, as patient-specific iPSCs can be differentiated and their derivatives can be used as therapeutic cells. The major advantage which comes with the use of such cells is that these cells can be transplanted into the patient, with minimal risk with respect to genetic incompatibility of transplanted cells or even immune rejection (Chanda et al. 2021). Using this technique, differentiation of these iPSCs into terminally differentiated myogenic cells was successfully achieved. Cationic lipid-mRNA with a poly(A) tract capped with ARCA, coding for the same four transcription factors mentioned above was successfully used to reprogram HFF, leading to the generation of iPSCs expressing alkaline phosphatase (AKP) enzymes and various embryonic stem cell markers (Yakubov et al. 2010).

Viral vectors (such as retroviral, lentiviral or even adenoviral vectors) can be used for generation of iPSCs, but there is a risk of genomic integration, which tends to limit the clinical application of iPSCs produced in such manner. In order to lessen the risks associated with viral vectors, a number of integration-free approaches have been developed, which include the Sendai virus, cell permeating recombinant proteins, non-integrating plasmids, or episomal DNA. Though these techniques presented minimal risk of genome integration, the observed efficiency of generation of iPSC was very low (Chanda et al. 2021).

9 Safety of mRNA Therapeutics

As seen across different studies, the clinical use of mRNA for therapeutic purposes has demonstrated that they are safe, tolerable, and pose no major risks for the subject (Sahin et al. 2014). In the majority of cases, for example, in the case of mRNA-based protein-replacement therapies, apart from studies by independent researchers, there have been no clinical trials conducted for checking the safety and efficacy in larger groups of organisms. Due to this reason, there is a lack of evidence that proves or shows the nature of safety problems and challenges that may be posed as a result of these therapies, hence leaving the scientific community at a point of doubt (Sahin et al. 2014). The production of IVT mRNA is relatively simple and cost-effective than all other methods documented to date. It is also known that the product quality is uniform in nature and also, quality control is easier in this case. Since the production of synthetic mRNA (IVT mRNA) does not include any cellular or animal component, the safety issues and other risks are far lower than alternative methods (Sahin et al. 2014). However, despite having an edge over other therapeutic methods, the risks should also be seriously evaluated.

9.1 The Safety Concern Over the Use of Non-Natural Nucleotides/Nucleosides in IVT mRNA

RNAses are found within extracellular spaces in abundance. These enzymes act as control mechanisms that are responsible for regulating the levels of RNA molecules (Sahin et al. 2014). To date, there are no reports of safety concerns associated with IVT mRNA consisting of unmodified nucleotides (natural nucleotides) with respect to their absorption, metabolism, excretion profile, etc. This is because the human body breaks down and excretes out much higher amounts of mRNA daily. But the aforementioned conditions may not apply to the IVT mRNA composed of non-natural nucleotides. There is still no significant data that explains the mechanism of the breakdown and excretion of such unnatural nucleotides. Also, the toxic effects and associated risks are still unknown. Besides the mRNA, compounds resulting from the breakdown of mRNA composed of unnatural nucleotides may be toxic to the cell as evidenced from their association with unusual mitochondrial toxicities (Lewis 2003) linked with the roles of nucleoside transporters (Sahin et al. 2014). Significant clinical toxicities such as myopathy, lactic acidosis, pancreatitis, lipodystrophy were found in HIV-positive patients treated with nucleoside reverse transcriptase inhibitors. Mitochondrial dysfunction was solely attributed to the deleterious effects of such unnatural nucleoside analogs on the DNA polymerase γ, leading to its inhibition and hence, blocking mitochondrial DNA replication (Sahin et al. 2014). Hence, safety aspects should be strictly looked upon after the administration of modified mRNA with unnatural nucleotides/nucleosides. The organs under high risk should be diligently monitored after the administration of such drugs for prolonged use.

9.2 Safety Considerations Regarding the Encoded Protein

Other safety concerns are related to the nature and application of the protein encoded by the mRNA. There should be strict and diligent monitoring of the action of these IVT mRNA molecules in vivo that it only performs and executes the function for which it was designed, on a case-specific basis. Another main area of concern is dosing. The dose for a specific inject should be carefully determined and should be decided in accordance with the need for achieving the objectives of a particular therapy (Sahin et al. 2014).

10 Conclusions

Various studies evidenced that IVT mRNA-based therapeutics hold the noteworthy potential to be used in medicine. Despite some safety concerns, they are now being looked upon as agents which hold tremendous potential to revolutionize the field of regenerative medicine. Their efficacy in expressing the proteins of interest in the host cell may be of great therapeutic significance and can be extended for wide use in humans after necessary clinical trials. Treatment of many genetic disorders, viral infections, cancers can now be visualized to be one step easier with the help of synthetic mRNA molecules. The advancement in delivery systems for IVT mRNA needs more inclusive research efforts, and many such studies are already in progress. Though a significant advancement in the utility of synthetic mRNA molecules has been made, the possible challenges posed by these molecules need to be evaluated more comprehensively in clinical trials.

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Hospital-Based RNA Therapeutics

Tulsi Ram Damase, Roman Sukhovershin, Min Zhang, Daniel L. Kiss, and John P. Cooke

Contents

Abstract Hospital-based programs democratize mRNA therapeutics by facilitating the processes to translate a novel RNA idea from the bench to the clinic. Because mRNA is essentially biological software, therapeutic RNA constructs can be rapidly developed. The generation of small batches of clinical-grade mRNA to support IND applications and first-in-man clinical trials, as well as personalized mRNA therapeutics delivered at the point-of-care, is feasible at a modest scale of cGMP manufacturing. Advances in mRNA manufacturing science and innovations in mRNA biology are increasing the scope of mRNA clinical applications.

Keywords Messenger RNA · Hospital-based mRNA therapeutics · Circular mRNA · Self-amplifying mRNA · RNA-based CAR T cell · RNA-based gene-editing tools

1 Introduction

A therapeutic revolution is underway. It is now quite clear that message RNA (mRNA) therapies will become a major therapeutic armamentarium against infectious diseases, as they provide a platform for a flexible and rapid response to

T. R. Damase · R. Sukhovershin · M. Zhang · D. L. Kiss · J. P. Cooke (✉)
Center for RNA Therapeutics, Houston Methodist Hospital, Houston, TX, USA
e-mail: jpcooke@houstonmethodist.org

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_4

pandemics. However, the scope of mRNA therapeutics is much broader. Because mRNA is essentially biological software, one can rapidly write the code for any protein and thereby create vaccines against infectious diseases, cancer, or any pathogenic protein where an immunogenic response is desired. One can also generate mRNA constructs encoding proteins that are deficient, such as in the congenital absence of a metabolic enzyme. In addition, it is possible to use mRNA to enhance cell therapies, or to biologically modify scaffolds used in regenerative medicine. The excitement about mRNA therapies is reflected in the high valuations of the companies that are early movers in this field, such as Moderna, BioNTech, and Curevac, each of which also has partnerships with large pharmaceutical companies. However, the future of mRNA therapies will not be the exclusive province of big pharma. Because mRNA therapies can be rapidly generated with manufacturing processes that are less complicated than recombinant proteins and have a simpler regulatory roadmap than new chemical entities, it becomes possible for small companies and academic groups to participate in therapeutic development and clinical application. Furthermore, hospital-based programs can fill gaps in personalized therapies and rare diseases that are not addressed by large pharmaceutical programs.

2 A Role for Hospital-Based Drug Development

Development of therapeutics within academic hospitals can expedite the development of novel and personalized therapies, particularly for rare diseases, and speed their entry into clinical trials. Such programs combine unique scientific expertise in certain field or technology with the translational and clinical infrastructure available on site. Notably, hospital-based programs can bring therapeutics from bench to bedside (Damase et al. 2021) and can facilitate the development of personalized treatments for rare diseases. With respect to nucleic acid therapeutics, Nationwide Children's Hospital, St. Jude Children's Research Hospital, and Boston Children's Hospital are examples of successful hospital-based gene therapy programs.

Nationwide Children's Hospital is known for Zolgensma and Golodirsen—gene therapies that have transformed care of patients with spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), respectively. SMA patients have a defective survival motor neuron 1 (SMN1) gene. A deficiency of survival motor neuron (SMN) protein leads to the death of motor neuron cells that control muscle function, thus affecting the patient's ability to move, swallow, and breathe. Zolgensma delivers a fully functional copy of the human SMN1 gene into the target motor neuron cells through a viral vector AAV9. A single infusion of Zolgensma generates sufficient SMN protein in motor neurons to improve muscle movement and function (Caccomo 2019; Waldrop et al. 2020; Damase et al. 2021).

Patients with DMD have a mutation in the gene that encodes dystrophin. The latter protects muscle cell membranes, and its loss causes myofibril damage and muscle atrophy in early childhood, dramatically shortening life expectancy. Golodirsen is an antisense oligonucleotide that binds to DMD pre-mRNA and affects its splicing

to skip the defective exon and generate a functional dystrophin protein (Kahn 2019; Damase et al. 2021; Scaglioni et al. 2021).

In St. Jude Children's Research Hospital, a gene therapy to treat severe combined immunodeficiency (SCID-X1) was developed. SCID-X1 is caused by mutations in the gene encoding IL2RG protein on the X chromosome, which is essential for immune system functioning. Patients without this protein have very few natural killer and T cells, as well as nonfunctional B cells. To correct IL2RG deficiency, the bone marrow stem cells are obtained from the patient and IL2RG gene is delivered ex vivo with lentiviral vectors. Genetically modified cells are infused back into the patient after bone marrow ablation (Mamcarz et al. 2019; Damase et al. 2021). Eight patients were treated so far and all tolerated and responded well to this therapy.

Boston Children's Hospital has pioneered the development of personalized gene therapy for a 7 year-old girl diagnosed with a novel mutation in CLN7/MFSD8, a subtype of Batten's disease. The mutations in CLN7 gene disrupt MFSD8 protein synthesis. Although its exact function is unknown, MFSD8 is believed to be involved in transport across the lysosomal membrane. The patient presented with an insidious onset of impaired vision, ataxia, seizures, and developmental regression. The presence of characteristic lysosomal inclusions on skin biopsy suggested the diagnosis which was confirmed by genetic testing. Standard genetic testing revealed heterozygosity for a single pathogenic mutation in CLN7. Because the disease is autosomal recessive, the suspicion that there was a second mutation was confirmed by whole-genome sequencing. Specifically, an SVA retrotransposon insertion was found that resulted in a cryptic splice acceptor site (i6.SA) that caused a mis-splicing of exons 6 and 7. To prevent mis-splicing, the antisense oligonucleotide milasen targeting the i6.SA site was designed and tested initially on the patient's fibroblasts in vitro. Milasen has the same backbone and sugar chemistry modifications as nusinersen, an FDA-approved drug for SMA, which changes the splicing pattern of SMN2 to resemble SMN1. In culture, milasen boosted the normal:mutant splicing ratios by a factor of 2.5–3. Its safety was confirmed in toxicity studies, and clinical trial was initiated within 1 year after the first contact with the patient, during which a clinical improvement was noted (Kim et al. 2019; Damase et al. 2021).

At Houston Methodist Hospital in the Texas Medical Center, we have established a hospital-based program for mRNA therapeutics. mRNA-based therapeutic technology is a disruptive technology as it facilitates rapid development and clinical translation of novel solutions for previously "undruggable" diseases. It can be more rapidly deployed by academic groups and small biotech start-ups. However, most of these small entities lack the crucial infrastructure required to bring their transformative therapy from the lab bench to the clinic. They need partners with expertise in RNA manufacturing, formulation, preclinical and clinical testing. Our program at Houston Methodist Hospital provides a translational pipeline for small entities such as biotechnology start-ups or academic groups with great ideas about RNA therapeutics. Houston Methodist Hospital (HMH) made a strategic investment in its research institute to build the infrastructure necessary to support preclinical and clinical translation of novel therapeutics. The RNAcore facility, a central pillar of our program, is housed within the research institute and is managed by a team of

scientists with expertise in engineering and manufacturing mRNA constructs for the scientific community. Within past 5 years, we have generated more than 100 unique constructs for more than 40 collaborators. Funded in part by the NIH and the Cancer Prevention and Research Institute of Texas (CPRIT), the RNA Core facilitates the development of RNA therapeutics globally, with a particular interest in supporting cancer research in Texas.

Our program encompasses all essential competencies that are required for development and translation of mRNA-based drugs (Fig. 1). Our RNA biology and bioinformatics groups innovate RNA design to enhance the stability and translational efficiency of our RNA constructs. Our nanomedicine group helps in the generation

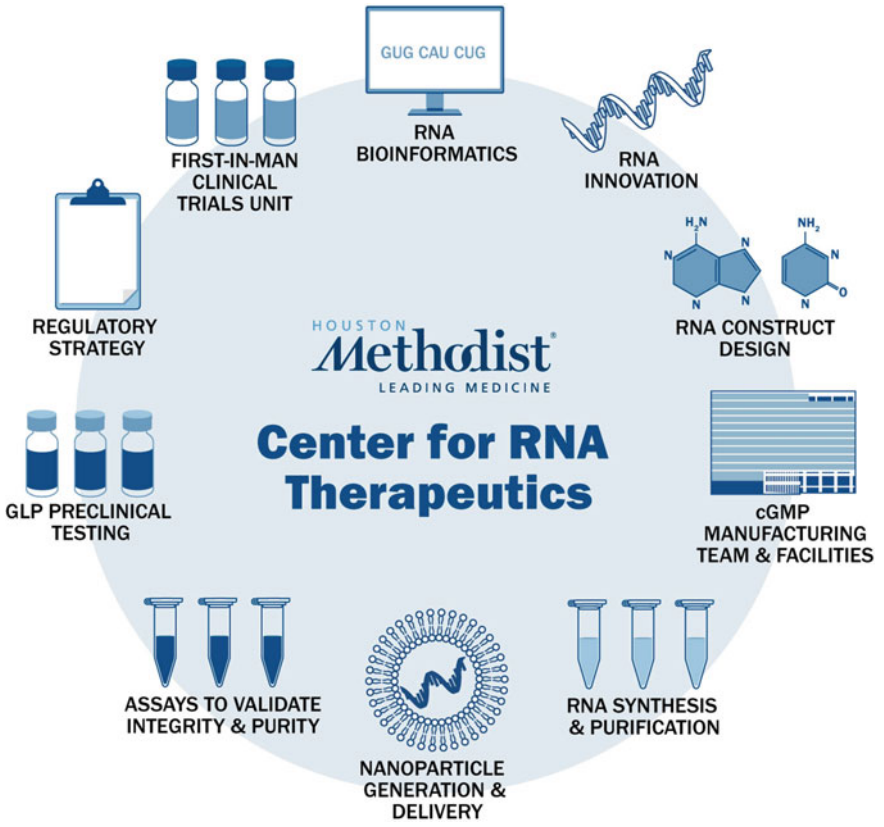

Fig. 1 Schematic of Houston Methodist Hospital center for RNA therapeutics. As typical of a hospital-based therapeutic program, we have a deep fund of knowledge in our therapeutic arena, and facilities for synthesizing clinical grade materials. We have expertise in RNA bioinformatics and innovation; with proprietary manufacturing methods for the synthesis, purification, validation, and encapsulation of mRNA; facilities and personnel for GLP preclinical studies in preparation for an IND; clean rooms and cGMP processes and personnel for generating clinical-grade mRNA and LNPS; a phase 1 unit for first-in-man clinical trials; and a large hospital system for doing later stage clinical trials

of lipid formulations and fabrication of lipid nanoparticles to deliver RNA. The Office of Translational Production and Quality (OTPQ) guarantees quality products for patients and provides in-house cGMP facilities, with clean rooms and calibrated equipment for manufacturing cGMP-grade RNA and lipid nanoparticles. The in-house quality resources lead to fast and cost-effective GLP-grade test and release of mRNA-based products for clinical use. An Office of Regulatory Affairs aids with the development of the regulatory roadmap and interactions with regulatory bodies. Our in-house Comparative Medicine Program assists with preclinical animal studies, including GLP studies for IND applications. Our Clinical Trial Center has skilled nurses and physicians that perform the first-in-man clinical trials.

Our hospital-based program can produce small batches of cGMP RNA therapeutics to support early phase clinical trials. For later stage clinical trials and commercialization, the project is carried forward by our partner VGXI Inc., based just outside of Houston. Together with VGXI we have scaled our manufacturing processes in their facility so that there is a seamless transition for mRNA drugs from small batch manufacturing for GLP preclinical studies and early phase clinical trials, to the large batch manufacturing that is required for products that are entering later stage clinical trials (Fig. 2). VGXI has prior expertise in large batch manufacturing of DNA therapies which facilitated the partnership. Currently, there is no other academic program

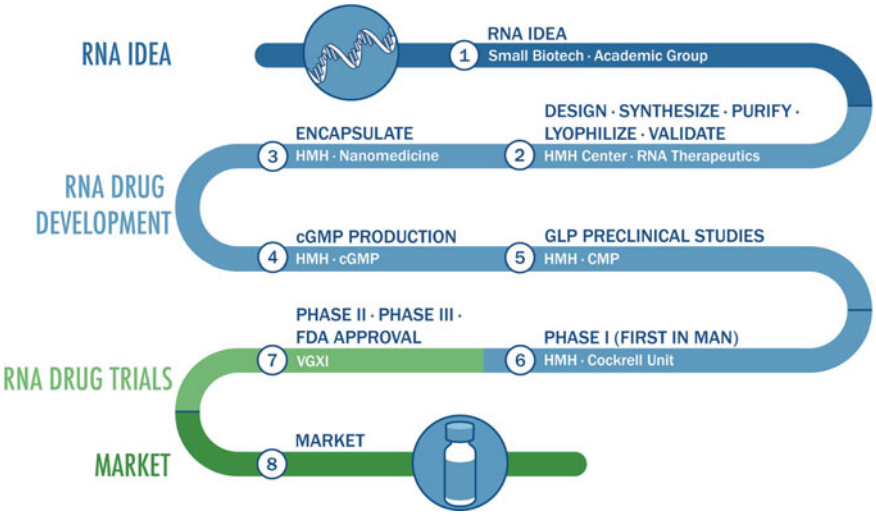

Fig. 2 Roadmap to the clinic. Our intent is to assist academic groups and small companies with a great RNA idea translate their technology to the clinic. Many small groups do not have some of the core competencies to traverse the gap from bench to bedside. We have processes and personnel to synthesize, purify, encapsulate, and validate the integrity, purity and strength of the product, as well as the facilities and experience for preclinical studies and early clinical trials. A corporate partner (VGXI Inc, Woodlands TX) uses our scaled-up processes for large batch manufacturing to support later stage clinical trials and commercialization

in the USA with comparable capabilities. However, we anticipate that the therapeutic revolution in mRNA, together with advances in manufacturing technology, and the need for personalized RNA drugs, will create the demand for such centers. These regional centers will provide a pathway for academic investigators and start-ups to translate their novel RNA therapeutics ideas from the bench to the bedside (Damase et al. 2021). Thus, the generation of small batches of clinical-grade mRNA to support IND applications and first-in-man clinical trials, as well as personalized mRNA therapeutics delivered at the point-of-care, is feasible at a modest scale of cGMP manufacturing within a hospital-based program.

3 Manufacturing of mRNA

In theory, it is possible to generate an RNA construct for any protein of interest. The production of mRNA is relatively straightforward, fast, and robust process. Moreover, a generic production workflow can be used to make broad variety of RNA constructs, as simple changes in RNA sequence will not affect the methods used in manufacturing.

mRNA for therapeutic applications is usually transcribed in vitro (IVT) from the DNA template using a bacteriophage DNA-dependent RNA polymerase (T7, T3, or SP6) and ribonucleotide triphosphates (NTPs) (Pardi et al. 2013). In a DNA template, a protein-encoding sequence should be placed downstream from the promoter sequence and flanked by 5′ and 3′ untranslated regions (UTRs). Kozak sequence significantly affects expression and should be added in front of protein-coding sequence (Kozak 1987). The poly A tail-related sequences can be included after 3′UTR (Pardi et al. 2013) to either transcribe the tail from the template directly or perform enzymatic polyadenylation after transcription (Pardi et al. 2013). If a plasmid is used as a template, then a unique restriction site should be incorporated downstream of the poly A tail-related sequence followed by T7 terminator sequence. This restriction site aids in cutting (or linearizing) the template at a specific site to ensure the polymerase terminates transcription to generate mRNA of defined length (Fig. 3). DNA templates can also be prepared by polymerase chain reaction (PCR).

To ensure RNA translation, 7-methylguanylate cap must be incorporated into mRNA either post-transcriptionally with vaccinia capping enzyme (Martin and Moss 1975) or co-transcriptionally through the addition of an anti-reverse cap analog (ARCA) or CleanCap reagent (Henderson et al. 2021). Different modified NTPs, such as pseudouridine or 5-methyluridine, may be incorporated into mRNA construct by adding them to IVT reaction mixture. After RNA is transcribed, enzyme DNase can be used to remove template DNA from the reaction mixture (Green and Sambrook 2019).

Replacing some standard RNA bases with the modified nucleosides mentioned above eliminated much of the innate immune stimulatory response that was triggered by IVT RNA. However, a low-level induction of interferons (IFNs) and inflammatory cytokines still remained (Karikó et al. 2011). These immune responses

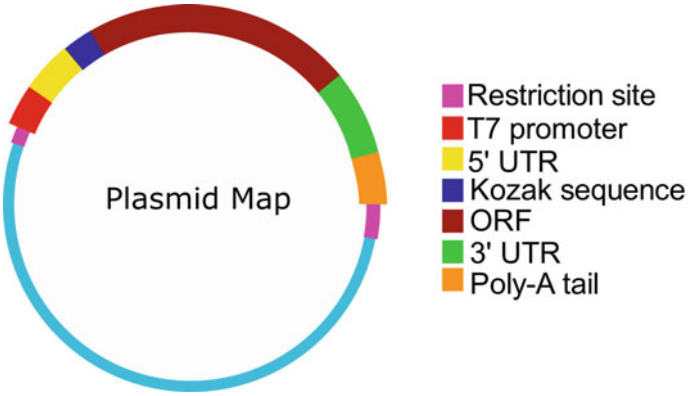

Fig. 3 Typical plasmid map. The typical DNA plasmid used in manufacturing will incorporate many restriction sites so as to easily incorporate different open reading frames encoding the protein of interest. The DNA plasmid is amplified using bacterial fermentation, purified, and linearized for the in vitro RNA transcription. 5′UTR = 5′ untranslated region. 3′UTR = 3′ untranslated region. ORF = open reading frame

were triggered by trace contaminants including double-stranded RNA (dsRNA), uncapped RNA, and short abortive RNAs which occur as byproducts of high-yield IVT reactions (Karikó et al. 2011). When combined with nucleoside modifications mentioned above, the efficient removal of these trace contaminants yields low-to-non-immunogenic IVT mmRNAs.

Purification methods have reduced the immunogenicity of IVT mmRNAs and have enabled the development of mRNA therapeutics that will be applicable to many diseases. Precipitation (Henderson et al. 2021) and liquid chromatography-based methods (Karikó et al. 2011) can be employed to purify RNA from enzymes, free NTPs, residual DNA, truncated RNA fragments, and double-stranded RNA. At small scales, preparative polyacrylamide gel electrophoresis is a powerful and commonly used tool to separate the desired RNA products from abortive products or other impurities (Green and Sambrook 2021). However, this method is only suitable for short RNA oligonucleotides or small coding or non-coding RNAs (up to a limit of ~ 500 nucleotides) (Summer et al. 2009). Further, as gel purification methods are both inefficient and are not scalable to large production lots, other purification alternatives were needed for mmRNA therapies. Fast-performance liquid chromatography (FPLC) is reported to efficiently remove small abortive RNAs but is limited in its ability to distinguish RNA molecules with similar size (McKenna et al. 2007). High-performance liquid chromatography (HPLC) has also been identified as an easily performed and scalable method that removes multiple contaminants from IVT mRNAs (Karikó et al. 2011). For larger scale manufacturing, methods that avoid the use of flammable organic solvents are preferred. These methods may include sorbent-based or nanochanneled monolith affinity chromatography, ion exchange and size exclusion, reversed phase chromatography, or reversed phase ion-paired chromatography. Purified RNA products must be stored frozen (preferably at − 80 °C),

unless lyophilized (Crommelin et al. 2021). It is critical to maintain an RNase-free environment while manufacturing RNA.

Quality control (QC) is an essential component of RNA manufacturing processes. The following quality attributes are commonly assessed in finished RNA products: identity (sequencing), concentration (UV absorbance), integrity (electrophoresis; RT-PCR), capping efficiency (LC–MS and others), residual protein (colorimetric methods), residual DNA (qPCR), double-stranded RNA (blotting, ELISA), residual solvents/buffer components (if used for purification; methods vary).

4 Innovations in mRNA Therapeutics

A greater understanding of RNA biology contributed to the emergence of RNA therapeutics. One of the major obstacles to the field was that synthetic RNA molecules triggered robust immune responses when introduced into mammalian systems (Alexopoulou et al. 2001; Damase et al. 2021). These immune responses thwarted the development of mRNA-based therapies, especially protein replacement strategies for many years (Karikó et al. 2005; Damase et al. 2021). A seminal insight that advanced the field was the finding that in vitro transcribed (IVT) mRNA activated innate immune signaling through toll-like receptor signaling (TLR3/7/8), retinoic acid-inducible gene I protein (RIG-1), or melanoma differentiation-associated protein 5 (MDA5) (Alexopoulou et al. 2001; Heil et al. 2004; Yoneyama et al. 2004, 2005). Subsequently, the development of more efficient capping methods, the incorporation of modified nucleosides, as well as specialized purification protocols, were found to substantively reduce the high immunogenicity of IVT mRNAs (Karikó et al. 2005; Damase et al. 2021).

4.1 Reducing Immunogenicity

Different modified RNA bases have been assessed for their potential to reduce the immunostimulatory side effects inherent within IVT mRNA. Previous studies reported that modified nucleosides including pseudouridine (Ψ), 5-methylcytidine (m5C), 5-methyluridine (m5U), N6-methyladenosine (m6A), 2-thiouridine (s2U), or N1-methylpseudouridine (m1Ψ) eliminate the majority of TLR stimulation (Karikó et al. 2005). Hartmann et al. also demonstrated that mRNAs incorporating both Ψ and s2U also suppressed or evaded RIG-1 signaling (Hornung et al. 2006).

Of these modifications, Ψ and m5C are the most commonly used to generate modified messenger RNA (mmRNA). The breakthrough experiments were performed by Kariko and Weissman and showed that incorporation of Ψ not only reduces recognition by innate immune sensors but it also increases the translational capacity of the mmRNA (Karikó et al. 2008, 2011). Recently, the m1Ψ substitution has been shown to outperform other modified nucleosides via a more efficient immune response

escape combined with a further increase in translational output (Parr et al. 2020). Consequently, Pfizer and Moderna replaced 100% of the uridine residues in their COVID-19 mRNA vaccines (Pfizer/BioNTech (BNT162b2) and Moderna's mRNA-1273) with m1Ψ to improve the performance of their vaccines (Jackson et al. 2020; Polack et al. 2020; Corbett et al. 2021). A combination of s2U and m5C was also found to reduce the immune signaling through abrogating mRNA interaction with TLRs and RIG-1 (Hornung et al. 2006). mmRNA therapeutic candidates containing this combination of modified bases have been employed to restore the expression of a deficient gene in a mouse model (Kormann et al. 2011).

4.2 Cell-Specific/Tissue-Specific Delivery and Translation

A current challenge in RNA therapeutics is to attain cell-specific or tissue-specific delivery and translation. Almost certainly, just as a combination of approaches was needed to effectively reduce the inflammatory properties of IVT mRNAs, a combination of approaches may address the need for tissue-specific expression.

Currently, lipid nanoparticles (LNPs) are most widely used as a delivery vehicle for mRNA. Indeed, the two mRNA vaccines for SARS-CoV-2 that are currently approved for use are each encapsulated in LNPs. The LNPs stabilize the mRNA by reducing its interaction with water, or with RNAases that are ubiquitous in the environment. As a vaccine, the mRNA LNP is simply injected into the deltoid muscle (as with the SARS-CoV-2 vaccine) or subcutaneous tissue, where it can be taken up by resident antigen-presenting cells to initiate an immune response. However, for delivery to other organs, the situation is complicated by the fact that LNPs circulating in the blood are avidly taken up by the reticuloendothelial system, i.e., the liver and the spleen. For this reason, hepatic diseases are an excellent target for current mRNA therapies. Other organs are more difficult to target. Approaching is to modify the LNPs with tissue-specific "addresses" such as antibodies directed against a tissue-specific antigen (Cheng et al. 2020; Molinaro et al. 2020). In a related approach, biomimetic particles that incorporate membrane proteins from leukocytes have been shown to target inflamed tissues (Cheng et al. 2020; Molinaro et al. 2020). These so-called leukosomes accumulate at sites of inflammation, where the endothelium is activated to express ligands that bind leukocyte counter-ligands that have been incorporated into the LNPs. Leukosomes have been shown to deliver the immunosuppressant rapamycin to the atheromatous aorta of hypercholesterolemic mice, to reduce the proliferation of macrophages in the vessel wall (Christian et al. 2020).

Another strategy to achieve cell-specific translation is by designing an mRNA construct so that it is preferentially expressed in a tissue. For example, one can include tissue-specific microRNA (miRNA) recognition elements in the mRNA drug. It has been showed that, by incorporating into the 3′ UTR a cardiomyocyte-specific recognition element, miRNA-induced degradation of their RNA construct in cardiomyocytes provided for preferential expression in non-cardiomyocytes (Magadum et al. 2020).

Hewitt et al. also included a miR-122 binding site into the 3′UTR of their therapeutic IL-12 mRNA to prevent the IL-12 protein production in hepatocytes without impairing expression in cancer cells (Hewitt et al. 2020). Importantly, the inclusion of miRNA binding sites is a negative expression strategy. Simply, this means that the IVT mRNAs are actively degraded in off-target tissues (those with high levels of the miRNA whose sites have been added to the 3′UTR of the IVT RNA). While early studies show that this strategy can be effective in achieving tissue-specific expression, it is limited by the miRNA profiles of targeted tissues.

Trigger RNAs (trRNA) represent another early stage innovation that could be developed further to introduce more selectivity and aid with the targeting of mRNA therapies (Green et al. 2014; Zhao et al. 2021). Simply, trRNAs are trans-acting RNA sequences that recognize and de-repress the translation blockade caused by adding a toehold regulatory RNA sequence prior to the coding sequence of an mRNA (Green et al. 2014; Zhao et al. 2021). Currently designed trRNA sequences work in concert with IRES sequences, so it remains to be seen if trRNAs can effectively regulate open reading frames driven by cap-dependent translation (Zhao et al. 2021). Furthermore, current designs call for this system to work in trans where the presence of two exogenous RNAs in the same cell would allow the expression of the protein of interest (Green et al. 2014; Hanewich-Hollatz et al. 2019). As independent delivery of the trRNA and its target are required for the regulation to be fully effective, two different RNAs would be required to target the same cells to confer the desired regulation of translation.

4.3 Increasing mRNA Stability and Expression

The comparatively short lifespan of mRNA and mmRNA in cells remains a limiting factor for the utility of mRNA therapeutics. Limited mRNA lifespans may require high doses of mRNA (which increases the risk of adverse effects, as well as the cost of goods) or a repeated dosing schedule (increasing the expense of the therapy) required for clinical effectiveness. Such regimens are often incompatible with long-term treatment strategies required for many clinical indications.

One approach to increase mRNA longevity derives from the mechanisms by which Zika virus exoribonuclease-resistant RNAs (xrRNAs) function in infected cells (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). Simply, xrRNA sequences fold into a stable pseudoknot structure that confers considerable exoribonuclease-resistance to the RNAs bearing them (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). One may surmise how the addition of xrRNA sequences to the 5'UTR could improve the lifespan of a therapeutic mRNA in vivo. However, the ability of cells to robustly translate mRNAs containing xrRNA sequences in their 5′ UTRs remains unknown, although their presence exclusively in viral 3′ UTRs hints that they could inhibit traditional cap-dependent translation mechanisms (Pijlman et al. 2008; MacFadden et al. 2018; Zhao and Woodside 2021). Further, since xrRNA sequences confer their functions by forming either tight

secondary structures and modified RNA bases often disrupt RNA base-pairing, the compatibility of xrRNA sequences with mmRNA therapeutics remains to be seen (Wesselhoeft et al. 2018; Mauger et al. 2019).

A very promising approach is to circularize the mRNA (circRNA) so that it is resistant to degradation. The promise of using circRNAs with an incorporated internal ribosome entry site (IRES) as a durable protein expression system has been known for decades (Collett and Faras 1976; Puttaraju and Been 1992; Ford and Ares 1994; Beaudry and Perreault 1995). The increased stability of circRNAs, also known as endless RNA, is mostly conferred by their circular structure. Simply, most cellular RNases attack and degrade RNAs by recognizing an unprotected 5′ or 3′ end (Yang 2011). Since circRNAs are continuous, they lack traditional RNA ends and evade most mechanisms that would degrade them resulting in an increased lifespan (compared to linear mRNAs) in vivo. Furthermore, with this increased RNA lifespan, a protein encoded by an IRES-initiated coding region within a circRNA will have a sustained expression pattern compared to a traditional mRNA. The biggest recent advance was made by the Anderson lab when they devised an efficient method to generate circRNAs of sufficient size to hold the open reading frame of a desired protein (Wesselhoeft et al. 2018, 2019). Their convincing findings show prolonged expression of different proteins from circRNAs in vivo (Wesselhoeft et al. 2018, 2019). Importantly, their method also succeeded in generating large circRNAs, including one encoding Cas9, which was over 4.9 kilobases (Wesselhoeft et al. 2018). Further, their subsequent work showed that their circRNAs were translated in vivo (Wesselhoeft et al. 2019). Indeed, several companies have capitalized on these properties and are developing different circRNA therapies.

Another approach lies in self- and trans-amplifying RNAs. In vivo replicating RNA technologies have been under development for many years (Geall et al. 2012; Bogers et al. 2015). These technologies consist of two components that can be contained either on a single or two RNAs. The first component is an open reading frame for the RNA-dependent RNA polymerase (RdRp) machinery of a virus, usually either Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEEV), or a chimera of VEEV and Sindbis virus replicons (VEE-SINV) (Blakney et al. 2021). Once those proteins are circulating in a cell, they can recognize the second component, which incorporates an RNA sequence encoding the open reading frame of the desired protein (Geall et al. 2012; Bogers et al. 2015).

Currently, two main types of replicating RNAs are being tested (Blakney et al. 2021; Bloom et al. 2021). First, self-amplifying RNAs (saRNA) are most similar to their parental viral sequences and have been under development for about a decade (Geall et al. 2012). As their name implies, saRNAs are single RNA transcripts that encode the viral RdRp machinery, RNA regulatory sequences that recruit the RdRp, and the open reading frame for the desired protein in a single RNA (Blakney et al. 2021; Bloom et al. 2021). Since saRNAs can contain five open reading frames, they are much longer than a traditional therapeutic mRNAs. As they are often exceed 10 kilobases, the large size of saRNAs does pose further challenges with manufacturing and packaging, but they have proven effective in limited studies (Geall et al. 2012; Bogers et al. 2015).

Trans-amplifying RNAs (taRNAs) are the second class of replicating RNA therapies in development (Blakney et al. 2021; Bloom et al. 2021). taRNAs work almost identically to saRNAs, but they are comprised of two distinct RNA strands that can be co-packaged into one delivery vehicle. Conceptually, and in practice, this change makes taRNAs a much more versatile platform technology with certain inherent advantages. First, with current technologies, manufacturing two ~ 5–6 kilobases RNAs is much simpler than efficiently manufacturing a single 10–12 kilobases RNA. Second, the modular design of taRNAs also offers manufacturers the ability to manufacture large lots of a single replicase RNA while allowing them to pair with different amplified cargoes. This modularity could allow manufacturers to bypass cost- and time-intensive optimization experiments required to optimize the conditions with individual saRNAs.

5 Applications of RNA Therapeutics

In 1990, it was demonstrated that naked exogenous mRNA injected into murine skeletal muscle can translate into a protein at the injection site (Wolff et al. 1990). In 1992, proof-of-principal for using mRNA as a therapeutic has provided. In this case, intracranial injection of mRNA encoding vasopressin in a rodent model of diabetes insipidus temporally reversed the disease (Jirikowski et al. 1992). In 1995, the possibility therapeutic utility of vaccination with mRNA encoding a carcinoembryonic antigen was demonstrated (Conry et al. 1995).

The vaccines against SARS-CoV-2 became the first RNA-based drugs approved by FDA with emergency use authorization in 2020 (Biointech/Pfizer's BNT162b2; and Moderna's mRNA-1273) followed by full approval in 2021. Both vaccines encode membrane-anchored full-length spike protein of the viral capsid. Two proline mutations were introduced into the sequence to increase protein stability. The spike RNA is encapsulated into lipid nanoparticles and delivered intramuscularly in two doses several weeks apart. Data obtained in phase III trials suggest that these vaccines are safe and effective: 95% efficacy for BNT162b2 and 94.1% efficacy for mRNA-1273 for the current SARS-CoV-2 strains (Polack et al. 2020; Baden et al. 2021) (at the time of this writing, the efficacy of the vaccines against the Omicron variant is unknown). It is worth noting that these vaccines were developed with unprecedented speed—for instance, mRNA-1273 was generated and administered to the first study participant just 63 days after the SARS-CoV-2 genome sequence was published (Moderna 2020). Currently, there are many preventive mRNA vaccines under development, including those aimed at protecting from rabies (Aldrich et al. 2021), cytomegalovirus infection (ModernaTX Inc. 2021a), HIV (International AIDS Vaccine Initiative 2020), Zika virus (ModernaTX, Inc. 2021b), influenza virus (Bahl et al. 2017), and others. In addition to a rapid development time, RNA vaccines have some other advantageous characteristics, such as being able to deliver an encoded antigen in an HLA-independent manner and to act as natural adjuvants due to their TLR7/8 ligand activity (Grunwitz et al. 2019).

RNA technology can also be used to make therapeutic cancer vaccines. In this case, RNA encodes tumor neoantigens, shared antigens or combination of both. For instance, CV9202 is a vaccine encoding six shared antigens commonly found in non-small cell lung cancer (Papachristofilou et al. 2019). mRNA-5671 is a vaccine against KRAS-positive cancers, such as pancreatic or colon cancer (Merck Sharp & Dohme Corp. 2020). The vaccine against HPV16 is another promising example of therapeutic RNA vaccines—it encodes E6 and E7 oncoproteins of HPV and is administered systemically to induce potent and durable $CD8^+$ T cell response (Grunwitz et al. 2019). Of note, the encoded antigens in this vaccine are fused to the MHC class I signal sequence and transmembrane and cytoplasmic domains for routing to the endoplasmic reticulum, resulting in increased presentation efficacy of MHC class I and II epitopes (Kreiter et al. 2008). Such optimization of vaccine design reflects the flexibility of RNA technology and is often implemented to increase antitumor efficacy. A vaccine encoding four shared melanoma-associated antigens, for instance, was enhanced by incorporating a RIG-I immunostimulatory sequence (Heidegger et al. 2019). Examples of personalized vaccines include mRNA-4157 and BNT122, which constructs neoantigens identified through genetic sequencing and bioinformatic analysis of a patient's tumor (van Dülmen and Rentmeister 2020; Jou et al. 2021).

Recently, the proof-of-concept for using RNA as tolerogenic vaccine was provided. In contrast to a vaccine augmenting immune responses against a pathogen, a tolerogenic vaccine is aimed at inducing tolerogenic responses to suppress or impair immunity against antigens, which in most cases are auto-antigens or allergens (Geng et al. 2015). Such vaccines may help in managing autoimmune diseases. Indeed, an RNA encoding a multiple sclerosis-related antigen suppressed the progression of this autoimmune disease in several mouse models. The treatment effect was associated with a reduction of effector T cells and the development of regulatory T cell populations, which executed strong bystander immunosuppression and thus improved disease induced by cognate and noncognate auto-antigens (Krienke et al. 2021).

RNA can also be used to enhance dendritic cell (DC)-mediated immunotherapy for cancer. In this approach, mRNA is delivered into patient's DCs ex vivo or in vivo to induce their maturation and enhance their ability to stimulate T cells. For example, TriMix is a cocktail of RNA encoding CD40L, CD70, and constitutively active toll-like-receptor 4 (caTLR4) that is transfected into DCs ex vivo. CD40L and caTLR4 promote maturation of cytokine-secreting DCs, whereas CD70 enhances T cell proliferation. When TriMix cocktail was co-transfected with melanoma antigens and co-cultured with autologous naïve CD8 + T cells, a 500-fold increase in antigen-specific CD8 + T cells was observed when compared with immature DCs, and a 200-fold increase when compared with cytokine cocktail-matured DCs (Bonehill et al. 2008). TriMix-based therapeutic vaccine, TriMixDC-MEL, in combination with ipilimumab, a cytotoxic T-lymphocyte-associated protein 4 blocker, induced an encouraging rate of highly durable tumor responses in patients with advanced melanoma (Wilgenhof et al. 2016).

RNA encoding chimeric antigen receptors (CAR) for T cells is another promising avenue for RNA technology. CAR can be designed to recognize any desired antigen on the cell surface so that cytotoxic T cell expressing the CAR will detect and destroy other cells bearing that antigen. Usually, T cells are expanded and transfected with CAR-encoding RNA ex vivo, then infused back into the patient (Krug et al. 2014; Foster et al. 2019). Descartes-08 is an RNA-based CAR T cell therapy to treat myasthenia gravis. In this autoimmune disease, pathogenic autoantibodies produced by plasma cells bind to and destroy neuromuscular synapses resulting in muscle weakness and rapid fatigue. Plasma cell clones producing autoantibodies also express B cell maturation antigen (BCMA). Descartes-08 can recognize and eliminate BCMA-expressing cells and is now in Phase 1/2 clinical trials (Cartesian Therapeutics 2021a; Cartesian). Similarly, Descartes-11 eliminates BCMA-expressing myeloma cells and is in development to treat newly diagnosed patients with high-risk multiple myeloma (Cartesian Therapeutics 2021b; Cartesian 2021). Recently, it has been demonstrated that CAR RNA can be delivered into T cells in vivo with lipid nanoparticles carrying anti-CD3 antibodies. Such particles accumulated in the organs enriched in T cells, such as spleen, thymus, and lymph nodes, and, when loaded with CAR RNA, could reprogram host T cells to recognize and eliminate leukemia cells and prostate cancer cells in animal models, prolonging survival significantly (Parayath et al. 2020).

RNA is also an attractive technology for genome editing as it can ensure transient expression of genome editing tools. Several preclinical and clinical studies using RNA encoding zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated protein 9 (Cas9) are ongoing (Zhang et al. 2019). For example, SB-728mR-T encodes ZFN targeting chemokine receptor (CCR5) gene. The latter is a surface protein present on the white blood cells which serves as a co-receptor for HIV virus and certain mutations of this receptor grant natural resistance to the virus. SB-728mR-T is designed to delete 32 bp in CCR5 gene making the recipient resistant to HIV. In currently ongoing clinical study, the drug is electroporated ex vivo into autologous hematopoietic stem/progenitor cells of HIV-1 infected patients preconditioned with busulfan (DiGiusto et al. 2016; City of Hope Medical Center 2020). Another RNA-based gene-editing drug, NTLA-2001, is targeted against transthyretin amyloidosis. This RNA construct encodes a CRISPR-Cas9 system, encapsulated into nanoparticles formulated to achieve preferential delivery to the liver. NTLA-2001 knocks out the hepatic TTR gene responsible for the production of misfolded transthyretin protein. A single dose of NTLA-2001 edits more than 70% of the genome in the liver of non-human primates and reduces serum transthyretin concentration more than 80% in patients with amyloidosis (Intellia Therapeutics 2020; Gillmore et al. 2021). Another application is the use of an mRNA CRISPR-Cas9 system, delivered in lipid nanoparticles, to knock out hepatic PCSK9 gene and thereby reduce LDL cholesterol levels in non-human primates. PCSK9 is a negative regulator of LDL receptors (LDLR) which bind to and clear LDL cholesterol from circulation (Musunuru et al. 2021). Both LDLR and PCSK9 are highly expressed in the liver, thereby making this organ the main target for RNA-based gene therapy of hypercholesterolemia.

Since RNA can encode any protein, the number of therapeutic applications for RNA technology is indeed countless and examples above represent only a small portion of RNA-based therapeutics under development (Damase et al. 2021). Gene replacement therapy with RNA can reverse a deficiency of key metabolic enzymes causing a disease process. For instance, ARCT-810, now in early clinical trials, is designed to supply a functional transcript of ornithine transcarbamylase (OTC) (Arcturus Therapeutics, Inc. 2020, 2021). This enzyme helps to eliminate ammonia through urea cycle in the liver. Genetic defects causing a deficiency of OTC result in hyperammonemia and subsequent neurological derangements. Promising preclinical data have also been reported for RNA-based replacement therapy in the models of α-1-antitrypsin deficiency (Connolly et al. 2018), citrin deficiency (Cao et al. 2019), lysosomal storage diseases (Zhu et al. 2019), and glycogen storage diseases (Cao et al. 2021).

In addition, RNA-enhanced cell therapies and bioengineering are on the horizon. As one example, we have shown that mRNA encoding human telomerase can extend telomeres in senescent human cells, increase replicative capacity, reverse the senescence-associated secretory phenotype, reduce DNA damage, and restore normal cellular functions (Ramunas et al. 2015; Li et al. 2017, 2019; Mojiri et al. 2021). This promising work has stimulated preclinical studies to assess the benefit of this mRNA therapy to enhance an FDA-approved cell therapy product for burn patients.

6 Conclusion and Future Perspective

The future is bright for the field of mRNA therapeutics. Current mRNA technology provides a platform to rapidly develop and deploy vaccines against infectious diseases; to personalize therapies for cancer and genetic diseases; to generate therapeutic proteins to address previously "undruggable" targets; to address the major illnesses that afflict modern society including cardiovascular and neurological disorders. Our hospital-based program is designed to help small companies and academic groups with a great RNA idea get to the clinic. Such programs will increase the number and the diversity of RNA solutions for disease. In addition, hospital-based programs will address current gaps in medical care for orphan diseases and will provide personalized RNA therapies for their patients. Advances in tissue targeting and in the stability and translational efficiency of mRNA will increase its application for a broad range of diseases. The opportunities for RNA therapeutics are almost limitless, as we stand on the threshold of a brave new world.

Acknowledgements This work was supported in part by funding from the George J. and Angelina P. Kostas Charitable Foundation, and grants to JPC from the National Institutes of Health (NIH R01 HL133254; R01 HL148338 and R01 HL157790); and from the Cancer Prevention and Research Institute of Texas (CPRIT RP200619). DLK and MZ were supported by NIH R35 GM137819 to DLK. Our gratitude to Rachael Whitehead for generating Figs. 1 and 2.

Disclosures Dr. Cooke is an inventor of patents, assigned to Stanford University and licensed to Cooke's company, which protects the use of mRNA telomerase for cellular rejuvenation. Dr. Sukhovershin has filed invention disclosures with Houston Methodist Hospital regarding the manufacturing and testing of mRNA constructs, which intellectual property has been licensed to VGXI Inc.

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Medical Use of mRNA-Based Directed Gene Delivery

A. C. Matin and Alexis Forterre

Contents

Abstract Systemically administered targeted gene therapy can benefit many diseases. This chapter focuses on mRNA-mediated gene delivery. We discuss why mRNA is superior to DNA for this purpose, especially for treating diseases like cancer, where it is necessary to kill also quiescent cells; and measures to increase mRNA stability. As vectors for directed mRNA delivery, lipid nanoparticles (LNPs) have many advantages, such as the ease of large-scale production and delivery of large molecules. Approaches to make them targeted include manipulating their chemical composition and charge. An example of the latter is successful CRISPR/Cas-mediated PTEN editing in targeted organs. LNP lipids can be immunogenic and toxic, but measures are being pursued to counter this. More recently, the extracellular vesicles (EVs, also called exosomes), "nature's antigen delivery system," have attracted much attention for being biocompatible and likely to be nonantigenic. While many small RNAs have been targeted using EVs, their loading

A. C. Matin (✉)
Department of Microbiology and Immunology, Stanford University School of Medicine, Fairchild Building, 299 Campus Drive, Stanford, CA 94305, USA
e-mail: a.matin@stanford.edu

A. Forterre
EVORA Biosciences, 24 Rue du Faubourg Saint Jacques, 75014 Paris, France
e-mail: aforterre@evorabio.com

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_5

with mRNA has only recently been accomplished; this has necessitated the use of special plasmids, although it has also now been done more directly. mRNA-loaded EVs displaying targeting peptides have, in preclinical studies, successfully treated implanted tumors in mice without side effects. The attachment of targeting peptides to EVs has been accomplished using, for example, the C1C2 domain of lactadherin, which not only binds tightly to EV lipid membranes but also, by masking their surface phosphatidylserine, increases their circulation time. Advantages of LNPs as vectors include the ease of their large-scale production and capacity to deliver large molecules; those of EVs are their biocompatibility, and relative non-toxicity and immunogenicity.

Keywords LNPs · Extracellular vesicles · Exosomes · Systemic administration · Targeted gene delivery · mRNA · Cancer · Gene replacement · Gene repair · Gene silencing

Abbreviations

18PA	1,2-Dioleoyl-sn-glycero-3-phosphate
ARCAs	Anti-reverse cap analogs
ApoE	Apolipoprotein E
APEs	Ionizable amino-polyesters
CNOB	6-Chloro-9-nitro-5-oxo-5H-benzo[a]phenoxazine
CB1954/tretazicar	5-(Aziridine-1-yl)-2,4-dinitrobenzamide
DC	Dendritic cells
DSB	Double-stranded break
EVs	Extracellular membrane vesicles
GDEPT	Gene-directed enzyme prodrug therapy
HEK293	Human kidney embryo 293 cells
IVT	In vitro Synthesized mRNA
IRES	Internal ribosome entry site
ITG	Integrin
LDL	Low-density lipoprotein
let7a	Let-7ainordertodeliverlet-7a
LNP	Lipid nanoparticles
m7G	Me-m7GpppG
MCHB	9-Amino-6-chloro-5H-benzo[a]phenoxazine-5-one
MPNQ	5-(Aziridine-1-yl)–2,4-N-acetoxy-2-nitrobenzamide
NTPs	Nucleotide triphosphates
PEG	Polyethylene glycol
PS	Phosphatidylserine
ROP	Ring-opening polymerization
SORT	Selective organ targeting

TTR	Transthyretin protein
UTR	Untranslated region

1 Introduction

It is widely recognized that the treatment of many diseases can greatly benefit by targeted delivery of a gene(s) specifically to the intended site. Examples include diseases resulting from inherited defective genes or those altered by unfavorable mutations after birth. Treatment of diseases, including cancer, can be made more effective by this approach. One example is treatment involving prodrugs. Prodrugs are innocuous in their native state but can be converted to highly toxic drugs by a bacterial or viral enzyme. If the delivery of the gene encoding the converting enzyme is confined to the cancer, the drug toxicity would be restricted to the tumor, rendering the drug effective at low doses and obviating the severe side effects that accompany conventional non-directed chemotherapy (Rautio et al. 2008; Thorne et al. 2009).

Gene delivery has generally focused on DNA**,** but its delivery via mRNA has many advantages and marked progress in mRNA-based gene delivery has recently been made. Examples are the mRNA vaccines for immunization against cancer and—currently of great relevance—against the SARS Cov2 virus.

This chapter will focus on disease treatment by *mRNA-based directed gene delivery following systemic injection* of the gene-carrying vehicle. Although lipid nanoparticles (LNPs) have been discussed in other chapters of this book, we will mention some examples of such directed therapy also with LNPs.

Recently, much interest has focused on the use of extracellular membrane vesicles (EVs; also called exosomes) for directed mRNA-based gene delivery (Jayasinghe et al. 2021; Forterre et al. 2020; Wang et al., 2018); this will be discussed in greater detail.

2 Gene Delivery with mRNA Versus DNA

2.1 The Comparison

But first, let us address the question of why use mRNA for gene delivery? As opposed to DNA, mRNA does not pose the danger of insertion into the host genome, which can result in harmful outcomes. Further, to be effective in generating the desired protein, DNA needs to be transported into the nucleus for transcription, while the mRNA can be translated right upon entry into the cytosol. DNA transport from the cytosol to the nucleus mainly occurs during mitosis and is inefficient, particularly in non-growing cells. Also, mRNA can be produced at a large scale by in vitro transcription (IVT) in a cell-free environment that dispenses microbes and cultured cells—it requires,

besides RNA polymerase, only the template DNA and the trinucleotides (TNPs); DNase treatment following manufacture eliminates the DNA template providing the mRNA in a pristine state.

But the DNA upon nuclear entry can generate several copies of mRNA. How do then the two nucleic acids compare in gene delivery? We focus here on non-growing cells—they are important, for instance, in cancer. Cancer cells grow rapidly, many becoming distant from the tumor blood vessels, and the resulting nutrient deprivation ushers them into the G0, non-growing, quiescent phase (Shibamoto and Streffer 1991). This phase is reversible and leads to cancer resurgence, which can be metastatic (Dudjak 1992): effective cancer treatment clearly must kill also non-growing cells.

Estimates of the proportion of quiescent cells in several cancers have been made; this includes the very serious ones, like melanoma and O771 adenocarcinoma. In one study, cytochalasin B was employed, which blocks cytoplasmic but not nuclear division. Scoring of multinucleated cells and the total number of nuclei and cells permitted estimation of non-growing cells: It showed that up to 67% of tumor cells can be quiescent (Shibamoto and Streffer 1991). So, how effective is mRNA versus DNA-based gene delivery in non-growing cells?

In primary neuronal cortical cells, which do not grow, luciferase reporter gene transfection was examined. mRNA-mediated transfection resulted in luciferase expression within 1 h, peaking at 5–7, and ending at 12 h. With DNA, no expression was seen until 7 h, but peak expression, which occurred at 36–48 h, was an order of magnitude greater (Zou et al. 2010). But if gene delivery is intended to kill the recipient cells, the delayed higher expression with DNA is irrelevant, as dead cells would have no expression. The results imply that mRNA would be better for killing non-growing cells by gene delivery.

Subsequent findings validate this. Bax gene delivery in malignant melanoma cells was examined (Okumura et al. 2008). Bax protein promotes apoptosis and cell killing. When liposome-Bax mRNA formulations were used for gene delivery to mice with this implanted cancer, Bax production occurred at 12 h; in contrast, with liposome Bax-DNA delivery, there was only a minor increase in Bax protein even after 24 h. Greater TUNEL-positive cells resulted with mRNA-compared to DNA-NLPs, and the apoptotic index (indicating the proportion of apoptotic cells) was 4.6-fold higher with mRNA. The tumor growth slowed significantly between 20 and 30 days with mRNA; with DNA-NLPs, only minor growth inhibition occurred at day 20 with no further mitigation. A similar finding has been made in prodrug cancer therapy, as is discussed below. *Thus, mRNA is superior, especially when treatment requires killing also of non-growing cells.*

2.2 Improving IVT mRNA for Clinical Use

This involves increasing mRNA stability and minimizing its antigenicity and toxicity.

2.2.1 Stability

mRNA can be highly unstable. Approaches to increase stability and expression include manipulation of mRNA five-prime cap (5′ Cap), its nucleotides, and its Poly(A) tail. The 5′ cap in eukaryotic RNAs is an altered nucleotide [7-methylguanosine (m7G)] at the 5′ end. It protects against mRNA degradation, promotes its translation, and mitigates its immunogenicity. Thus, for IVT mRNA synthesis, the DNA templates used incorporate anti-reverse cap analogs (ARCAs) along with NTPs and RNA polymerase. Examples of the ARCAs are 3′-O-Me-m7GpppG (Sahin et al. 2014) and the modified cap analogues, phosphorothiolate and imidodiphosphate; the latter two also render mRNA resistant to decapping enzymes, enhancing its stability (Wojtczak et al. 2018).

As regards the mRNA 5′- and 3′-untranslated regions (UTRs) and nucleotides, incorporation of β-globin and/or the TEV start site in the UTRs (Russell and Liebhaber 1996; Adibzadeh et al. 2019), and substituting uridine by pseudouridine and/or cytidine by 5-methylcytidine promote stabilization and enhance translation (Khan et al. 2009; Gallie 2001; Steinle et al. 2017). Reversible addition–fragmentation chain transfer (RAFT) polymerization is another promising approach: triblock copolymers are used to mediate mRNA condensation enhancing stability, biocompatibility, and cytosolic entry (Cheng et al. 2012).

Increasing the IVT mRNA in the producer cells by enhancing its entry in them can boost mRNA content in the EVs generated by the cells. Complexing the mRNA with lipofectin and using a nonlipid cationic reagent such as TransMessenger can accomplish this (Weissman et al. 2000).

It is thought that increased length of the polyA tail of mRNA enhances translation. However, many mRNAs that are efficiently translated have short tails, indicating that the optimal length may be transcript specific. This may thus need to be determined for a given mRNA and the required tail length incorporated in the DNA template (Holtkamp et al. 2006).

Self-amplifying circular RNA (circRNA) holds great promise. It lacks the free ends that the nucleases for mRNA degradation utilize and therefore has a longer half-life than its linear counterpart (Wesselhoeft et al. 2019). Linear mRNA encodes only the therapeutic protein but circRNA encodes also proteins which enable mRNA replication (Vogel et al. 2018). An internal ribosome entry site (IRES) allows translation, so high levels and prolonged protein synthesis can result (Daijogo and Semle 2011).

2.2.2 Immunogenicity

Although, as mentioned above, the 5′cap m7G minimizes mRNA immunogenicity, it does not eliminate it. RNAs can interact with RNA sensors in humans such as the toll-like receptors, RIG-I, and PKR (Yu and Levine 2011). Danger signals are activated by this, which interfere with mRNA translation. mRNA can also activate type I interferons and proinflammatory cytokine production (Freund et al. 2019;

Dammes and Peer 2020). Some of the measures mentioned above for increasing mRNA stability can also minimize this propensity. In addition, the use of pseudouridine and methylpseudouridine, and chemical modification of the phosphate backbone and mRNA termini also minimize immunogenicity.

3 Targeted LNPs

As is noted in other chapters of this book, lipid nanoparticles (LNPs) are widely used in mRNA delivery. They are easy to make –mixing a lipid solution in ethanol with mRNA in water and using microfluidic devices. Further, LNPs have the capacity to deliver large molecules (Hou et al. 2021). Given the theme of our chapter, we mention salient examples of *targeted mRNA-based* gene delivery by systemically administered LNPs to therapeutic effect.

Measures to target LNPs include manipulating their chemical composition. For example, cholesteryl oleate incorporation confers selectivity on LNPs for liver endothelial cells as opposed to hepatocytes and this specificity is enhanced by oxidative changes in the cholesterol tail. And manipulation of alkyl length of a lipid can direct the LNPs to liver or spleen (Hou et al. 2021). A library of ionizable amino-polyesters (APEs) was synthesized by ring-opening polymerization (ROP) of lactones using tertiary amino-alcohols; the number of repeating monomer units was controlled to generate degradable polymers (Kowalski et al. 2018). Mixing APEs with the appropriate lipids and mRNA generated mRNA-APE-LNPs. The APE-LNP library was tested for uptake capacity in HeLa cells; top-performing APEs contained four and two amines (A-TD3, B-DD3 and I-DD3). LNPs containing this had organ selectivity: ATD3 for spleen, B-DD3 for lungs.

A notable accomplishment is the targeted NLP-mediated delivery of clustered regularly interspersed palindromic repeats enzyme system (CRISPR/Cas) for gene manipulation. Cas is an endonuclease which, when directed by a guide RNA (sgRNA), can introduce a DNA double-stranded break (DSB) at essentially any site in the genome; this gap can be replaced with a desired DNA fragment by flanking it with sequences homologous to the DSB region. The technology can precisely edit genes, correct disease-causing mutations, and eliminate aberrant protein expression. mRNA-instead of DNA-based delivery of Cas is preferable, as the former is easy to produce, results in rapid expression whose transience minimizes off-target cleavage.

Selective organ targeting (SORT) approach was used for targeted delivery of mRNA-LNPs to deliver the CRISPR/Cas system. As LNP charge can affect their organ tropism, SORT molecules were added to traditional LNPs (‘mDLNPs’) to make them organ-specific (Cheng et al. 2020). Increasing molar percentage of the SORT molecule, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, a cationic lipid), altered organ tropism of the NPs. While the base mDLNPs without DOTAP targeted liver, the SORT-LNPs containing 10–15% DOTAP targeted spleen, and those containing 50% of this lipid targeted the lungs. Incorporation of 10–40% of the negatively charged 1,2-dioleoyl-sn-glycero-3-phosphate (18PA), resulted in highly

selective targeting of the spleen. SORT-LNPs successfully edited phosphatase and tensin homolog (PTEN), a tumor suppressor expressed in most cells. Cas9 mRNA and sgPTEN co-loaded in SORT-LNPs were i.v. injected in mice, and PTEN deletion was quantified. Both base mDLNPs and 20% DOTAP SORT-LNPs caused PTEN editing in liver, but not in spleen or lung, while 50% DOTAP SORT-LNPs-mediated PTEN editing only in the lungs. No off-target editing was seen (Cheng et al. 2020; Rosenblum et al. 2020). Mechanical properties of the liposome core can affect LNP stiffness; layer-by-layer (LbL) NPs with controlled stiffness can have enhanced circulation, tumor penetration, and accumulation (Kong et al. 2021).

An mRNA-based LNP drug, NTLA-2001, which utilizes the CRISPR-CAS system is currently in clinical trial to treat transthyretin amyloidosis, a life-threatening disease caused by the accumulation of misfolded protein transthyretin (TTR) in nerves and cardiomyocytes. Apolipoprotein E (ApoE)-targeted LNPs can transduce liver hepatocytes by binding to low-density lipoprotein (LDL) receptors. These were loaded with Cas-encoding mRNA and the corresponding guide RNA. Their intravenous injection resulted in CRISPR/Cas-mediated inactivation of TTR followed by DNA repair by non-homologous end-joining. Serum TTR in patients was reduced by ca. 96%, which may potentially lead to disease amelioration (Gillmore et al. 2021). In this relatively short-term 28-day study, no adverse effects were seen but as the LDL receptor is present also on other cells, possible off-target effects require careful monitoring.

Incorporation of polyethylene glycol (PEG; 'pegylation') in LNPs is another means of making directed LNPs by linking specific antibodies to PEG; pegylation also promotes avoidance by LNP of mononuclear phagocytes and renal filtration, thus increasing circulation residence time. This approach enabled targeted mRNA-LNPs to treat inflammatory bowel disease as well as cancer (Rosenblum et al. 2020). Neoantigens, which are usually specific to cancers, can thus also be used for targeted delivery (Kowalski et al. 2019).

There are, however, safety and other issues with LNPs. PEG-lipids stimulate the complement system inducing hypersensitivity, and the antigenic response can also result in shortened circulation time with accelerated blood clearance, mitigating therapeutic efficacy. Attaching PEG molecules on LNP surface through labile bonds sensitive to, e.g., serum albumin, to promote slow de-pegylation, and manipulation of PEG surface density/chain length are possible countermeasures (Abu Lila et al. 2013a, b). Lipid components can cause lung and liver injuries in mice; the solution may be to improve LNP biocompatibility by using biodegradable lipids (Sedic et al. 2018).

4 Extracellular Vesicles (EVs, Aka Exosomes)

The use of extracellular vesicles (EVs, also called exosomes; Fig. 1) for nucleic acids and drug delivery has engendered considerable excitement. EVs are constitutively generated by body cells.

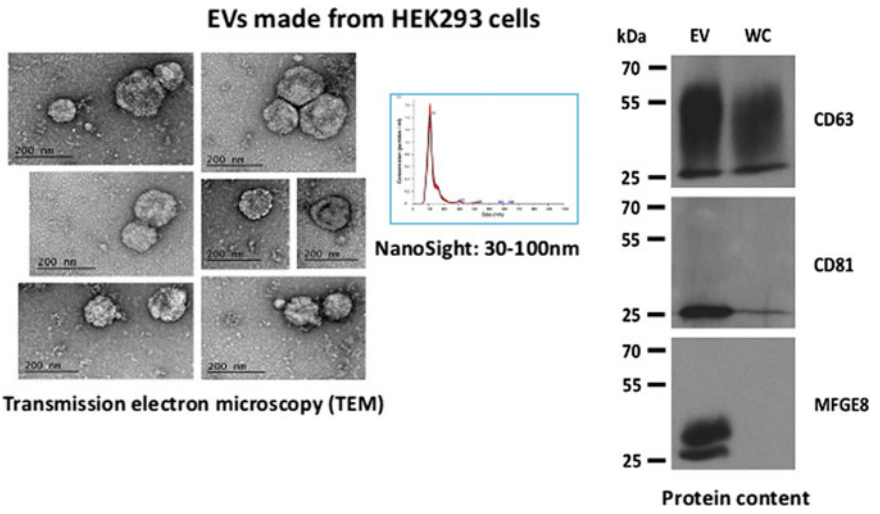

Fig. 1 Left: TEM of EVs; center: their NanoSight sizing; right: presence in them of proteins, which conform to standards of EV definition. The EVs were made by differential centrifugation: [600 xg (to remove cells); 2,000 xg (to remove apoptotic bodies); 100,000 xg (to pellet EVs). *Source* (Wang et al., 2018). Reproduced by permission of the corresponding author and the Journal

They are lipid bilayers and contain nucleic acids, proteins, and other biomolecules whose identity depends on their cellular origin. This 'native' content has been shown to be transferred to the cytoplasm of the neighboring or distant recipient cells. Thus, EVs serve as natural antigen delivery systems and are therefore likely to be biocompatible and minimally immunogenic/toxic: indeed, exosomes of human kidney embryo 293 (HEK293) cells (the 'work horse' of EV research) are harmless in mice (Wang et al. 2018; Zhu et al. 2017; Forterre et al. 2020). EVs derived from mesenchymal stem cells (Lee et al. 2021) or from patient's own, e.g., dendritic cells (DC), may be completely safe for gene/drug delivery; indeed, DC-derived EVs have been found to be safe in human clinical trials (Pitt et al. 2016).

As regards targeting, EVs have the advantage of intrinsic tissue tropism due to their membrane proteins. For example, integrin (ITG) αVβ5 possessing EVs bind specifically to liver Kupffer cells and EVS with ITGα6β4 and ITGα6β1expression have affinity for fibroblasts and epithelial cells in the lung, respectively (Capasso et al. 2020). Their natural ability to extravasate through fenestrations in tumor blood vessels makes them suitable vectors also in treating cancers in general. However, these inherent advantages are by themselves not sufficient for effective targeted therapy; this requires display on the EV surface of specific directing moieties. Examples of directing molecules resulting in successful treatment by EVs are as follows. Av-integrins [doxorubicin delivery to tumors (Tian et al. 2014)]. Epidermal growth factor receptor-targeting moiety [let-7ainordertodeliverlet-7a (let7a) delivery to breast cancer in mice (Kooijmans et al. 2016; Ohno et al. 2013)]. And asialoglycoprotein hepatocyte receptor-targeting ligand [delivery of siRNAs to blood cells for

selective silencing of genes responsible for disease causation (Wahlgren et al. 2012)]. These examples also underscore the fact that EVs can evade the lysosomal–endosomal pathway well enough to deliver the required therapeutic agent to the targeted cells in sufficient strength to treat diseases.

The native EV content as well as non-EV biomolecules that may co-isolate with them can be affected by their method of preparation (Veerman et al. 2021). These might be co-delivered along with the intended therapeutic agent to the recipient cells. No harmful effects have so far been reported resulting from such unintended co-delivery, but future studies may point to the need for specific EV preparation methods for particular therapeutic ends.

4.1 mRNA Loading of EVs

As stated, EVs have been successfully used for targeted delivery of si- and miRNAs. But loading them with foreign larger molecules, such as mRNA, proved challenging. Electroporation did not work. EVs generated by HEK293 producer cells transiently expressing Luc–RFP contained the mRNA of this reporter, but the mRNA was degraded in the recipient cells (Kanada et al. 2015). A bacteriophage protein bridge between the EVs and mRNA also succeeded in loading EVs with mRNA, but again it was nonfunctional in the recipient cells (Hung and Leonard 2016).

The first successful EV-mediated delivery of functional mRNA to treat cancer was accomplished with *HchrR6* mRNA, which encodes an improved and humanized version of *Escherichia coli* nitroreductase (*HchrR6*) (Barak et al. 2006, 2008). This enzyme is therapeutically important, as it reductively activates several prodrugs used to treat cancer, such as CNOB which is converted to the drug 9-amino-6-chloro-5H-benzo[a]phenoxazine-5-one (MCHB) (Fig. 2); and CB1954 (tretazicar), which is transformed to the drug 5-(aziridine-1-yl)–2,4-N-acetoxy-2-nitrobenzamide (MPNQ) (Patel et al. 2009). [Activation of a harmless prodrug to a toxic drug by a bacterial or viral enzyme for treatment is termed gene-directed prodrug therapy (GDEPT)].

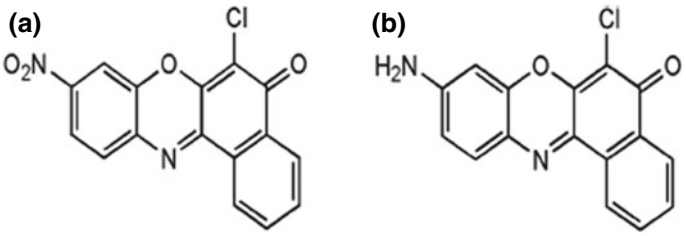

Fig. 2 **a** Prodrug 6-chloro-9-nitro-5-oxo-5H-benzo[a]phenoxazine (CNOB), and **b** its reduced product, the drug 9-amino-6-chloro-5H-benzo[a]phenoxazine-5-one (MCHB). *Source* (Thorne et al., 2009). Reproduced by permission of the corresponding author and the Journal

Both MCHB and MPNQ cause DNA intercalation and kill both growing and non-growing tumor cells, which is advantageous, as discussed above, for cancer treatment. Both also have an excellent bystander effect (BE), meaning that the cytotoxic drug can readily leak out from the cells and thus also kills the neighboring cells not expressing the required enzyme-encoding gene. As no method of gene delivery is effective enough to transfect all cells in a tumor and given that the targeted receptor/ligand is often not expressed by all cancer cells (Filho et al. 2021). BE is important for GDEPT success.

CNOB is a new prodrug (Thorne et al. 2009), not yet clinically tested. But it has the useful feature that the drug it generates, MCHB, (Fig. 2B) by the activity of *HchrR6* enzyme is highly fluorescent, and is easily visualizable in vitro and in living mice; this is shown for the latter in Fig. 3 (Thorne et al. 2009; Wang et al. 2016). This facilitated the development of GDEPT approaches discussed below in ensuring that the targeted EVs activated the prodrug in the tumor. Tretazicar's activated drug, MPNQ, requires involved methods for detection, but it has been clinically tested with its safe dose established (Patel et al. 2009); it is thus a prime candidate for GDEPT transfer to the clinic; the MCHB fluorescence has paved the way for this transfer as is discussed below. It should be noted that no reductive prodrug has as yet been approved by the FDA for cancer treatment.

Native mRNAs in EVs contain a common sequence, called the 'zipcode' (Bolukbasi et al. 2012), and this was utilized in loading EVs with *HchrR6* mRNA. Two tandem (DNA counterparts of) zipcode sequences were inserted in the UTR of the *HchrR6* DNA under a constitutive promoter, and the construct was cloned into the System Biosciences 'XPort' plasmid (Wang et al. 2018). The resulting plasmid (named 'pXPort/*HchrR6* mRNA') was used to transfect the HEK293 producer cells, which generated *HchrR6* mRNA containing EVs; these were made targeted to the HER2 receptor of BT474 human $HER2^{+}$ cells as described below. (The mRNA-loaded, directed EVs are termed, EXODEPTs.) When the EXODEPTs were mixed

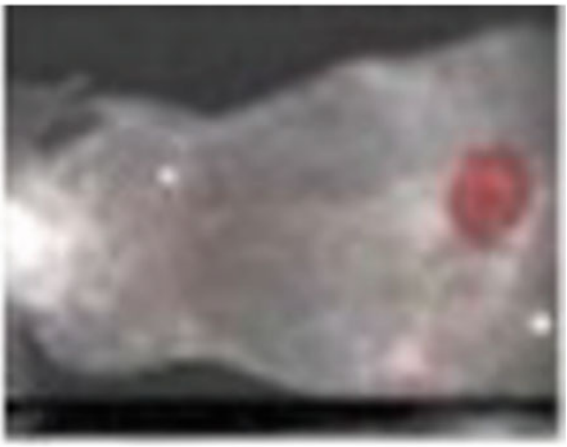

Fig. 3 Non-invasive visualization in living mouse of localized conversion of CNOB to MCHB in implanted tumor. Reproduced by permission from Thorne et al. (Mol Cancer Ther, 8(2), 333–341))

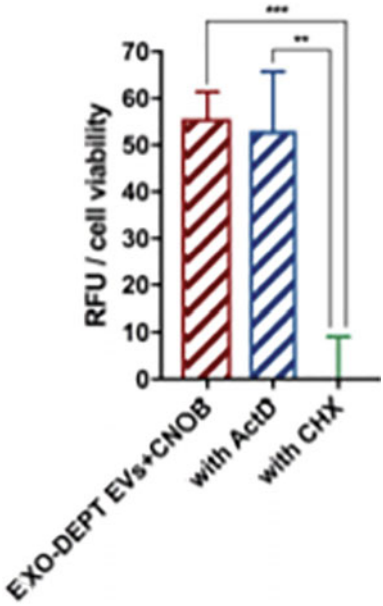

Fig. 4 mRNA transferred to HER2$^+$ BT474 cells via the EXODEPTs is functional. Upon CNOB addition, MCHB is generated, as detected by its fluorescence. This process is actinomycin D (ActD) resistant and is abolished by cycloheximide (CHX), showing that it's the *HchrR6* mRNA that was transferred to the BT474 cells, which translated it to the *HchrR6* enzyme making them capable of CNOB activation. *Source* (Wang et al., 2018). Reproduced by permission of the corresponding author and the Journal

with the (HER2$^+$) BT474 cells, the latter acquired the ability to activate the prodrugs; this is shown for CNOB activation in Fig. 4: the activation could be easily 'seen' due to MCHB fluorescence. Acquisition of this capability was not affected by actinomycin D ('ActD', transcription inhibitor) but was by cycloheximide ('CHX', protein synthesis inhibitor; Fig. 4). Thus, it was the *HchrR6* mRNA that the EVs transferred, which the recipient cells translated into the *HchrR6* enzyme.

In a related study, several plasmids were used to generate EVs capable of transferring catalase-encoding mRNA to recipient cells (Kojima et al. 2018). However, the catalase activity of the producer cells was not reported; nor whether catalase expression in the recipients was insensitive to actinomycin D. Thus, the catalase activity in the recipient cells could have resulted from the transfer of catalase-encoding plasmid and/or the catalase protein itself, rather than the catalase-encoding mRNA.

An additional method for loading EVs with mRNA relied on the application of transient electrical pulses to a small area of the recipient cell's membrane (Yang et al. 2020, 2021). A cellular nanoporation (CNP) biochip was used to cultivate various producer cells, including embryonic fibroblasts and the DC cells. An array of nanochannels in the chips provided the electrical pulse, which shuttled PTEN plasmids from the buffer into the cell monolayers attached to the CNP surface. The authors report that the method increased mRNA loading into the EVs by 2000–10,000-fold. How the electric pulse enhanced plasmid entry into the nucleus for transcription of the mRNA that got loaded into the EVs was not clarified. The resulting EVs were made capable of targeted delivery of PTEN to a murine glioma model (see below).

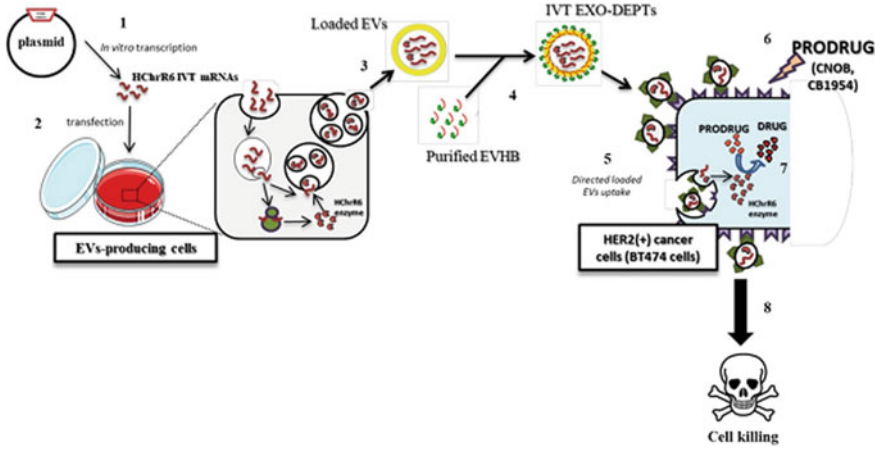

Fig. 5 Steps in making and using the IVT-EXODEPTs. *Source* (Forterre et al. 2020). Reproduced by permission of the corresponding author and the Journal

The above methods relied on transfection of producer cells with plasmids, which were likely transferred to the EVs that the cells generated, and from the EVs into the recipient cells. This is potentially problematic for clinical EV use, as plasmid introduction into patients can have unpredictable effects. Moreover, the use of plasmids to generate mRNA in the producer cells for transfer to their EVs is constrained by the need of the plasmid entry into the nucleus for transcription which, as noted, is inefficient, diminishing mRNA loading of the EVs (see below). Therefore, the plasmid use was replaced by direct loading of the EVs with vitro transcribed (IVT) *HchrR6* mRNA (Forterre et al. 2020). This required several steps (Fig. 5); naked mRNA being prone to instability, these workers ensured the mRNA's functionality through these steps by a facile method described below.

4.2 Strategies to Target mRNA-Loaded EVs and Their Therapeutic Use

Making EVs effective therapeutic agents requires display on their surface of specific targeting moieties, as mentioned above. This has been accomplished by constructing fusion proteins consisting of a targeting domain and an EV anchor domain. Salient examples of the EV anchors are as follows.

4.2.1 Lamp2b

An early example of the anchoring approach is the use of Lamp2b protein, which is abundant in EVs. It was fused to the N-terminal of neuron-targeting rabies viral glycoprotein. EVs displaying this fusion delivered siRNA to the brain and caused significant knockdown in mice of *BACE1*, a target in Alzheimer's disease (Alvarez-Erviti et al. 2011). Others, however, had problems with this approach, such as degradation of the peptides fused to Lamp2b upon EV-mediated transfer (Hung and Leonard 2016; Wang et al. 2018). It is now recognized that incorporation of a glycosylation motif in the Lamp2b fusions can protect the peptides (Hung and Leonard 2016). This approach can therefore be effective also in targeted mRNA delivery, although no such attempts have so far been published.

4.2.2 Lactadherin C1C2 Domain

Lactadherin is a secreted protein with a C1C2 domain at the C-terminus. This domain binds to lipid membranes with high affinity, especially when phosphatidylserine (PS) is present, as is the case on the EV surface. PS is 'eat me' signal to eliminate apoptotic cells by phagocytosis. Use of this domain for targeting fusions in EVs thus has the advantage of masking PS, thereby mitigating EV phagocytosis, and enhancing their potential to reach the intended target (Dammes and Peer 2020; Jayasinghe et al. 2021). This approach was used by the Matin group (Wang et al. 2018; Forterre et al. 2020) to treat implanted orthotopic $HER2^+$ breast cancer (BC) tumors in mice. The prodrugs CNOB and CB1954 were used in separate studies with the *HchrR6* enzyme (that can, as mentioned, activate both). $HER2^+$ BC has poor prognosis and results from dysregulation of tyrosine kinase signaling network due to HER2 gene amplification. Drugs like trastuzumab and lapatinib have been effective in treating it, but a 10-year follow-up study shows that $\geq$ 25% of early-stage patients treated with trastuzumab relapse, often with distant metastatic disease that does not respond to this drug (Cameron et al. 2017), highlighting the need for additional therapeutic approaches.

Using appropriate source plasmids, a new plasmid, pEVC1C2HER, was constructed (Wang et al. 2018). Transfection of HEK293 producer cells with this plasmid yielded EVs displaying the protein fusion termed EVHB (Fig. 6); MW, 68 kDa). It has lactadherin leader sequence (LS; to enable the protein to migrate to the EV surface), an scFv termed ML39, with high affinity to bind the HER2 receptor [K(d)10^9 mol/L], and a flexible linker that connects it to the C1C2 domain. Isolated and purified EVHB protein was mixed with *HchrR6* mRNA-loaded EVs. In vitro, the resulting EXODEPTs displaying EVHB selectively targeted the $HER2^+$ BT474 BC cells, but not the $HER2^-$ MCF7 cells, and converted the former to CNOB-activating agents, as measured by MCHB fluorescence. The EVs needed to be displaying EVHB—to be EXODEPTs in effect—to convert the recipient cells into CNOB activators (Fig.4). As noted above, Fig. 4 documents that the acquired CNOB-activating

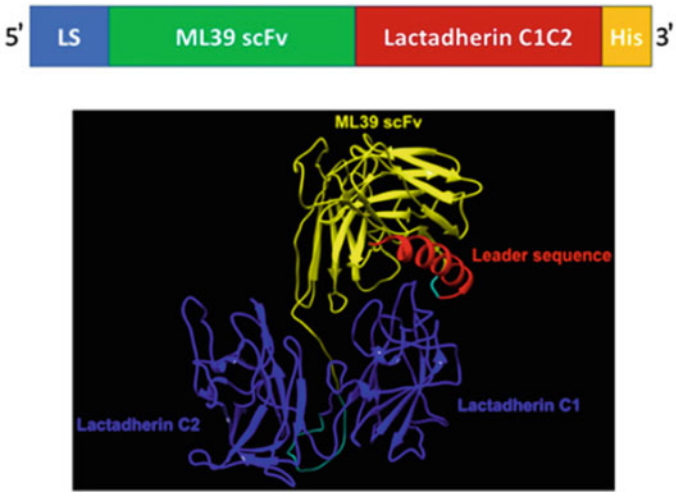

Fig. 6 Top: components of the EVHB chimeric protein; Below: its predicted structure. Colors: green, scFv antibody; red, C1C2 domain; blue, leader sequence; yellow, His tag. (Note colors in the predicted structure are different; the various components are identified within this figure). *Source* (Wang et al., 2018). Reproduced by permission of the corresponding author and the Journal

capability in human BT474 cells was not inhibited by actinomycin D and therefore was due to the EV-delivered mRNA to these $HER2^+$ cells (Fig. 4).

Use of IVT mRNA and Tretazicar

As mentioned, loading EVs with the IVT mRNA required a multistep process (Fig. 5); IVT mRNA avoids the use of plasmids potentially harmful to patients and tretazicar has proven safe in humans in phases I/II clinical trials, as mentioned above. Thus, using IVT-EXODEPTs + tretazicar enhances the prospect of clinical transfer of this GDEPT. Tretazicar-activated drug, MPNQ, is difficult to detect and so a facile indirect strategy was used for ensuring that the mRNA remained competent to activate tretazicar through the multistep process of IVT loading into the EVs (Fig. 5). This relied on the fact that *HchrR6* can activate both CNOB and CB1954. So, it was hypothesized that if the mRNA-translated product at various steps of IVT-EV preparation and uses (Fig. 5) generated MCHB fluorescence from CNOB, it would indicate its competence to activate tretazicar as well. At every step indicated in Fig. 5, the mRNA did indeed encode the protein that generated MCHB from CNOB; and as hypothesized, also activated tretazicar.

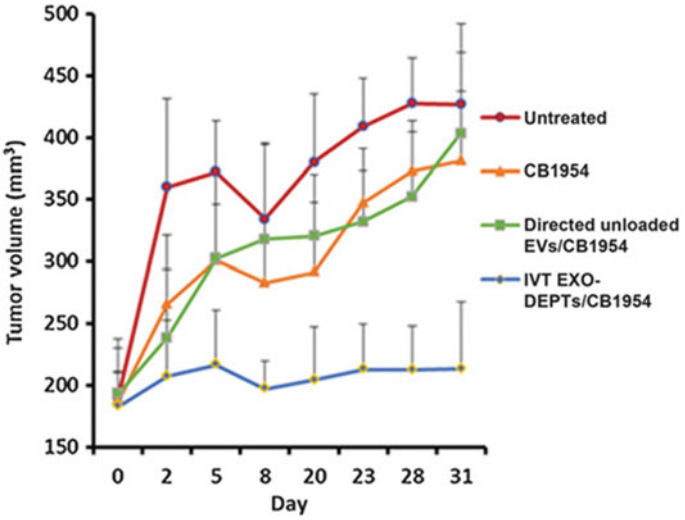

Fig. 7 In vivo effectiveness of systemic IVT EXODEPTs + tretazicar. Implanted orthotopic human HER2$^+$ BC tumor volumes in mice, as measured by caliper. The treatment likely killed the tumor—see text for further information. *Source* (Forterre et al. 2020). Reproduced by permission of the corresponding author and the Journal

Systemic Administration

Until the Matin group studies (Forterre et al. 2020; Wang et al. 2018), cancer prodrug treatment pre-clinical studies had required injection of the therapy directly into the tumor. As not all cancers, especially multiple sites of metastatic cancer, are accessible to direct injection, this limited GDEPT applicability. The EXODEPT/CNOB and EXODEPT/tretazicar therapy overcame this problem, as these treatments were effective in vivo upon systemic injection. This is shown for the IVT EXODEPT/tretazicar treatment of implanted BT474 human HER2$^+$ tumors in mice (Fig. 7).

CB1954 was used at its safe dose (determined in its phases I/II clinical trials; see above) and a total of 2.8×10^7 mRNA copies were delivered per mouse via IVT-EXODEPTs. In mice receiving this treatment, there was minimal growth of the tumor and it ceased by day 8 with no growth resumption over the next 21 days; the experiment was stopped on the 29th day in conformance with the approved animal use protocol. As the last dose was on day 7, it is reasonable to conclude that the treatment had killed the tumor. Untargeted, but mRNA-loaded, EVs also caused significant arrest of the xenograft growth but some 50% less than with the EXODEPTs. This illustrates the facts that EVs can be effective because of the enhanced permeability retention (EPR) effect, but that for full effectiveness they need to be directed. Untreated controls sowed vigorous tumor growth. It should be noted that nearly three orders of magnitude fewer IVT-EXODEPTs were as effective in treating the cancer as the pXPort/*HchrR6* mRNA plasmid-EXODEPTs described above; this was ascribed

mainly to the limitation imposed on the latter by the need for the plasmid to enter the nucleus of the producer cells for mRNA generation (Forterre et al. 2020).

Trastuzumab and similar drugs are effective in treating the $HER2^+$ cancer because they interact with the HER2 receptor and inhibit its signaling. The EXODEPTs displaying the anti-HER2 receptor scFv may have been effective, at least in part, for the same reason. This, however, was not the case, because administration of directed EVs (displaying the scFv) not containing *HchrR6* mRNA had no effect on tumor growth (Forterre et al. 2020).

EVs temporarily colonize organs like pancreas, spleen, and liver, and the ML39 scFv of the EXODEPTs might also have recognized mouse ErbbB2-expressing normal cells and by delivering the mRNA might have enabled them to activate the CB-1954. This would have caused general injury besides treating the cancer. However, a comprehensive investigation of organ histopathology (namely of liver, spleen, kidney, lung, heart, and the brain), hematology, and serum chemistry indicated no deleterious effects. Thus, the therapy was cancer curative without side effects (Forterre et al. 2020).

The above studies were conducted in immune-deficient mice. The authors are planning to extend them to immune-competent FVB/NJ mice. These contain an oncogenic form of human HER2—HER2Δ16 (Turpin et al. 2016)—resulting in spontaneous development of $HER2^+$ BC (inducible by Dox). The therapy discussed above (Forterre et al. 2020; Wang et al. 2018) includes elements of human origin, viz. the C1-C2 lactadherin domain and the anti-HER2 scFv, ML39. An immune response to these, while possibly deleterious can, however, bolster the therapy instead. There was no off-target toxicity of the regimen, as discussed, indicating that the EXODEPT location was confined to the tumor. Thus, the immune rejection of these human elements of EXODEPTs might, by being directed to the tumor, further promote its eradication. HER2 tumor ablation evokes a strong anti-HER2 immune response (Milani et al. 2014), which too can reinforce the therapy.

A recent study involving an innovative chimeric protein to concomitantly activate two prodrugs, ganciclovir and tretazicar, has been reported (Kanada et al. 2019). The genes encoding the activating enzymes were cloned into minicircles (miniaturized plasmids, which are more efficient in gene delivery than the parent plasmid). Injection of the two prodrugs and 5.5×10^9 minicircle plasmids encoding the hybrid gene directly into breast cancer xenografts in mice resulted in 54% killing of the tumor. With EV-mediated mRNA gene delivery in the EXODEPT approach mentioned above, over two orders of magnitude fewer gene copies sufficed to kill the tumor despite systemic administration and the use of only one prodrug. This may be because DNA for gene delivery is less efficacious in cancer treatment than mRNA, as discussed above.

4.2.3 CD47

This is an abundant EV surface protein, and as opposed to PS, which needs to be masked to prevent EV phagocytosis, CD47 suppresses phagocytosis, and so a

strategy opposite to that described for C1C2 fusions was followed that increased CD47 expression on the EVs (Yang et al. 2020). EVs containing the PTEN mRNA generated, as described above, were made to display increased CD47 fused to glioma targeting peptides ('Exo-T' EVs). Exo-Ts had increased circulation time and were specifically taken up by U87 and GL261 cells. Their use in orthotopic PTEN-deficient glioma mouse model inhibited tumor growth and resulted in increased survival.

4.3 Improving EVs for Clinical Use

A major step needed for this is scaling up of EV production. The electrical pulse method described above is a step in this direction. Among other approaches being pursued are placing cells in a nitrogen cavitation vessel under pressure (350–400 psi) and its quick release to disrupt the cells. This generates large numbers of what are referred to as 'NC EVs.' These EVs significantly attenuated acute lung inflammation (Gao et al. 2017). Another method involves the use of sulfhydryl blocking reagents, such as formaldehyde and a reducing agent such as dithiothreitol during EV production; it generated in one hour the amount of EVs that conventional methods (e.g., differential centrifugation) take 12 h to produce (Li et al. 2017) (https://patents.google.com/patent/WO2018102608A1/en). Increasing intracellular calcium also resulted in increased EV release (Savina et al. 2003), and it has been reported that incubation of liposomes with cells enhanced EV secretion several fold (Emam et al. 2018).

Other needed measures are: 1. To standardize EV engineering protocols. 2. To prevent rapid clearance of EVs from circulation to permit effective binding to the targeted cells. The use of CD47 fusions discussed above is conducive to this. The need for high residence time in circulation can be decreased by display on the EVs of high affinity directing molecules that can bind to the target rapidly after systemic injection; the use of the EVHB protein (see above, Fig. 6) is an example of this. 3. Avoiding potential immunogenicity. As discussed, being natural means of biomolecule exchange, this may not be a major problem with EVs. Further, their localization to the tumor can direct any anti-EV immune response against the tumor, reinforcing the therapeutic effect (see above). And finally, the use of stem cell EVs and those made from patients' own, e.g., DCs is unlikely to evoke an immune response.

5 Conclusion

Directed, systemically administered mRNA-based gene delivery has the potential of revolutionizing therapy of disease like cancer and others. Both LNPs and EVs are highly promising vehicles for such delivery, each with its own unique advantages.

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SARS-COV-2 and Other mRNA Vaccines

Nicholas Jackson

Contents

Abstract Advances in our biological understanding of mRNA and ionizable-lipid-based nanoparticles (LNP) for delivery have allowed their application as vaccines for the prevention of SARS-COV-2 disease following unprecedented speed of development through to emergency use licensure in around three hundred days from virus sequence availability. Case studies of three SARS-COV-2 mRNA vaccines for which field clinical efficacy data are available are examined and related to mRNA/LNP attributes where possible. The status of other SARS-COV-2 and non-SARS-COV-2 mRNA vaccines in clinical development and select future prospective innovations are reviewed.

N. Jackson (✉)
Clover Biopharmaceuticals, Research and Development, London, UK
e-mail: nicholas.jackson@cloverbiopharma.com

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_6

Keywords mRNA · LNP · Vaccines · Infectious disease · SARS-COV-2 · COVID-19 · Clinical development · Efficacy · Effectiveness · Safety surveillance · Immunogenicity · Reactogenicity

1 Introduction: The Successes of MRNA-LNP Vaccines for SARS-COV-2

This chapter focusses on three SARS-COV-2 mRNA-LNP vaccines, the licenced products of Pfizer-BioNTech and Moderna, and the clinical phase 3 tested CureVac candidate vaccine (Fig. 1). The following sections will review clinical trial and field effectiveness data and relate back to mRNA-LNP attributes, where possible. For completeness, other SARS-COV-2 and non-SARS-COV-2 candidate vaccines will be summarized. Future scientific challenges and opportunities for mRNA vaccines will conclude the chapter.

2 Pfizer-BioNTech—BNT162b2 (Comirnaty)—mRNA-LNP SARS-COV-2 Vaccine

BNT162b2 received Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA) on December 11th, 2020, for subjects 16 years of age and older; that was subsequently extended to include 12–15-year-olds on May 10th, 2021. On August 23rd, 2021, the U.S. FDA granted full approval, indicated for active immunization to prevent COVID-19 caused by SARS-COV-2 in individuals over 16 years of age. The vaccine also continues to be available under EUA for 12–15-year-olds and for the administration of a third dose in certain immunocompromised individuals. On the December 31st, 2020, the World Health Organization (WHO) approved the vaccine for Emergency Use Listing (EUL).

2.1 Clinical Phase 1 Study in Adults (19–55 years), Germany

Following early development activities, two phase 1/2 studies were initiated in Germany (NCT04380701, first dosing April 23rd, 2020) and in the U.S. (NCT04368728, first dosing May 5th, 2020). In the German phase 1 study, BNT162b2 was tested in healthy adults aged 19–55 years of age, who received one of the four different doses of mRNA (1, 10, 20 and 3 μg) formulated with LNP by IM injection. The mRNA expressed the full-length SARS-COV-2 S protein, genetically stabilized in a pre-fusion state, as the immunogen. Healthy subjects with no previous history of COVID-19 received vaccinations on days 1 and 22 (Sahin et al. 2020a).

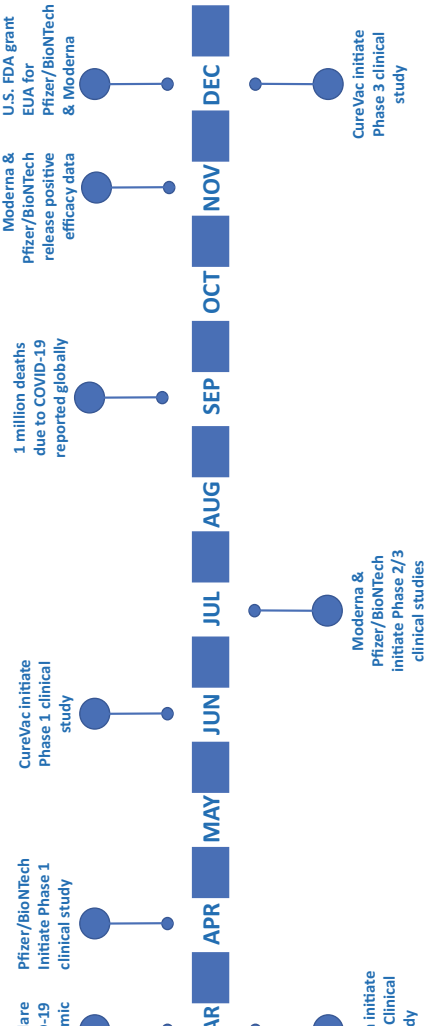

Fig. 1 The unprecedented development timelines from the availability of the SARS-COV-2 genetic sequence to the U.S. F.D.A. Emergency Use Authorization (EUA) of the Pfizer/BioNTech and Moderna COVID-19 mRNA/LNP vaccines. Major clinical development milestones for Pfizer/BioNTech, Moderna and CureVac are shown

There were no Serious Adverse Events (SAE) reported during the trial. Pain at the injection site was the predominant local reactogenicity adverse event (AE) that was mild to moderate in severity and comparable after both doses. The most common systemic AEs were fatigue followed by headache. Generally, AEs resolved within 2–3 days. A decrease in blood lymphocyte counts was detected in vaccines 2 days after vaccination that returned to baseline on day 7. A transient increase in C-reactive protein, a marker of inflammation, was also seen. Neither were associated with any clinical sequalae. As described, mRNA vaccines can trigger innate immune sensing that induce inflammatory responses able to regulate the circulation of lymphocytes (Kamphuis et al. 2006). One can postulate that the vaccine triggered an innate response sufficient to temporarily direct systemically circulating lymphocytes to the sources of mRNA-associated inflammation. Overall, the investigators concluded that the vaccine was well tolerated and there were no apparent safety issues (Sahin et al. 2020b).

Neutralization (or killing) of the SARS-COV-2 virus, in vitro, is currently considered the gold-standard assay by the regulatory authorities for the evaluation of vaccine-elicited immunity, complemented by a pseudovirus (a different virus, in this case Vesicular Stomatitis Virus [VSV] engineered to present the SARS-COV-2 S protein) neutralization assay and the measurement of binding IgG antibody by enzyme-linked immunosorbent assay (ELISA). After the first dose, only a few responders had a neutralizing response in the three highest doses and there were no responders to the 1 μg dose. However, the immune system was primed because the second dose (at 10, 20 or 30 μg) substantially increased neutralization responses that were comparable in magnitude to the reference sera of convalescent SARS-COV-2-infected individuals. On day 43 (21 days after the second dose), vaccinated participants who received the 10 and 30 μg doses of mRNA had 50% virus neutralizing geometric mean titres (GMTs) between 108 and 166 that remained stable up to day 85 (63 days after the boost). Vaccination elicited robust IgG binding responses, including the low dose group, which suggests the low dose can elicit humoral responses that are less functional in terms of neutralization. At that time, mutant forms of the SARS-COV-2 virus were less understood, and the current spectrum of variants of concern (VOC) had not yet emerged (GISAID 2021). Nevertheless, the pseudovirus neutralization assay included the dominant variant D614G of that period and sera collected after the second dose showed diminished but largely comparable neutralization titres as those seen against the ancestral strain used to derive the vaccine S protein immunogen sequence. Vaccination after two doses also elicited SARS-COV-2 S protein-specific CD4 + (39/39, 100%) and CD8 + (35/39, 89.7%) T cell responses. Overall, two doses of the vaccine at 10, 20 and 30 μg of mRNA elicited robust humoral and cellular immune responses (Sahin et al. 2020a; VRBPAC/Pfizer-BioNTech 2020).

2.2 Clinical Phase 1 Study in Adults (18–55 and 65–85 years), U.S.

In parallel to the phase 1 study in Germany, a placebo-controlled, dose-escalation study was performed in the US, in healthy adults 18–55 and 65–85 years of age (195 participants). Subjects receiving the vaccine were given either 10, 20, 30 or 100 μg of mRNA by IM injection. All study groups received two doses with the same 21-day interval as the German phase 1 study, except one group that received a single dose of the 100 μg formulation due to a study amendment (see below) (Walsh et al. 2020).

No SAEs were reported in the study. Pain at the injection site was the most common local reactogenicity in all ages, and the majority of all local reactogenicity was mild to moderate in severity. Regarding systemic AEs, subjects 18–55 years of age had most frequently fatigue that was elevated after the second dose, and dosage dependent; reported in 33, 58 and 75% of subjects after a second dose at 10, 20 or 30 μg, respectively. Fever was reported in 17% of subjects who received the 30 μg dose after both doses (Falsey et al. 2021). In another phase 1 study in adults 18–55 years of age, using the same mRNA-LNP technology but expressing a truncated form of the S protein as an immunogen, after the first dose with 100 μg of mRNA, 100% of subjects reported injection site pain (mostly mild to moderate and 1 severe) and 50% fever (≥38 °C), as well as high percentages of fatigue, headache and chills (Mulligan et al. 2020). The sponsor decided for that study, and the US study, to stop the administration of a second dose at 100 μg because of the high reactogenicity and the apparent lack of an increased immunogenicity benefit after a single dose compared to 30 μg. This exemplifies the potential for high doses of intramuscularly delivered mRNA-LNP vaccines to stimulate an unacceptable level of local and systemic reactogenicity without an immunology benefit to justify the higher dosage. One can hypothesize that the high level of mRNA triggered a deleterious inflammatory response, and/or the higher molar ratio of LNP contributed some level of toxicity.

Focussing on live virus neutralization, in both age cohorts, responses were low or absent after the first dose. However, in 18–55-year-olds after a second dose, neutralizing GMTs increased significantly at all dose levels and peaked at day 28 (7 days post-dose 2). In the 30 μg dose, the GMT was 361, and overall, the responses were in the upper quartile range of the convalescent sera comparative panel. In the 65–85-year-olds, the neutralizing response was overall less than 18–55-year-olds, but nevertheless robust and peaked at day 36. The 30 μg dose elicited the highest neutralizing titres in the older cohort with a peak GMT of 206 which was also in the upper quartile range of the comparative sera panel (Walsh et al. 2020).In conclusion, the BNT162b2 vaccine elicited robust SARS-COV-2 neutralization responses in both age groups. The two phase 1 studies allowed the dose selection of 30 μg for the subsequent pivotal efficacy trial given acceptable tolerability and a robust immunogenicity profile in both age groups (VRBPAC/Pfizer-BioNTech 2020).

2.3 *Clinical Phase 2/3, (16 Years of Age or Older) Multi-Country*

On July 28th, 2020, Pfizer-BioNTech initiated a phase 2/3 study that expanded upon the phase 1 studies and included efficacy endpoints. The international trial was designed as a pivotal efficacy study in persons 16 years of age or older in a 1:1 ratio to receive two doses, 21 days apart, of either the BNT162b2 vaccine candidate with 30 μg of mRNA per dose or a placebo. 152 sites in the United States, Argentina, Brazil, South Africa, Turkey and Germany enrolled and randomized a total of 43,448 participants: 21,720 received BNT162b2 and 21,728 received placebo (Polack et al., 2020). One study amendment included younger adolescents 12–15 years of age but they were primarily included for tolerability and safety evaluation (VRBPAC/Pfizer-BioNTech 2020).

The study had two co-primary efficacy endpoints: firstly, the incidence of virologically confirmed symptomatic COVID-19 disease with an onset at least 7 days after the second dose in subjects without serological or virological evidence of past SARS-COV-2 infection before and during vaccination regimen; the second primary end point was efficacy in participants with and without evidence of prior infection. The primary safety endpoints were evaluated in participants receiving at least 1 dose of study intervention. Secondary endpoints included, among others, the assessment of efficacy against more severe forms of disease. Immunogenicity was evaluated in the phase 2 portion of the study in 360 persons and was comparable to phase 1 immunogenicity profiles (VRBPAC/Pfizer-BioNTech, 2020).

A subset of the trial, 8183 participants, were monitored for reactogenicity. The overall profile was grossly comparable to the earlier clinical trials. Pain at the injection site was the most common local AE with more events reported in the younger age range of 16–55 years. The majority were mild to moderate in nature with less than 1% severe across all ages. Systemic events were also reported more frequently in the younger age range, and there were more events after the second dose. Fatigue was the most common systemic event followed by headache: (59% and 52%, respectively, after the second dose, among younger vaccine subjects; 51% and 39% among older subjects). These numbers should be noted against significant percentages reported by placebo recipients, for example, 23% fatigue after the second dose in the younger age range. For fever ($\geq$ 38 °C), the frequency of events increased with the number of doses (first dose versus second dose): younger group (4 versus 16%) compared to older group (1 versus 11%). Severe fever (> 38.9–40.0 °C) was reported in the BNT162b2 group after the first dose at a rate of 0.2% and after the second dose at 0.8%, and in the placebo group after first and second doses at a rate of 0.1% (Polack et al. 2020). Overall, the rate of related AEs for 43,252 participants post-dose 2 was 21% in vaccines and 5% in placebo recipients. The percentage in vaccines is not atypical for a phase 3 vaccine trial and was largely driven by reactogenicity. Lymphadenopathy, swollen LNs, was reported in 0.3% of vaccines and < 0.1% in placebo recipients, likely clinically manifestations of the postulated inflammatory response to vaccination described above in the phase 1 trial. The investigators

confirmed 4 related SAEs in vaccinees: shoulder injury related to vaccine administration, right axillary lymphadenopathy, paroxysmal ventricular arrhythmia and right leg paresthesia. It is important to note that the EUA licensure relied on safety data with a median follow-up time of 2 months after the second dose for half the trial. This was agreed with the regulators to accelerate licensure because of the status of the COVID-19 pandemic. Continued safety monitoring of the trial (two years post-dose 2) for all subjects and subsequent post-licensure safety monitoring therefore remain essential (see below) (Polack et al. 2020).

For the primary efficacy endpoint, in those with no evidence of prior infection, the mRNA-LNP vaccine provided 95% (95% CI 90.3–97.6) protection against symptomatic virological-confirmed disease, of any severity, after the second dose. Efficacy was comparable (94.6%) in those with and without prior evidence of an infection. The estimates of efficacy did not differ by age, 95.6%, 93.7% and 94.7% in 16–55, > 55 and ≥ 65 years of age, respectively. For severe disease, although the number of cases were low after the first dose (N: 10), the vaccine provided 88.9% (95% CI 20.1–99.7) protection. Counting cases between the first and second dose, to give an estimate of efficacy provided after a single dose, a point estimate of 52% (95% CI 29.5–68.4%) protection was derived.

In totality, the primary endpoint of the efficacy trial was successfully met, demonstrating very high protection against SARS-COV-2 with the mRNA-LNP vaccine. The vaccine was also highly efficacious against severer disease, and there was evidence of significant protection even after a single dose. Data from approximately 38,000 participants randomized 1:1 to receive either vaccine or placebo with a median of 2 months of follow-up after the second dose of vaccine was safe and well tolerated in participants ≥ 16 years of age. Thus, the regulators were able to agree that the benefit of the vaccine against SARS-COV-2 outweighed any known or theoretical safety risks and therefore granted EUA. Later additional data have supported the U.S. FDA's extension of the indication for 5 years of age and older under the authorization.

2.4 Post-licensure Effectiveness, Safety Surveillance and Boosting

Prior to this authorization and during initial roll-out of BNT162b2, the virus had accumulated mutations associated with ongoing adaptation to circulation in human populations, resulting in a spectrum of variants of concern (VOC) and variants of interest (VOI) that have certain phenotypic advantages such as increased transmission (Salleh et al. 2021). In an effort to understand the consequences on vaccine protection, numerous studies have demonstrated that BNT162b2 vaccine-elicited antibodies can maintain neutralization of VOC/VOI identified to date, comparable or varying modest reductions versus the ancestral strain, or more significantly reduced against the Beta (B1.351) variant (Planas et al. 2021; Liu et al. 2021a, 2021b). However, field effectiveness trials assessing the protection of BNT162b2 against these variants

after mass vaccination campaigns have demonstrated that the mRNA-LNP vaccine provides substantial protection against variants, especially against severe disease (IVAC 2021). It remains to be seen whether the virus will mutant further because of immune pressure from vaccinated or infected populations and escape vaccine protection entirely.

In addition to studying the effectiveness of vaccines, in real-world post-licensure settings, safety surveillance is paramount to capture any rare events associated with vaccination. It is particularly important for mRNA given that it is a new technology platform granted emergency use licensure prior to full licensure. Robust pharmacovigilant surveillance is highly dependent upon a country's resources and ability to identify any safety events after vaccination, and many countries lack sufficient capabilities (Chandler 2020). However, a leading U.S. health plan network (Kaiser Permanente: Colorado, Northern California, Northwest, Southern California and Washington; Marshfield Clinic; HealthPartners; and Denver Health) using a reliable Vaccine Safety Datalink, a collaboration between US health plans and the U.S. Centers for Disease Control and Prevention (CDC), has reported important surveillance findings (Klein et al. 2021). From December 2020 to June 2021, weekly analysis in individuals 12 + years of age covered 3,539,611 million first doses of BNT162b2 administered, and 3,214,737 s doses, for a 21-day risk interval following dosing. The investigators concluded that the incidence of selected serious outcomes was not statistically higher 1–21 days of postvaccination compared with 22–42 days of postvaccination. Notwithstanding the statistical conclusions, the highest estimates of excess cases per million doses were 7.5 (95% CI, − 0.1–14.0) for venous thromboembolism, 1.2 (95% CI, − 6.9–8.3) for acute myocardial infarction and 1.2 (95% CI, − 2.1–3.3) for myocarditis/pericarditis. Thromboembolic outcomes with thrombocytopenia have been linked to licenced SARS-COV-2 viral vector vaccines (Hippisley-Cox et al. 2021; Pottegård et al. 2021; US/CDC 2021; EMA/News 2021).

Looking beyond the all-age findings above, the surveillance did however identify a significant clustering of myocarditis/pericarditis within the first week after vaccination, especially after the second dose, associated with mRNA vaccines (both Pfizer-BioNTech and Moderna) in younger individuals (Klein et al. 2021). During the observable period postvaccination, there were a total of 34 cases (n = 7 myocarditis, n = 6 pericarditis, n = 21 myopericarditis) among individuals aged 12–39 years, of whom 53% were aged 12–24 years, 85% were male, 82% were hospitalized (median length of stay, 1 day), and nearly all were recovered at case review. Of special note, cases were significantly clustered within the first 5 days after vaccination ($P < 0.001$). These events equate to an estimated 6.3 (95% CI, 4.9–6.8) additional cases per million doses in days 0 through 7 after vaccination in 12–39 years of age. The elevated risk was highest after the second dose, with an additional 11.2 (95% CI, 8.9–12.1) cases per million doses in days 0 through 7 for individuals aged 12–39 years. Other surveillance studies have confirmed these observations (Barda et al. 2021). The U.S. FDA revised the product labels of the Pfizer-BioNTech and Moderna mRNA vaccines in June 2021 to cite a risk of these events (Blumenthal et al. 2021).

As time passes after the immunization of subjects, it is important to understand the duration of protection offered by vaccines, which can vary from short (1–2 years)

to long term (5–10 + years) for multiple reasons. Initial studies have suggested that the protection elicited by BNT162b2 may be waning over time against symptomatic disease and/or against the Delta variant but protection against the severe forms of disease nevertheless remains high. This may not be surprising for any vaccines after receiving only two doses; several licenced vaccine platforms require three doses during a primary immunization series, or later boosting, for optimal protection. Thus, Pfizer-BioNTech have accessed the value of a booster dose and demonstrated a substantial increase in neutralizing antibody responses after a third dose (VRBPAC/Pfizer-BioNTech 2020). For example, in 18–55-year-olds in the phase 1 given a boost (30 μg mRNA) at a median interval of 6.8 months since the primary series had 25- and 39-fold higher neutralizing GMTs 1 month after the boost compared to their responses before boosting against the ancestral and Beta virus strains, respectively. In the 65–85-year-olds, the same parameters were 50- and 78-fold higher, respectively (Falsey et al. 2021). On the 22nd September, the U.S. FDA amended the EUA for the BNT162b2 vaccine to allow its additional use as a single booster dose, to be administered at least six months after completion of the primary series in persons ≥ 65 years of age and 18–64 years with certain high-risk conditions.

3 Moderna—mRNA-1273 (Spikevax) mRNA-LNP SARS-COV-2 Vaccine

Moderna received EUA from the U.S. FDA on the December 18th, 2020, for the use of their mRNA-1273 vaccine for active immunization to prevent COVID-19 in individuals 18 years of age and older. On the April 30th, 2021, the WHO listed the mRNA-1273 vaccine for emergency use, making it the fifth vaccine at that time to have received emergency validation from the WHO.

3.1 Clinical Phase 1 Trial in Adults and Older Adults, U.S.

The clinical development plan begun on the March 16th, 2020, in the U.S. with a Phase 1 dose-ranging study (NCT04283461), to evaluate two vaccinations, 28 days apart, with 25, 100 or 250 μg of mRNA in adults aged 18–55 years (Jackson et al. 2020). These doses were notably higher than Pfizer's BNT162b2 mRNA vaccine.

The most common solicited local AE was pain at the injection site and generally mild to moderate in severity. There was slightly more injection site pain reported after the second dose. For systemic AEs, post-dose 1, headache and fatigue were most frequently reported. There were significantly more systemic events after the second dose at all dosage levels, particularly in the 250 μg group were all subjects reported systemic events and 3 out of 14 had one or more severe events. Fever was

reported by 40% who received 100 μg and 57% for 250 μg (1 severe fever in the latter). There were no SAEs reported (Jackson et al. 2020).

Focussing on neutralizing antibody responses, using a pseudovirus assay, after the first dose there were a low number of responders with low titres of antibody across all doses. However, after a second dose, significant neutralization titres were detected at all doses suggesting that the first dose had primed the adaptive immune response. The 100 and 250 μg mRNA doses elicited the highest responses, with peak Geometric Mean Responses (GMRs) of 256 and 343 in the 100 μg dose at day 36 (7 days post-dose 2) and day 43 (14 days post-dose 2), respectively, and 373 and 332 for the 250 μg dose. These responses were in the upper quartile range of the comparative convalescent sera reference panel that had a GMR of 109. T cell immunity was also evaluated in a subset of participants, and Th1-biased CD4 + responses were elicited by the 25 and 100 μg doses and low-level CD8 + responses after the second vaccination in the 100 μg dose group (Jackson et al. 2020). All considered, this phase 1 study suggested that the 100 μg mRNA dose had a more optimal balance between sufficient immunogenicity and a more favourable reactogenicity profile in adults.

The sponsor then expanded the above trial to include older adults (56–70 years or ≥ 71 years of age) to assess the performance of the vaccine given as two doses of either 25 or 100 μg of vaccine administered 28 days apart. The decision had been made to remove the 250 μg dose presumably based on its elevated reactogenicity profile and no significant immunological benefit over 100 μg, as well as the lower number of doses that could be manufactured if 250 μg was required. No SAEs were reported, and solicited AEs were predominantly mild or moderate in severity and, commonly included fatigue, chills, headache, myalgia and pain at the site of injection. These local and systemic events in the older adults were dose-dependent and more frequent after the second dose (Anderson et al. 2020).

The majority of vaccinees were non-responders after the first dose, except a few responders (≤ 5 persons) in the 100 μg dose who were ≥ 71 years of age. However, robust neutralization responses were elicited in all subjects after the second dose and the highest titres achieved in the 100 μg mRNA group. Peak responses across all participants were generally seen at day 43 (7 days after the second dose). In the 100 μg group, there was little difference in the neutralizing GMTs by age: 402 and 317 at day 43 in 56–70 and ≥ 71-year-olds, respectively. These post-dose 2 GMTs were also largely comparable to those seen in 18–55-year-olds. Again, the investigators concluded that the optimal dose was 100 μg of mRNA (Anderson et al. 2020).

3.2 Clinical Phase 2 Trial (18 Years of Age and Older), US

Although the evidence from the above phase 1 evaluations suggested that 100 μg of mRNA was the optimal dose, Moderna were evidently concerned that this was still a significantly high dose in terms of manufacturing and thereby reduce production yields. A phase 2 study was conducted to provide additional safety data to support the

initiation of the large-scale phase 3 study and assess a 50 μg dose (VRBPAC/Moderna 2020). Healthy adults in the U.S., 18 years of age and older, were randomized 1:1:1 to receive either a placebo, 50 or 100 μg of the mRNA-1273 vaccine administered as two IM injections 28 days apart. Two age cohorts were analysed, ≥ 18 to < 55 and ≥ 55 years of age (300 subjects per age cohort) (Chu et al. 2021). Switching to a live virus microneutralization assay for this study, responses were detected after a single dose of either 50 or 100 μg in both age cohorts and these neutralizing GMTs were significantly enhanced after a second dose to 1733 at 50 μg and 1909 at 100 μg in younger adults (at day 43) and 1827 at 50 μg and 1686 in older adults (at day 43). It should be noted that one cannot compare these titres to those derived in the prior trials with a different assay format. These phase 2 GMTs were around 5–sixfold higher than those of the comparative convalescent sera panel (GMT 321). Overall, post-dose 2, there was no significant difference in neutralization responses, or the kinetics of the responses, between the 50 and 100 μg groups, or between the two age cohorts. Nevertheless, the sponsor selected a 100 μg dose for the phase 3 clinical trial.

3.3 Clinical Phase 3 Efficacy Trial in Adults (18 Years of Age or Older), U.S.

On July 23rd, 2020, Moderna initiated dosing in their pivotal phase 3 efficacy trial with their mRNA-1273 vaccine formulated with LNP. The U.S. only study was designed as a randomized, placebo-controlled trial in persons at high risk of SARS-COV-2 infection or its complications, randomly assigned in a 1:1 ratio to receive two IM injections of mRNA-1273 (100 μg) or placebo, 28 days apart. The primary efficacy end point was prevention of SARS-COV-2 disease with onset at least 14 days after the second dose in participants who had not previously been infected with SARS-COV-2 (Baden et al. 2021). The primary endpoint for safety was the assessment of any events after immunization.

The study enrolled a total of 30,420 participants (15,210 in each intervention group). 2.2% had evidence (serologic, virologic or both) of SARS-COV-2 infection at baseline. A total of 17,774, 5065 and 7512 were enrolled who were 18 to < 65 (not at risk), 18 to < 65 (at risk) and ≥ 65 years of age, respectively. The most common local reactogenicity was pain at the injection site and was mostly mild to moderate in nature, and there were slightly more incidences after the second dose. Headache and fatigue were the two most common systemic AEs, mostly mild to moderate, and occurred more frequently after the second dose (79% systemic reactogenicity in vaccinees versus 36% in placebo). Systemic reactogenicity severity also increased after the second dose (15.8% grade 3), compared to the first (2.9% grade 3). Overall, younger adults reported more AEs than the older age range. SAEs occurred at the same rate in both study arms (0.6%) (Baden et al. 2021).

The primary efficacy endpoint was successfully met with 94.1% (95% CI, 89.3 to 96.8%; $P < 0.001$) protection against virologically confirmed symptomatic SARS-COV-2 disease after two doses. Point estimates of efficacy were comparable across other endpoints: 18 to < 65 years (95.6%), ≥ 65 years (86.4%) and sex (male 95.4% and female 93.1%). Assessing efficacy from 14 days after the first dose showed 95.2% protection. This should not be confused with Pfizer-BioNTech's reported efficacy between the first and second dose (52%) which is an actual measurement of efficacy after just a single dose. Thirty cases of severe disease were captured during the study and all occurred in the placebo arm, equivalent to 100% efficacy (Baden et al. 2021). Given the demonstration of high efficacy, only transient local and systemic reactions and no safety concerns identified based on a 2-month median exposure safety database from the phase 3 study in 15,000 vaccines, the U.S. FDA granted Emergency Use Authorization.

3.4 Post-Licensure Effectiveness, Safety Surveillance and Boosting

Neutralizing responses against VOC/VOI using sera from persons vaccinated with Moderna's mRNA-1273 have been assessed and are generally comparable to the ancestral strain or variably diminished against particular variants, most significantly against Beta (van Gils et al. 2021; Wu et al. 2021). However, comparable to the situation with BNT162b2, field effectiveness studies have consistently demonstrated that mRNA-1273 provides high levels of protection against the Beta strain and other prominent variants (IVAC 2021). Overall, across multiple effectiveness studies in different countries, different populations and an array of different virus strains, mRNA-1273 demonstrates a high level of protection particularly against severer disease.

In parallel, Moderna nevertheless generated a new VOC candidate vaccine with the same mRNA and LNP technology, by switch in the S protein of the Beta variant (called mRNA-1273.351). Clinical trial participants who previously received a two-dose primary series of mRNA-1273 approximately 6 months earlier entered an open-label phase 2a study (NCT04405076) to evaluate the safety and immunogenicity of a single booster dose of mRNA-1273 (50 μg), modified mRNA-1273.351 (20 or 50 μg) or a bivalent mRNA-1273.211 (a 1:1 mix of mRNA-1273 [25 μg] and mRNA-1273.351 [25 μg]) (Choi et al. 2021). Using a pseudovirus neutralization assay, the immune responses after boosting were compared to the levels of neutralization after the primary schedule and just prior to boosting. Overall, all three booster mRNA vaccines elicited strong anamnestic responses, suggesting the existence of a robust memory response generated after the first two priming doses given 6 months earlier. These increased titres were achieved regardless of the variant sequence used to derive the S protein immunogen. Interestingly, the mRNA-1273 vaccine (50 μg)

with the original S protein immunogen performed well as a third dose booster eliciting significantly higher GMTs against all VOC compared to levels just prior to boosting: Beta 32-fold, Gamma 43.6-fold and Delta 42.3-fold higher. This suggests that although recipients of the mRNA-1273 vaccine had neutralizing responses that had waned after the primary two-dose schedule, particularly against the Beta strain, a third booster dose with mRNA-1273 was able to drive the breadth of neutralization in addition to elevated levels of functional antibody, hallmarks of immune maturation and memory (Choi et al. 2021). Importantly, mRNA-1273 at 50 μg (half the dose of the primary schedule) as a third booster dose was able to elicit potent neutralizing responses against the globally dominant Delta strain (GMTs 1268 two weeks post-dose 3 versus 30 six months after the primary series) (Choi et al. 2021). On the October 14th, 2021, Moderna received approval from the U.S. FDA for their booster third dose with 50 μg of mRNA for individuals aged 65 and older as well as individuals aged 18 through 64 at high risk of contracting COVID-19 (Moderna/Press-release 2021).

Subsequent to Emergency Use Authorization, mRNA-1273 has been monitored through surveillance networks to capture any safety events not detected during the clinical trials (Klein et al. 2021). mRNA-1273 has been associated with an elevated risk of myocarditis/pericarditis related to age and gender, although it should be noted the age indication is from 18 years of age, whereas Pfizer-BioNTech's BNT162b is from 5 years of age (US/CDC/VAERS 2021). The U.S. C.D.C. continues to recommend that mRNA vaccines against SARS-COV-2 be used given that the benefits far outweigh the potential risks of having a rare adverse reaction to vaccination, including the possible risk of myocarditis or pericarditis (US/CDC/Clinical-considerations 2021).

4 CureVac CVnCoV

On March 15th, 2020, CureVac officially entered the race for a SARS-COV-2 vaccine using their unmodified mRNA in an LNP formulation, called CVnCoV, that expresses a stabilized form of SARS-COV-2 spike S protein. Their development proceeded slower than Pfizer-BioNTech and Moderna and received authorization from the Belgian and German regulators to initiate a phase 1 trial on June 17th, 2020. Their phase 2b/3 efficacy trial commenced on December 14th, 2020 and provided data around six months later on June 30th, 2021. However, on October 12th, 2021, the company decided to withdraw CVnCoV from regulatory review claiming overlapping approval timelines for a second-generation candidate vaccine in development, although the decision was likely driven by additional considerations related to their efficacy results discussed below (Curevac/Press-release 2021).

4.1 Clinical Phase 1 Trial (18–60 Years of Age), Germany and Belgium

The phase 1 study in healthy participants (aged 18–60 years) assessed two IM administrations 28 days apart of 2, 4, 6, 8 and 12 μg of unmodified mRNA in an LNP formulation or placebo. Tolerability and safety monitoring did not identify any concerns during the trial. Focussing on seronegatives at baseline to SARS-COV-2, there was a dose-dependent increase in local and systemic AEs in both incidence and severity. The majority of local events were mild-to-moderate injection site pain. The frequency of systemic AEs was comparable after the first and second doses but severity increased after the second dose, notably in grade 3 severe events in the 12 μg group (3 out of 28 post-dose 1 versus 9 out of 26 post-dose 2). The most common solicited systemic AEs were headache (39–88% of vaccine groups versus 33% of placebo) and fatigue (34–88% of vaccines versus 42% of placebo) after the first doses, and rates were comparable after the second dose. Fever was most frequently reported in the two highest dose groups, after the first dose 29% (3% severe) and 38% (4% severe) in the 8 and 12 μg groups, respectively, and after the second dose 34% (9% severe) and 52% (13% severe) in 8 and 12 μg groups, respectively. As observed during the Pfizer-BioNTech phase 1 trial in Germany (NCT04380701), there was evidence of transient lymphopenia in the majority of a subset analysed the day after vaccination (Kremsner et al. 2021).

Looking at functional antibody responses, measured using a microneutralization assay with live virus, there were mostly non-responders or low-level responders after a single dose. After the second dose, titres were higher and peaked at the day 43 sampling time point (7 days of post-dose 2) but overall, the neutralizing responses were underwhelming at all doses and there was little evidence of a dose–response curve. Compared to a convalescent sera reference panel (GMT 113), post-dose 2 responses at all vaccine doses had lower GMTs: 48 (2 μg), 40 (4 μg), 20 (6 μg), 57 (8 μg) and 57 (12 μg). A significant proportion of response levels at each dosage level were in the lower quartile ranges of the reference panel measurements. S-specific IgG titres appeared more robust, suggesting that only a subset of these antibodies was driving functional neutralizing responses (Kremsner et al. 2021). An older adult age group (> 60 years) were not included in this phase 1 trial. In the absence of a correlate of protection at that time, a basic predictive model assessed available field efficacy data against neutralization responses calibrated as a ratio against human convalescent sera panel measurements (Earle et al. 2021). Overall, the notably lower neutralization responses elicited by CVnCoV in the phase 1 study, judged against this predictive model, raised questions in the field whether or not the vaccine would ultimately result in the high efficacy seen with mRNA-1273 and BNT162b. CureVac concluded on a dose selection of 12 μg of mRNA for the subsequent phase 2b/3 trial.

4.2 Clinical Phase 2b/3 Efficacy Trial (18 Years of Age and Over), Multi-Country

The pivotal phase 2b/3 efficacy trial was a placebo-controlled study in 10 countries in Europe and South America. Healthy SARS-COV-2 seronegative adults aged 18 years and over were randomized 1:1 to receive either two doses of CVnCoV containing 12 μg of mRNA or placebo 28 days apart, intramuscularly (Kremsner 2021). There were two co-primary endpoints, to demonstrate the efficacy of a 2-dose schedule of CVnCoV in the prevention of first episodes of virologically confirmed SARS-COV-2 cases of any severity in SARS-COV-2 naïve subjects and to demonstrate the same for confirmed moderate-to-severe cases (Curevac/Protocol 2021). A total of 19,783 participants received CVnCoV and 19,746 received a placebo. The first 4000 participants were enrolled in the phase 2b part of the trial, and the first 600 participants in each age group (18–60 and ≥ 61 years) were included in the immunogenicity subset (data not yet available).

For the primary efficacy endpoint, protection was 48.2% (95% CI: 31.0–61.4) in the overall population (Kremsner 2021). By age, 18–60-year-olds, the point estimate for efficacy was 52.5% (95% CI: 36.2–64.8). For subjects over ≥ 61 years of age, the authors claim there were too few cases reported (CVnCoV: 12, placebo: 9) to evaluate vaccine efficacy but the case split is suggestive of low efficacy. Efficacy against moderate-to-severe COVID-19 was 70.7% (95% CI: 42.5–86.1) in the overall population. The number of severe cases observed (CvnCoV: 4, placebo: 10) was considered insufficient to evaluate vaccine efficacy (Kremsner 2021).

During the follow-up period of disease case capture, there was an evolving regional and global dynamic for the circulation of SARS-COV-2 variants and the protocol was amended accordingly to address the occurrence of confirmed cases caused by VOC/VOI. Vaccine efficacy was 55% [95% CI: 24–74]) against Alpha, 67% [95% CI: 30–85] against Gamma, 53% [95% CI: 8–76] against Lambda and 41.5% against B1.621 variants (Kremsner 2021). Given that neutralization responses elicited by CvnCoV would probably be reduced against viral variants and therefore have a greater sensitivity to any reduction given already lower neutralization responses against the reference strain, and because there were only 7 cases (out of 204 adjudicated cases with virus sequence data) caused by the reference strain during the efficacy trial, provides in totality a hypothesis for the observed efficacy rates (Cromer et al. 2021).

Solicited AEs were assessed in the phase 2b part of the trial, and the primary endpoint for both trials was to assess the safety of CVnCoV in all subjects. Injection site pain was the most common local AE (84%) and fatigue (80%)/headache (77%) the most common systemic AEs. Although the incidence of solicited AEs was higher in the CVnCoV arm, they were transient and mostly mild to moderate in nature. Overall, solicited AEs with a grade 3 severity were reported in 27% of vaccines and 3% of placebo. There was no increase in solicited reactions between the first and second CVnCoV dose. SAEs, still blinded in the publication, were detected at a rate of 0.4% in vaccines and 0.3% in placebo recipients (Kremsner 2021).

In conclusion, two doses of the unmodified mRNA/LNP CVnCoV candidate vaccine had an acceptable tolerability and apparent safety profile and offered modest protection against SARS-COV-2 of any severity and a higher point estimate of efficacy against moderate/severe manifestations of disease. Vaccine efficacy by viral variant was largely comparable to the overall point estimate of protection given the confidence interval ranges.

5 Overall Interpretation of Safety and Efficacy Data for mRNA-LNP Vaccines Against SARS-COV-2

Unfortunately, comparing estimates of vaccine efficacy between clinical trials is tenuous given a variety of variables between studies such as different protocols, primary endpoints, case definitions, study populations, participant risk exposure characteristics and statistical methods. Therefore, any attempt to make comparisons between our three mRNA/LNP vaccine examples should be considered notional and further confounded by multiple unknown aspects related to the actual mRNA designs and LNP formulations. With this in mind, one can argue that the point estimates of efficacy for the modified mRNA/LNP vaccines, after two doses, produced by Moderna and Pfizer-BioNTech, 94% (95% CI: 89–97) and 95% (95% CI: 90–98), respectively, are higher than all other available efficacy results with other platform technologies: Sinovac (inactivated killed whole virus vaccine, 2 doses) 83.5% (95% CI: 65–92), Gamaleya (heterologous viral vector vaccines, 2doses) 91% (95% CI: 84–95), AstraZeneca (viral vector vaccine, 2 doses) 74.% (95% CI: 65–80.5), J&J Janssen (viral vector vaccine, 1 dose) 67% (95% CI: 59–73), Sinopharm (inactivated killed whole virus vaccine, 2 doses) 78% (65–86%) and Novavax (protein / adjuvant vaccine, 2 doses) 89.7% (95% CI: 80–95) (Tanriover et al. 2021; Logunov et al. 2021; Falsey et al. 2021; Sadoff et al. 2021; Al Kaabi et al. 2021; Heath et al. 2021). However, the 95% confidence intervals around the estimates all overlap with Pfizer-BioNTech and Moderna's efficacy result except for AstraZeneca, Sinopharm and J&J (one dose schedule). The unmodified mRNA/LNP candidate vaccine, after 2 doses, produced by CureVac, has the lowest point estimate of efficacy, 48.2% (95% CI: 31.0–61.4); it is tempting to conclude that the difference in the modified and unmodified nucleotide approaches is the cause for the differences in efficacy among our mRNA vaccine examples, but there are many other known and unknown potential confounding factors about the CureVac mRNA construct and the LNP formulation that prevent any definitive conclusion on the value of modification.

In totality, the modified mRNA vaccines of Pfizer-BioNTech and Moderna, formulated with comparable LNPs, both expressing a full-length stabilized S protein immunogen, were able to elicit robust neutralizing humoral immune responses, that were associated with significant protection against SARS-COV-2. The vaccines can elicit T cell immunity, but these appear relatively modest compared to other vaccine platforms (Gilbert 2012).

The unmodified mRNA vaccine of CureVac, formulated with an LNP using an ionizable lipid, elicited markedly lower neutralizing responses, although it should be stated that comparing results from different assays in different laboratories is questionable at best. Nevertheless, it does allow one to hypothesize that the lower functional humoral responses compared to the convalescent sera panel used in the same assay translate into the lower point estimates of efficacy for this candidate mRNA vaccine. Future correlates of protection studies will help elucidate this topic.

For tolerability and safety assessments, there is a remarkable similarity; in that, all three of the mRNA/LNP vaccines of Pfizer-BioNTech, Moderna and CureVac, all result in injection site pain, fatigue and headache as the most frequent AEs, all transient and generally short lived. There is a gross pattern for increased AEs after the second dose compared to the first, suggesting a more potentiated inflammatory immune response to the later dose. The younger age ranges also appeared to have a greater sensitivity to these responses. There were examples in which the highest doses of mRNA tested (100 μg—Pfizer, 250 μg—Moderna) were stopped during clinical development because the elicited AEs were judged unacceptable against no clear justifiable immunological advantage. Lymphopenia was seen soon after mRNA immunization in certain trials, and this corroborates the hypothesis that significant innate responses to vaccination induce a temporal redirection of systemic lymphocytes to the point(s) of vaccine-associated inflammation. There were SAEs reported during these clinical trials that were considered related to vaccination but there were no major imbalances or clusters that prevented EUA or full approval in the case of Pfizer-BioNTech.

However, post-licensure safety surveillance has revealed an excess of myocarditis/pericarditis following mRNA/LNP vaccination using the Pfizer and Moderna vaccines particularly clustered in younger males after a second dose. Myocarditis is an inflammatory disease of the myocardium caused by a variety of infectious and non-infectious conditions, while pericarditis is inflammation of the pericardium surrounding the heart (Caforio et al. 2013). The precise pathophysiology of these conditions is not fully understood but potential causes include hyper-inflammation, dysregulated immune pathways, molecular mimicry and immunopathology (Furqan et al. 2021; Huber 2016). There is no proven mechanism of action to explain the association with mRNA-LNP vaccination. One can postulate that despite the modified mRNA design of these vaccines to reduce innate sensing, certain individuals with rare existing predisposition(s) that sensitize them to aberrant innate and adaptive immune response reactions are prone to the mRNA vaccine-related cascade of systemic immunological events that ultimately reach and disrupt the myocardium and/or pericardium (Bozkurt et al. 2021; Larson et al. 2021). The U.S. Advisory Committee on Immunization Practices concluded that the benefit of using the SARS-COV-2 mRNA-LNP vaccines still outweighed the risk posed to younger individuals (Gargano et al. 2021).

6 Other mRNA SARS-COV-2 Candidate Vaccines

There are at least ten mRNA candidate SARS-COV-2 vaccines in clinical development, that have not yet demonstrated efficacy or late development immunological bridging for the purposes of seeking licensure (Table 1). The two most advanced Walvax/Abogen and Gennova/HDT Bio are developing NRM and SAM candidate vaccine, respectively. All the developers appear to be using LNPs, except for Gennova that are uniquely using a proprietary lipid inorganic nanoparticle that attaches the mRNA to the nano-lipid carrier's surface through adsorption chemistry (Gennova, 2021). Several SAM technologies are under evaluation at varying stages of development; however, the available clinical data to date suggest a lower level of humoral immunogenicity elicited against SARS-COV-2 compared grossly to NRM (Pollock 2021; Low et al. 2021).

Arcturus's phase 1/2 trial compared 1 or 2 doses (28 days apart) of their SAM/LNP at varying doses of mRNA and demonstrated a high proportion of non-responders or responders with low antibody titres using a live virus neutralization assay. The second dose, at 5.0 μg of mRNA, showed a trend for increased titres compared to the first. The highest peak GMT (51.8) elicited in the trial was seen 14 days after a second 5.0 μg dose in younger adults (Low et al. 2021). The comparator convalescent sera panel gave a significantly higher range of titres. Imperial College evaluated their SAM/LNP candidate vaccine in health young adults who received two IM injections at six different dose levels of mRNA, 0.1–10 μg, given four weeks apart. Measuring antibody neutralization with a pseudovirus assay, there were a significant proportion of non-responders or responders with low titres. For example, at the highest three doses, 2.5, 5 and 10 μg, four weeks after the second dose, the serological responder rate/GMTs were 43%/79, 48%/40 and 52%/124, respectively. The range of titres were low compared to the titre range of the convalescent sera panel (Pollock 2021). The prevailing theory in the field to explain these current observations with SAM versus NRM is that the former triggers a higher proportion and/or magnitude of intracellular innate sensing pathways that rapidly shut down any self-amplification, ultimately resulting in a low level of immunogen expression. These results are also a reminder of the ongoing challenge of translating more promising preclinical results to clinical studies (McKay et al. 2020).

7 Clinical-Stage mRNA Candidate Vaccines Against Other Non-COVID-19 Infectious Diseases

Beyond SARS-COV-2, there are at least 17 clinical studies that have, or are, investigating mRNA vaccines against other infectious diseases (Table 2). It is likely that several of these candidate vaccines are no longer actively advancing through development and are legacy projects. There are no clinical stage mRNA vaccines yet against bacterial diseases, and this partly relates to the highly complex nature of their outer

Table 1 Other mRNA candidate SARS-COV-2 vaccines in clinical development yet to complete late development

Developer(s)	mRNA technology	Delivery technology	Immunogen	Dose	Clinical development stage	Clinical trial ID
Walvax/Abogen	NRM	LNP	Receptor binding domain	15 μg	Phase 3	NCT04847102 ChiCTR2000034112 ChiCTR2000039212 ChiCTR2100041855
Gennova/HDT bio	SAM	Lipid inorganic nanoparticle	Full-length S	Not known	Phase 2/3	CTRI/2021/04/032688
Arcturus	SAM	Lipid-mediated	Full-length S[a]	5 μg	*Phase 1/2*	NCT05012943
Translate bio/Sanofi Pasteur	NRM (unmodified)	LNP	Full-length S	7.5, 15, 45 μg	Phase 1/2	NCT04798027
Takeda	Moderna's mRNA	Moderna's LNP	Full-length spike	Not known	Phase 1/2	NCT04677660
ChulaCov19/Chulalongkorn University	NRM (modified)	LNP	Full-length spike	10 μg, 25 μg, 50 μg	Phase 1/2	NCT04566276
Providence therapeutics	NRM (modified)	LNP	Full-length spike	40 μg	Phase 1/2	NCT04765436
Imperial College	SAM	LNP	Full-length S	0.1-10 μg	Phase 1	ISRCTN17072692, EudraCT 2020–001,646-2
Gritstone Bio	SAM	LNP	Full-length spike + T cell Epitopes	1, 3, 6, 10 & 30 μg	Phase 1	NCT04776317
GSK	SAM	LNP	Full-length spike	1 μg	Phase 1	NCT04758962

[a] ARCT-021 ancestral strain and ARCT-154 targeting variants of concern or variants of interest

Table 2 mRNA candidate vaccines in clinical development against non-SARS-COV-2 infectious diseases

Developer(s)	mRNA technology	Delivery technology	Target indication	Clinical development stage	Trial identifier(s)
Moderna	mRNA-1647 NRM (modified)	LNP	CMV	Phase 2	NCT04975893 NCT03382405 NCT04232280
Moderna	mRNA-1893 NRM (modified)	LNP	Zika	Phase 2	NCT04917861 NCT04064905
Moderna	mRNA-1010 NRM (modified)	LNP	Seasonal influenza quadrivalent	Phase 1/2	NCT04956575
Moderna	mRNA-1440 NRM (modified)	LNP	Pandemic influenza (H10)	Phase 1	NCT03076385
Moderna	mRNA-1851 NRM (modified)	LNP	Pandemic influenza (H7)	Phase 1	NCT03345043
Translate Bio/Sanofi	MRT5400 and MRT5401 NRM (unmodified)	Two differing LNPs	Seasonal influenza monovalent	Phase 1	Not known[a]
Moderna	mRNA-1345 NRM (modified)	LNP	RSV	Phase 1	NCT04528719
Moderna/Merck	mRNA-1777 NRM (modified)	lipid nanoparticle (Merck)	RSV	Phase 1	Not Known[b]
Moderna/Merck	mRNA-1172 NRM (modified)	lipid nanoparticle (Merck)	RSV	Phase 1	Not known[c]
Moderna	mRNA-1653 NRM (modified)	LNP	hMPV/PIV-3	Phase 1	NCT03392389 NCT04144348
Moderna	mRNA-1325 NRM (modified)	LNP	Zika	Phase 1	NCT03014089
Moderna	mRNA-1944[c] NRM (modified)	LNP	Chikungunya	Phase 1	NCT03829384
Moderna	mRNA-1388 NRM (modified)	LNP	Chikungunya	Phase 1	NCT03325075

(continued)

Table 2 (continued)

Developer(s)	mRNA technology	Delivery technology	Target indication	Clinical development stage	Trial identifier(s)
GSK	CV7201 NRM (unmodified)	Complex with RNActive	Rabies	Phase 1	NCT02241135
CureVac	CV7202 NRM (unmodified)	LNP	Rabies	Phase 1	NCT03713086
CureVac	GSK3903133A SAM	Cationic emulsion	Rabies	Phase 1	NCT04062669
Moderna	mRNA-1644 NRM (modified)	LNP	HIV-1	Phase 1	NCT05001373

[a]Sanofi, Vaccines Investor Event, Part 2, 'Leading with innovation,' December 1st, 2021
[b]Moderna Press Release, Moderna Announces Updates on RSV Vaccine Programme, October 8, 2020
[c]Encodes an antibody not an immunogen and is delivered by IV injection. It is not clear if prophylactic application is being targeted

membrane proteins and/or the need for chaperone proteins. However, there are several preclinical staged mRNA vaccines targeting bacteria not covered here (Blakney et al. 2021). All the clinical stage studies are investigating NRM, except one SAM against Rabies. All are IM delivered, except one given by IV administration because it was expressing an anti-cytomegalovirus (CMV) antibody and therefore required much higher amounts of mRNA to be administered. Arguably, the most advanced candidate vaccine beyond SARS-COV-2 is currently Moderna's CMV vaccine being tested in a large phase 2 study in healthy adults (3 dose schedule). A similar amount of mRNA is being tested (50 versus 100 μg) as that explored and licenced in their SARS-COV-2 programme. The company is preparing for a phase 3 trial in at least 8000 participants (Moderna/Press-release 2021a). It is also important to note that their CMV vaccine requires 6 mRNA constructs formulated in LNP to express all the required immunogen components, making it one of the most complex high valency mRNA vaccines in the field. Moreover, it is a lyophilized formulation claimed to have over 18 months shelf-life when stored at 5 °C (Modern/Investor-relations 2021a). Overall, given the momentum behind mRNA as a technology for vaccines it is very likely that the volume of clinical stage candidates will substantially increase in the coming years.

8 Future mRNA Technology Advances

The totality of data in the vaccine field has demonstrated the remarkable scientific, technical and medical benefits of mRNA and LNP technologies as 'tools' for prophylaxis. Nevertheless, several important areas have been identified that need to be improved, or confirmed, to allow an even greater utility and acceptance of this vaccine platform against infectious diseases: superior thermal stability, higher manufacturing productivity, reduced bottlenecks in raw materials, lower doses, less frequent immunizations and an improved safety profile. From a development perspective, there is also an important need to generate sufficient data that will allow regulators to approve the use of mRNA as a true platform technology adaptable for multiple disease indications.

One of the highest priorities for manufacturing is superior productivity to drive down costs and ensure greater access to mRNA vaccines for all vulnerable populations (WHO/Joint-Press-Release 2021). Multiple factors impact overall productivity, namely the amount of mRNA per dose, the scale of production, yields, raw material availability and costs, downstream purification losses, capital investment and vial or container costs (Kis et al. 2020). General benchmarks for production yields are around 3–5 g of mRNA per litre of reaction, and future innovations could increase this to + 10 g per litre (Baronti et al. 2018). Given the current wide array of different methods used today for purification that all have certain limitations, it is anticipated that the field will improve purification methods that increase end yields (Lukavsky and Puglisi 2004; Rosa et al. 2021; Baiersdörfer et al. 2019). An area likely to offer improved efficiencies is the application of continuous manufacturing through the complete end-to-end integration of operations. In a commercial setting, this offers distinct flexibility in batch sizes to align with volume needs, reduced lead times, high compatibility with automation and overall proficiency (Kapoor et al. 2021). Innovation in the sourcing and re-cycling of raw materials has significant potential compared to the current use of such materials. As discussed above, these advances in manufacturing will likely be associated with optimized formulations and processes to improve thermal stability of mRNA, for example, through lyophilization or alternative methods of drying mRNA/LNP formulations (Uddin and Roni 2021).

The tolerability of mRNA/LNP vaccines appears acceptable for potential new infectious disease targets but the excess of myocarditis/pericarditis especially in adolescent or young adult males after a second dose will need to be addressed by vaccine developers (Mevorach et al. 2021; Witberg et al. 2021). While the benefit/risk ratio is acceptable in the context of SARS-COV-2, the safety observations are likely to hinder application against other pathogens when other platforms can prevent the disease without the same risk. One area of investigation may be reduced doses of mRNA.

New carrier delivery approaches to improve or replace LNPs are emerging (Hassett et al. 2019; Pan et al. 2019; Igyártó et al. 2021). Iterative investigations of novel ionizable lipids are likely to incrementally enhance the efficiency of LNPs, and the promising incorporation of ligands into LNPs may improve cellular and tissue

targeting (Witzigmann et al. 2020; Tam et al. 2013). Relevant for vaccines, the use of a specific ligand on liposomes facilitated their specific uptake by Langerhans cells in vitro (Schulze et al. 2019). Mannosylated and histidylated lipopolyplexes have shown superior uptake in vitro by a murine dendritic cell line (Perche et al. 2011). New routes of delivery, such as intranasal administration, may promote engagement of the mucosal arms of the immune system against certain respiratory pathogens (Buschmann et al. 2021).

With high certainty, one can expect to see further efforts to optimize mRNA constructs based on the pioneering understanding of how important aspects of the mRNA sequence, structure and design can improve performance. Innovation in circularized mRNA (circRNA) illustrates these opportunities. An unmodified circRNA was able to circumvent RNA sensing in RIG-I and TLR competent cells, as well as in mice, and promote stable expression (Wesselhoeft et al. 2019, 2018). Already in the context of a SARS-COV-2 vaccine, circRNA encoding a trimeric form of the receptor-binding domain with an LNP elicited humoral and cellular immune responses in mice (Qu et al. 2021).

225 years represent the period from the advent of modern vaccination in 1796 when Edward Jenner used a crude biopsy sample from a cowpox lesion to inoculate and prevent smallpox, to the 2020 demonstration that mRNA formulated with a LNP can protect against SARS-COV-2. The profound public health benefit of vaccination has acquired a new proven platform technology of important future potential.

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Pulmonary Delivery of Messenger RNA (mRNA) Therapeutics for Respiratory Diseases

Yingshan Qiu, Michael Yee-Tak Chow, and Jenny Ka-Wing Lam

Contents

Abstract With the advances in biotechnology, messenger RNA (mRNA) therapeutics have provided new and exciting opportunities for the treatment and prevention of a wide range of respiratory diseases. The potential applications of mRNAs include lung infections, genetic disorders (e.g., cystic fibrosis), inflammatory diseases (e.g., asthma), and vaccines. However, the development of mRNA therapeutics is hindered by its delivery and cellular uptake due to its large molecular size, high negative charge, hydrophilicity, and poor stability. Currently, the administration of mRNA therapeutics is limited to parenteral injection. Pulmonary delivery offers the advantages of non-invasive route of administration and direct application of mRNA at the site of action in the lung so that a lower dose is required with reduced systemic toxicity. A variety of delivery platforms are being developed to deliver mRNA therapeutics for lung diseases through the pulmonary route. This chapter summarizes and discusses (i) different delivery strategies, including lipid nanoparticles (LNPs), polymers, peptides, and chemical modification for pulmonary mRNA delivery; and (ii) the potential clinical applications of inhaled mRNA therapies.

Y. Qiu · M. Y.-T. Chow · J. K.-W. Lam (✉)
Department of Pharmacology and Pharmacy, LKS Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR
e-mail: jkwlam@hku.hk

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_7

Keywords Inhalation · Lipid nanoparticles · Modified mRNA · Pulmonary delivery · Vaccines

1 Introduction

Respiratory diseases such as lung infections and severe asthma are enormous global health and economic burdens, and there is no effective treatment for many of these including cystic fibrosis (CF), a genetic disorder that affects mainly the lung. Messenger RNA (mRNA) is a powerful biomolecule with huge potential for the treatment of various respiratory diseases. It can induce or augment the production of therapeutic proteins, for example, regulatory protein to reduce airway inflammation for the treatment of severe asthma (Zeyer et al. 2016), or cystic fibrosis transmembrane conductance regulator (CTFR) for the treatment of CF (Haque et al. 2018; Robinson et al. 2018). mRNA can also be used for the treatment or prophylaxis of lung infections by encoding neutralizing antibodies to block the entry of the pathogens (Van Hoecke and Roose 2019) or by encoding antigens (Haabeth et al. 2021) to elicit immunity against the pathogens. With the advancement of gene editing technology, mRNA encoding Cas13 protein for RNA editing can be employed to produce antiviral effect (Freije et al. 2019). However, delivery remains one of the major obstacles for the translation of mRNA therapy from bench to bedside, which involves the transportation of RNA molecules to the sites of action in a safe, effective, and reproducible manner. Lipid nanoparticles (LNPs) are the most widely studied RNA delivery systems in clinical applications, including the two approved mRNA vaccines against COVID-19, tozinameran (COMIRNATY®, developed by BioNTech/Pfizer) and elasomeran (SPIKEVAX®, developed by Moderna), and the first small interfering RNA (siRNA) therapeutics, patisiran (ONPATTRO®, developed by Alnylam Pharmaceuticals) for the treatment of polyneuropathy caused by hereditary transthyretin amyloidosis. However, the administration of these approved RNA therapeutics is limited to parenteral injection, which often has a poor distribution to the lung, rendering the delivery strategy suboptimal for the treatment of lung-related diseases. In contrast, pulmonary delivery can increase local concentration of RNA therapeutics in the airways to enhance efficacy in a non-invasive manner while minimizing systemic exposure, and thereby side effects. This chapter gives an overview of the latest development of various mRNA delivery systems that are specifically designed for pulmonary delivery, and the use of inhaled mRNA strategy for the treatment and prevention of different respiratory diseases.

2 Pulmonary RNA Delivery Systems

For successful pulmonary delivery of mRNA, there are a series of extracellular and intracellular barriers in the lung to be overcome (Chow et al. 2021; Sanders et al.

2009). Human respiratory tract is highly branched which promotes premature particle deposition in the upper airways through mechanical impaction. The presence of pulmonary lining fluid comprising of primarily mucus and pulmonary surfactants in the airway may interact undesirably with mRNA molecules and their delivery systems (Duncan et al. 1997). Mucociliary clearance and cough clearance serve as innate defense mechanisms to remove inhaled particles. Upon deposition, mRNA therapeutics must be recognized and taken up by target cells, as they are simultaneously subjected to enzymatic degradation and phagocytosis by alveolar macrophages, both of which result in mRNA inactivation. After entering the cells, mRNA molecules must escape from endosomal/lysosomal degradation and be released in the cytoplasm where mRNA is translated into the target protein. The high negative charge and large molecular size of mRNA prevent it from permeating through the cell membrane. Thus, an efficient and safe delivery system is one of the crucial factors in achieving successful mRNA transfection in the lung by promoting its cellular uptake and protecting it from premature nuclease degradation. Delivery systems of nucleic acids can be broadly classified into viral vectors and non-viral vectors. As nuclear entry is not required for mRNA translation, non-viral vectors are preferred due to the better safety profile, reduced immunogenicity, and lower production cost compared to their viral counterparts.

2.1 Lipid-Based Delivery System

Lipid-based delivery systems are the most popular delivery systems investigated for mRNA delivery due to their good biocompatibility, high transfection efficiency, and relatively simple synthesis. Cationic lipids with permanent positive charges have been used for nucleic acid transfection for a long time. They interact with the negatively charged nucleic acid to form lipoplexes with good in vitro delivery efficiency. However, lipoplexes tend to have large particle size, poor stability, and high toxicity, rendering them unsuitable for in vivo applications (Lv et al. 2006). Liposomes, which are defined as spherical vesicles of at least one phospholipid bilayer with an aqueous core, have been studied for the delivery of a wide range of therapeutics including mRNA. However, their RNA encapsulation efficiency is generally poor.

With the recent success of LNPs in delivering RNA therapeutics including the mRNA vaccines COMIRNATY® and SPIKEVAX®, and siRNA therapeutics ONPATTRO®, LNPs have become the focus of mRNA delivery system development. LNPs are evolved from liposomes but are structurally distinct from them. The key difference is that LNPs does not consist of a lipid bilayer surrounding an aqueous core. LNPs typically consist of four major components: (i) ionizable lipids to package mRNAs through electrostatic interactions; (ii) neutral helper lipids (e.g., zwitterionic phospholipids) to promote cell binding and transfection efficiency; (iii) cholesterol to maintain structural stability; and (iv) PEGylated lipids to provide colloidal stability and reduce opsonization (Fig. 1) (Guevara et al. 2020; Cullis and Hope 2017). The ionizable lipid is a critical component of LNP, and it determines the efficiency of

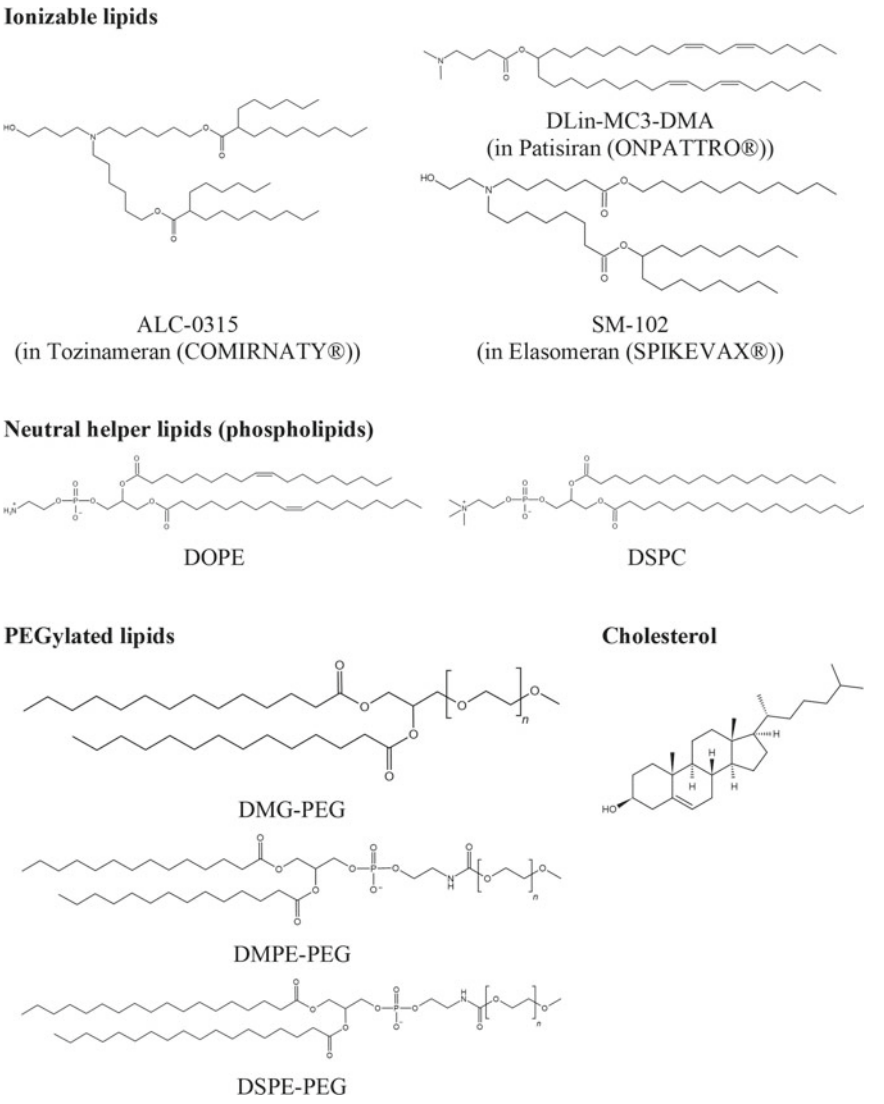

Fig. 1 Examples of lipids used in the preparation of LNPs and their chemical structures. ALC-0315, [(4-Hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate); DLin-MC3-DMA, [(6*Z*,9*Z*,28*Z*,31*Z*)-heptatriaconta-6,9,28,31-tetraen-19-yl] 4-(dimethylamino) butanoate; DMG-PEG, 1,2-dimyristoyl-sn-glycero-3-methoxypolyethylene glycol; DMPE-PEG, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxypolyethylene glycol; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxypolyethylene glycol; SM-102, 9-heptadecanyl 8-{(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino}octanoate

LNP. Instead of having a permanent cationic charge, the ionizable lipid is protonated at low pH to become positively charged while remaining neutral at physiological pH. This improves the biocompatibility of the lipids by reducing interaction with the cell membrane, with the additional advantage of promoting escape from the acidic endosomal environment (Hou et al. 2021). 1,2-dilinoleyloxy-*N*, *N*-dimethyl-3-aminopropane (Dlin-DMA) is an early generation of ionizable lipid designed for siRNA delivery. Through multiple rounds of optimization, [(6*Z*,9*Z*,28*Z*,31*Z*)-heptatriaconta-6,9,28,31-tetraen-19-yl] 4-(dimethylamino)butanoate (Dlin-MC3-DMA), also known as MC3 lipid, is developed with excellent transfection efficiency. MC3 lipid is employed in the formulation of patisiran (ONPATTRO®), the first siRNA therapeutics approved by the FDA. More recently, biodegradable lipids are introduced to improve the safety of LNPs, which are particularly important when multiple doses of mRNA are required. Examples of biodegradable ionizable lipids include [(4-Hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) (ALC-0315) and 9-heptadecanyl 8-{(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino}octanoate (SM-102), which are used in the COVID-19 mRNA vaccine formulations tozinameran (COMIRNATY®) and elasomeran (SPIKEVAX®), respectively.

In the past, LNPs were produced by a variety of methods including thin-film hydration, reverse phase evaporation, and double emulsion, which often require further particle size reduction and homogenization. These methods are time-consuming with relatively low entrapment efficiency (Barba et al. 2019). Nowadays, LNPs are usually produced by rapid mixing, in which lipids are first dissolved in ethanol phase whereas the RNAs are prepared in aqueous phase. With appropriate mixing conditions (e.g., pH and flow rate), high RNA loading efficiency with small nanoparticle size (below 100 nm) can be achieved. Recent advance in microfluidic technology enables LNPs to be prepared rapidly in a controllable, reproducible, and scalable manner (Shepherd et al. 2021).

While LNPs have enjoyed success in clinical applications, their administration is limited to parenteral injection and their effectiveness for pulmonary delivery is less well understood. Since many of the LNPs developed for RNA delivery are designed for systemic delivery and liver targeting, the rules for optimizing LNPs may not apply to pulmonary delivery. For instance, inhaler device such as nebulizer is required for delivering therapeutics to the lung. The introduction of high shearing force during nebulization may damage the integrity of the LNPs and mRNA, and the extent of the effect may depend on the condition and the type of nebulizer used. It is also speculated that the presence of pulmonary surfactants in the airways may influence the stability and the fate of inhaled LNPs (Garcia-Mouton et al. 2019). It was reported that lipoplexes consisting of a cationic lipid 1,2-dioleoyl-3- trimethylammonium-propane (DOTAP) and cholesterol at 2:3 molar ratio exhibited effective mRNA transfection in vivo following intratracheal administration (Van Hoecke et al. 2020). However, such a simple composition has led to poor colloidal stability. The particle size of these nanoparticles displayed a considerable increase from 183 to 339 nm after one hour of incubation in bronchoalveolar lavage fluid (BALF).

To explore the stability and delivery efficiency of LNPs for pulmonary administration through nebulization, a library of LNP compositions was screened using Design of Experiments (DOE) approach (Zhang et al. 2020). All the investigated LNPs consisted of the four main lipid components including ionizable lipid, phospholipid, cholesterol, and PEGylated lipid. The size and zeta potential of all formulations remained stable after 14 days of storage at 4 °C. However, the size of LNPs significantly decreased with increasing PEGylated lipid composition, which could be due to increased repulsion by the hydrophilic PEG. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-methyoxypolyethylene glycol (DSPE-PEG) was found to impair the stability of LNPs during nebulization whereas 1,2-dimyristoyl-rac-glycero-3- methoxypolyethylene glycol (DMG-PEG) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxypolyethylene glycol (DMPE-PEG) did not significantly affect LNP stability. On the other hand, increasing the molar ratio of PEGylated lipids significantly diminished the transfection efficiency, both before and after nebulization. In a separate study, a cluster-based approach was employed to identify the optimal composition of LNPs for nebulized mRNA delivery to the lung (Lokugamage et al. 2021). The study reaffirmed the importance of PEGylated lipids in stabilizing LNPs. In addition, it was observed that the interaction between helper lipids and PEG is critical in nebulized delivery. A low molar ratio of PEG improved the efficiency of LNPs that contained neutral helper lipids, whereas a high molar ratio of PEG was required for LNPs with cationic helper lipids. Overall, these studies have provided important insights on the use of LNPs for pulmonary mRNA delivery, which requires careful optimization of the lipid composition.

2.2 Polymer-Based Delivery System

Cationic polymers have been extensively investigated for mRNA delivery due to their high versatility and excellent transfection efficiency. However, toxicity remains one of the major concerns for their clinical application in the lung. Polyethylenimine (PEI) (Fig. 2) is one of the most promising synthetic cationic polymers that demonstrated effective transfection of nucleic acids (Ding et al. 2018; Okuda et al. 2019; Xie et al. 2016). Due to its high charge density, PEI has a strong affinity to nucleic acid to form polyplexes. Its wide buffering capacity enables the polyplexes to escape from endosomes through proton sponge effect although this hypothesis is still heavily debated (Benjaminsen et al. 2013). Nonetheless, its clinical development is hindered by its toxicity and concerns over accumulation in the body due to its nondegradable nature (Griesenbach et al. 2015). One attempt to overcome the toxicity issue is by PEGylation of PEI but with limited success. Although biocompatibility was improved and mucus clearance was minimized, PEGylation also led to the reduction of nucleic acid affinity, stability, and transfection efficiency (Kubczak et al. 2021).

A new class of biodegradable and cationic branched polymer called poly(beta-amino esters) (PBAEs), which consist of diacrylates, hydrophilic amines, and end-capping diamines as monomers, was developed for nebulized mRNA delivery (Patel

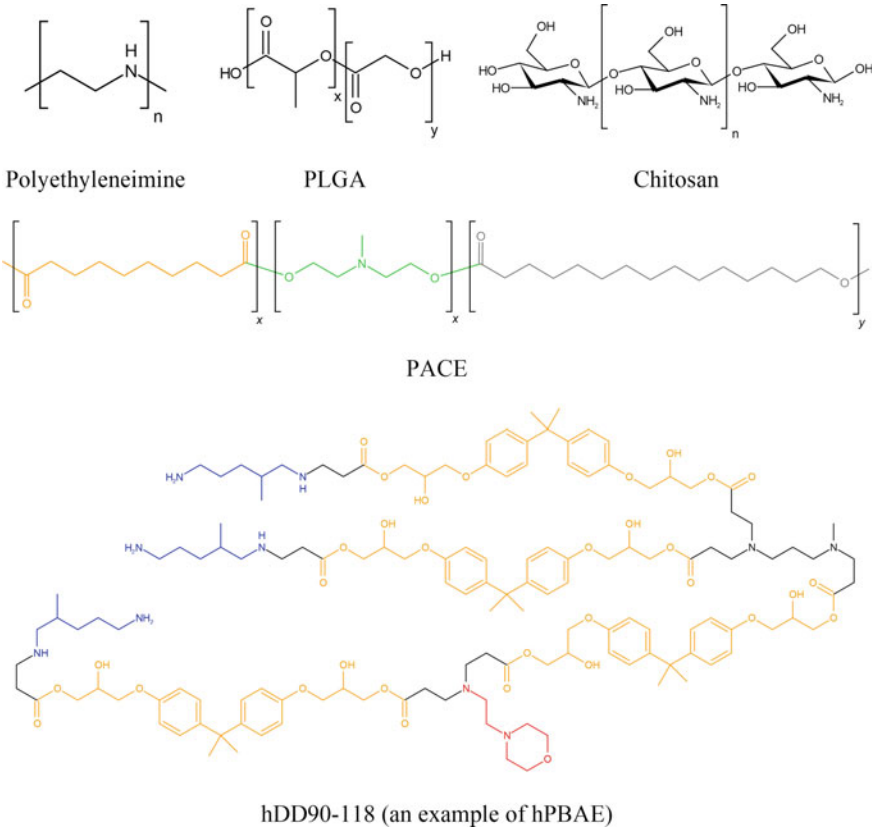

Fig. 2 Chemical structures of polyethyleneimine (PEI), poly(lactic-co-glycolic acid) (PLGA), chitosan, poly(amino-co-ester) (PACE), and hDD90-118, an example of hyperbranched poly(beta-amino ester) (hPBAE). PACE is made of monomers of sebacic acid (orange), methyldiethanolamine (green), and pentadecanolide (gray). hPBAE is made of monomers of diacrylates (orange), hydrophilic amines (red), and end-capping diamines (blue)

et al. 2019). The hyperbranched PBAE (hPBAE) (Fig. 2) nanoparticles could withstand high shearing force of nebulization and remain stable with particle size below 200 nm at high concentration without aggregation. These features are extremely crucial for nebulization therapy which often requires high drug concentration. The hPBAEs also demonstrated significantly higher luciferase mRNA expression in the lung of mice compared to branched PEI when delivered by nebulizer connected to a whole-body chamber. Moreover, hPBAEs were well tolerated in mice after repeated dosing of 1 mg of mRNA every three days for nine days. This delivery platform was also applied to the gene editing clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system by delivering mRNA encoding Cas13a and RNA guides to the lung of mice through nebulization

(Blanchard et al. 2021). Another biodegradable cationic polymer, poly(amine-co-esters) (PACEs) (Fig. 2) which are made of three types of monomers namely sebacic acid, methyldiethanolamine, and pentadecanolide, have also been investigated for pulmonary delivery of mRNA (Grun et al. 2021). PACE and PACE-PEG polymers were mixed at various ratios to investigate the effect on stability of the polyplexes and mRNA transfection. Similar to the observation with LNPs, PEGylation improved polyplexes stability but reduced transfection efficiency in vitro. Interestingly, when administered intratracheally to the lungs of mice, a small amount of PACE-PEG could indeed improve the level of gene expression, possibly due to the ability of PEG to overcome the mucus barrier in the airways. This work highlighted the unpredictable in vitro–in vivo correlation (IVIVC) in pulmonary mRNA delivery, and the significance of PEG and its optimization.

Chitosan (Fig. 2), which is derived from crustacean shells, is one of the most popular natural cationic polymers for RNA delivery because of its good safety profile and excellent biodegradability. It is a linear polysaccharide of randomly distributed *N*-acetylglucosamine and glucosamine units, with different degrees of deacetylation. Chitosan is generally insoluble in water. It is protonated in acidic environment to become soluble and cationic, enabling it to form polyplexes with the anionic mRNA through electrostatic interactions (Li et al. 2018). Its mucoadhesive and mucosa penetration properties have been utilized to facilitate the transport of RNA across the mucosal surface in the airways (Nielsen et al. 2010). Because of the limited delivery efficiency on its own, chitosan has been employed as coating polymer to enhance RNA transfection of other polymers in the lung (Mura et al. 2011; Haque et al. 2018). Poly(lactic-co-glycolic acid) (PLGA) (Fig. 2) is a neutral, synthetic, biocompatible, and biodegradable copolymer that has been widely used clinically in drug delivery due to its tunable degradation rate by manipulating the polylactic to polyglycolic acid ratio and the molecular weight, and its capacity for sustained release of the incorporated drug molecules (Makadia and Siegel 2011). PLGA nanoparticles have also been investigated for mRNA delivery, but due to its charge neutrality, they are often coated with cationic moiety such as chitosan to facilitate their interaction with the anionic membrane to promote cellular uptake in the lung (Mahiny et al. 2015; Haque et al. 2018). Using PLGA of 75:25 (polylactic to polyglycolic acid) ratio and chitosan with 83% of deacetylation, chitosan-coated PLGA nanoparticles were prepared using emulsion diffusion evaporation method and the mRNA was loaded by mixing with the nanoparticles. Successful mRNA transfection was observed in the lung of mice following intratracheal administration, but the efficiency was inferior to intravenous administration when the same formulation was used. The authors reasoned that the nanoparticles were not optimized to overcome the mucus and macrophages barrier in the airways. Nevertheless, the study demonstrated that the nanoparticles could be administered topically to the lungs safely without triggering any immune response although further optimization is required to enhance the delivery efficiency.

2.3 Peptide-Based Delivery System

Peptides have been increasingly recognized as a versatile mRNA delivery system. Based on their functions, peptides can be categorized into cell-penetrating peptides (CPPs), endosome disrupting peptides, and targeting peptides (Tai and Gao 2017). Peptides can either be used alone or more commonly, conjugated with other molecules such as lipids and polymers to improve their performance (Qiu et al. 2019; Guan et al. 2019). When used alone, peptides are positively charged by including lysine and arginine residues to allow the formation of complexes with the nucleic acids.

KL4 peptide is a synthetic cationic peptide with 21 residues containing repeating units of leucine and lysine (Cochrane et al. 1996; Saenz et al. 2011). This peptide was originally developed as a synthetic pulmonary surfactant (sinapultide) by mimicking the hydrophobic and hydrophilic pattern of human surfactant protein B. KL4 peptide was repurposed for pulmonary RNA delivery by taking the advantage of its cationic nature and known safety profile in pulmonary administration (Qiu et al. 2017). While successful RNA transfection was achieved in lung epithelial cells, the poor aqueous solubility of KL4 peptide, due to the presence of a high proportion of the hydrophobic leucine, has limited its potential as delivery vector. PEGylation of KL4 peptide with 12 units of monodispersed PEG was able to improve water solubility as well as enhancing the mRNA transfection efficiency. This PEG_{12}KL4 peptide demonstrated effective luciferase mRNA expression in the lung via pulmonary delivery in mice (Qiu et al. 2019). Instead of nebulization, the peptide system was formulated into an inhalable dry powder using spray drying or spray freeze drying techniques. Inhalable dry powder can be easily administered with the potential to improve the long-term stability and extend the shelf-life of mRNA therapeutics, an area yet to be properly explored.

Multi-modular synthetic peptide is another approach to deliver mRNA with high efficiency. Poloxamines are amphiphilic copolymers consisting of poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) blocks that display excellent biocompatibility but poor transfection efficiency. A peptide consisted of three functional moieties, namely an anchor moiety to interact with hydrophobic blocks of poloxamines, a cationic moiety to condense mRNA, and a targeting moiety to direct the mRNA to specific cells or organelles, was developed (Guan et al. 2019). The peptide formed compact ternary complexes with poloxamine-based copolymers to deliver mRNA to the lung. Following intratracheal administration to mice, the mRNA transfection efficiency of the peptide-poloxamine nanoparticles in the lung was significantly superior to peptide or poloxamine alone with negligible toxicity.

2.4 Modified Naked mRNA

As illustrated above, various types of delivery systems were developed to mediate mRNA transfection in the lung. Nonetheless, some studies opted to deliver mRNA in its naked form (without delivery vectors) because of its simplicity and potentially better safety profile (Kormann et al. 2011; Tiwari et al. 2018; Zeyer et al. 2016). Although naked mRNA has demonstrated successful transfection in the lung, the exact mechanism of how naked mRNAs penetrated the cell membrane in the airway remains unclear. It is speculated that the presence of pulmonary surfactants including the cationic surfactant proteins in the airways may play a role in mediating the cellular uptake of mRNA. However, there is a concern of large variation of transfection efficiency of naked mRNA, which may depend on the physiological and pathological conditions of the respiratory tract of individual patients (Chow et al. 2020). Single-stranded mRNA is also highly unstable in the serum with high innate immunogenicity. Chemically modified mRNA is therefore developed to overcome these issues alongside enhanced transfection efficiency with substantial success (Gao et al. 2021). This is particularly important when naked mRNA is used.

Common chemical modifications of mRNA include cap modification and nucleotide modification (Fig. 3). Capping of 5′ end of mRNA is an important structural modification that allows efficient mRNA translation. In native eukaryotic mRNA, the cap structure at the 5′ end consists of a 7-methylguanosine triphosphate group (m^7GpppG), which is crucial for protecting the mRNA from degradation and mRNA export from the nucleus. To minimize immunogenicity, it is important that the synthetic mRNA mimic the 5′ cap structure of non-immunogenic endogenous mRNA, as the uncapped RNAs may be recognized as foreign to the host, triggering an innate immune response. Nucleotide modification is another important modification, as Toll-like receptors (TLRs) can recognize the unmodified nucleotides of synthetic mRNA easily, rendering it highly immunogenic (Dalpke and Helm 2012). It has been demonstrated that the modification of nucleotide at the bases such as pseudouridine, N^1-methylpseudouridine, N^6-methyladenosine, and 5-methylcytidine can effectively evade TLRs activation to prevent immune response (Elkhalifa et al. 2022). Nucleotide modification can also take place at the ribose sugar. The absence of proton at 2′-OH group in the ribose can reduce TLR-mediated immune response, with the methylation at 2′-OH (2′-OMe) represents one of the most common RNA modifications (Elkhalifa et al. 2022). However, how effective can these chemical modifications enhance mRNA transfection and stability in the airways remain to be investigated.

3 mRNA Strategies and Respiratory Diseases

mRNA can be used in different approaches for the treatment or prophylaxis of a range of respiratory diseases by encoding proteins such as ion channels, regulatory proteins, enzymes, antigens, and antibodies for diverse therapeutic effects. Various

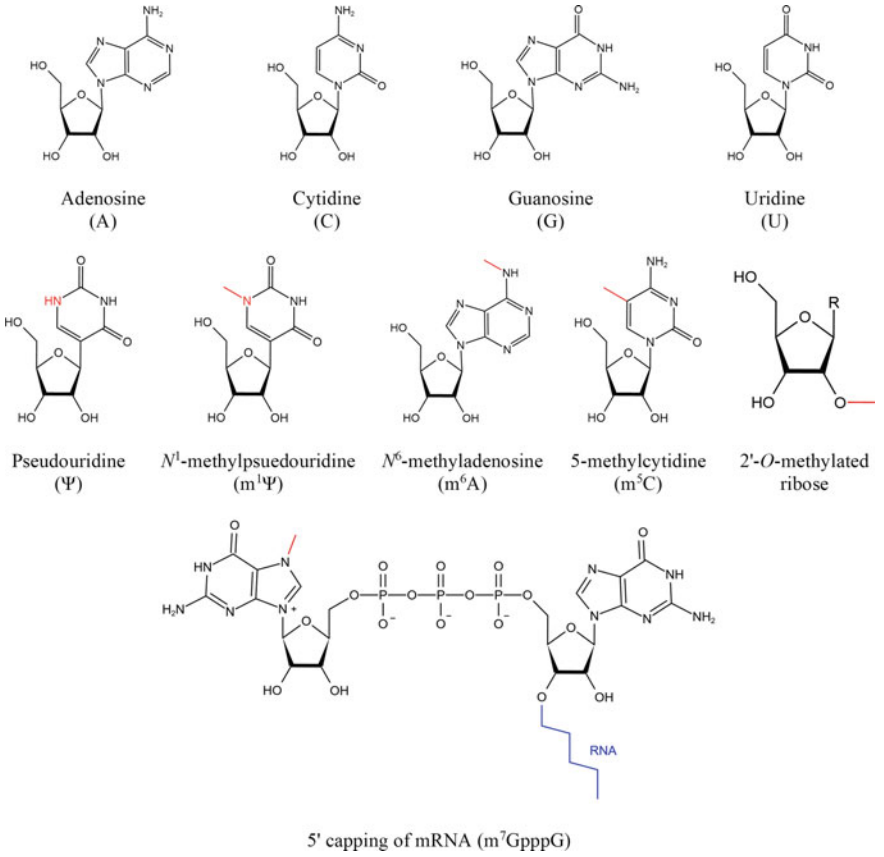

Fig. 3 Chemical structures of nucleosides (adenosine, cytidine, guanosine, and uridine) and common mRNA modifications. Nucleotide modifications (pseudouridine, N^1-methylpsuedouridine, N^6-methyladenosine, 5-methylcytidine, and 2′-*O*-methylation in the ribose), and cap modification (5′ capping with 7-methylguanosine triphosphate)

mRNA therapeutic strategies for pulmonary delivery being investigated are reviewed here.

3.1 Cystic Fibrosis

CF is a genetic disorder caused by mutations within the coding region of the *CFTR* gene (Rafeeq and Murad 2017). CTFR protein is an ion channel that has an important role in regulating fluid homeostasis and transporting ions including chloride and bicarbonate ions across the epithelial surface. Dysfunctional or absence of CTFR protein leads to mucus accumulation and compromised mucociliary clearance of

the airways. As a result, patients with CF are susceptible to lung infections and inflammations. Current treatment options for CF focus on symptom management, such as the use of antibiotics to control airway infections and mucolytic agents to modulate mucus viscosity (Miah et al. 2019).

CF could potentially be treated with gene therapy, which has become the direction of new CF therapy development. Supplement of CFTR protein with mRNA is a promising approach, and the success of pulmonary delivery of CFTR mRNA was demonstrated both in animal models and clinical trials. The administration of CFTR mRNA in LNPs intranasally to CFTR knockout (CFKO) mice at a daily dose of 0.1 mg/kg for 14 days was reported (Robinson et al. 2018). The LNPs consisted of DLin-MC3-DMA as ionizable lipid, DSPC as helper lipid, cholesterol, and DMG-PEG (Fig. 1). Nasal potential difference measurement showed that the net chloride efflux characteristic to the nasal airway epithelium was restored on day three of CFTR mRNA treatment, recovering up to 55% of that of healthy mice. In another study, CFTR mRNA was administered intratracheally to CFKO mice twice three days apart at a dose of 40 μg per mouse (Haque et al. 2018), with chitosan-coated PLGA nanoparticles employed as delivery vector. CFTR protein level significantly increased six days after mRNA treatment, and the treated CFKO mice efficiently restored their lung functions close to the level of the healthy control mice, as evaluated by the forced expiratory volume, airway resistance, and compliance. Another mRNA candidate encoding fully functional CFTR protein, MRT5005, has entered clinical trials in May 2018 (NCT03375047), using LNPs for delivery. It is the first inhaled mRNA candidate that is administered to patients with CF through nebulization and has received both the orphan drug and fast track designation by the FDA. Early results indicated that multiple doses of MRT5005 were well tolerated with no serious adverse events, and the phase 1/2 clinical trial is still ongoing at the time of writing (Translate Bio 2021).

3.2 Asthma

Asthma is characterized as a chronic airway inflammatory disease. Because of its heterogeneous characteristics, the goals of treatment have been focused on symptom management with the use of bronchodilators and anti-inflammatory agents (Castillo et al. 2017). However, some patients showed limited responses to the currently available treatment options. This prompts the seeking for alternative treatment strategy including mRNA.

For instance, allergic asthma is caused by the imbalance of T helper cell responses, resulting in the subsequent release of proinflammatory cytokines. FOXP3 is a regulatory T cell transcription factor. The intratracheal administration of chemically modified FOXP3 mRNA could reduce airway inflammation in animal model by modulating T helper cell responses (Mays et al. 2013). Another approach involves the TLRs, which belong to a family of pattern recognition receptors that activate innate and adaptive immunity by recognizing conserved molecular patterns of a series of

pathogens (Nie et al. 2018). TLRs affect T cell polarization and development, and polymorphisms in TLR1, TLR6, and TLR10 are able to form heterodimers with TLR2, offering protective effects on allergic asthma (Kormann et al. 2008). It was demonstrated that the intratracheal administration of chemically modified TLR1/2 or TLR2/6 mRNA combination in asthma model resulted in significant reduction in lung inflammation (Zeyer et al. 2016). Both studies suggested that pulmonary delivery of mRNA to the site of inflammation is a promising strategy for the treatment of allergic asthma.

3.3 Respiratory Syncytial Virus Infection

Respiratory syncytial virus (RSV) infection is the most common cause of acute respiratory tract infections in infants and is associated with acute exacerbation of chronic obstructive pulmonary disease (COPD) in adults (Griffiths et al. 2017; Mehta et al. 2013). Currently, aerosolized ribavirin is the only approved antiviral drug for the treatment of RSV infection (Griffiths et al. 2017), while palivizumab, administered by intramuscular injection, is a monoclonal neutralizing antibody licensed for the prevention of RSV infection in high-risk population (Goldstein et al. 2021). Both antiviral agents have their limitations. For instance, ribavirin is associated with severe adverse effects whereas palivizumab is only effective in infants < 29 weeks (Anderson et al. 2017).

Pulmonary delivery of mRNAs encoding both the whole and the single domain of palivizumab was investigated to prevent RSV infection (Tiwari et al. 2018). Initially, modified naked mRNA as well as commercially available PEI derivatives including Viromer® RED (VR) and in vivo-jetPEI® were examined for mRNA transfection efficiency in the lung of animals (Tiwari et al. 2018). When the mice were intratracheally administered with 40 μg of mRNA targeting membrane-anchored palivizumab as prophylaxis, followed by RSV infection, all three groups showed significant reduction in RSV F copy numbers, but only naked and VR-delivered mRNA could significantly reduce RSV viral titers in the lung. Since in vivo-jetPEI® was more toxic to the mice and induced immune response at 24 and 48 h post-administration, modified naked mRNA was chosen for subsequent study to minimize immunological risk. It is noted that both VR and in vivo-jetPEI® were off-the-shelf transfection systems and neither of them is specifically designed for pulmonary delivery. To further evaluate the safety of pulmonary mRNA delivery, modified naked mRNA was administered intratracheally at a dose of 100 μg per mouse followed by RSV inoculation. The mRNA-treated mice had significantly reduced RSV F copies compared with those treated with negative mRNA controls, with no significant difference in baseline cytokine levels. Overall, the results suggested that the expression of neutralizing antibodies in the lung through mRNA delivery is a novel approach to prevent RSV infection.

3.4 Antiviral Effect Using Cas13

The discovery of CRISPR-Cas gene editing technology is one of the biggest breakthroughs in the modern world of biotechnology (Jinek et al. 2012). Cas13 is an RNA targeting enzyme that serves as a powerful platform for RNA modulation. Pulmonary delivery of mRNA encoding Cas13a was investigated for the treatment of various respiratory viral infections (Blanchard et al. 2021). The type III and VI CRISPR systems can target single-stranded RNA (ssRNA) substrates with Cas13 proteins functioning as RNA endonuclease to cleave RNAs complementary to its CRISPR RNA (crRNA) (East-Seletsky et al. 2016). As about two-thirds of viruses that cause infections in humans have ssRNA genomes including influenza virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Cas13 can potentially act as an effective antiviral agent for these ssRNA viruses (Freije et al. 2019). The Cas13a mRNA and guide RNAs were delivered using a PBAE-based polymer to influenza and SARS-CoV-2-infected animals using a nose-cone nebulizer at a dose of 100 and 125 μg of mRNA per animal, respectively (Blanchard et al. 2021). Treatment of Cas13a mRNA demonstrated effective antiviral activity in both animal models with significant improvement in body weight and reduction in lung viral loads compared with non-targeted control group. This approach is demonstrated to be an exciting new strategy against various types of respiratory viral infections.

3.5 Vaccines Against Respiratory Viral Infections

mRNA vaccines have emerged as one of the most promising vaccination strategies against COVID-19 due to their effectiveness and rapidly scalable production. Two major approaches of mRNA vaccine are being studied, the non-replicating mRNA vaccine which encodes the antigen of interest, and the self-amplifying RNA which encodes not only the antigen of interest, but also the alphaviral replicase which allows the intracellular replication of RNA to enhance protein expression (Pardi et al. 2018; McKay et al. 2020). Only the former approach is currently used in the clinic for the protection of COVID-19, including COMIRNATY® and SPIKEVAX® (Liu et al. 2021), which use LNPs as mRNA delivery vectors for intramuscular injection (Pardi et al. 2015; Baden et al. 2021). Both mRNA vaccines showed good protection efficiency against COVID-19 (Polack et al. 2020; Dagan et al. 2021). Apart from COVID-19 vaccines, mRNA vaccines against influenza have also entered clinical trials and gained promising results. Two phase I clinical studies of mRNA vaccine expressing H10N8 and H7N9 antigens were evaluated in healthy adults. Similar to the COVID-19 mRNA vaccines, both studies employed LNPs as mRNA delivery vector and administered through intramuscular injection. Both vaccines showed favorable safety profiles and elicited robust humoral immune response (Feldman et al. 2019). More recently, mRNA vaccines against RSV, employing LNPs for intramuscular injection, have also been investigated (Aliprantis et al. 2021; Espeseth et al. 2020).

Although current intramuscular immunization of mRNA vaccines is a very promising approach in offering protection against various respiratory viral infections, it is limited by its invasive administration procedure. Inhaled mRNA vaccine, a type of mucosal vaccine by delivering mRNA to the pulmonary mucosa, has been proposed as an alternative non-invasive vaccination strategy with the potential advantage of having the virus-specific antibodies being released at the mucosal surface, where they can neutralize the viruses at the early stage of infection. Furthermore, mucosal mRNA vaccine can induce the secretion of immunoglobulin A, which serves as the front line of defense against infections (Kim et al. 2021; Yeo and Ng 2021). Majority of the mucosal mRNA vaccine studies have been focused on intranasal route possibly due to the relatively easy administration. The requirement of inhalation device could be a barrier for the widespread distribution of the vaccines. Nevertheless, pulmonary delivery of mRNA vaccine is definitely an encouraging strategy worth further investigation, especially as a dry powder dosage form to increase stability and prolong shelf-life (Chow et al. 2020; Heida et al. 2021).

4 Summary and Future Prospects

mRNA is an extremely powerful molecule with diverse approaches for a plethora of clinical applications. A vast number of studies have been initiated to explore the delivery of mRNA through the pulmonary route, which offers a non-invasive way to apply mRNA locally to the site of action for the treatment or prophylaxis of respiratory diseases. While LNPs have attracted tremendous attention in the field of mRNA delivery because of their approval in several injectable RNA products, other delivery strategies including polymers, peptides, and modified naked mRNA are also investigated for their safety and transfection efficacy in the airways. This chapter has reviewed a number of clinical applications of inhaled mRNA therapies that have reached the pre-clinical stage. The treatment of many other lung-related diseases such as COPD and lung cancer could also benefit with pulmonary mRNA delivery strategy. Apart from delivery system, an effective inhalation device is also a critical factor for successful inhaled mRNA therapy. Most studies use liquid formulations which require nebulization for clinical use. Perhaps, inhalable dry powder formulation of mRNA is an area that deserves further investigation for better stability.

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Synthetic mRNA Gene Therapies and Hepatotropic Non-viral Vectors for the Treatment of Chronic HBV Infections

Dylan Kairuz, Prashika Singh, Tiffany Smith, Patrick Arbuthnot, Abdullah Ely, and Kristie Bloom

Contents

Abstract Hepatitis B virus (HBV) infection remains a global health challenge with an estimated 296 million people chronically infected, leading to a high incidence of HBV-associated liver cancer and cirrhosis. Currently, there is no cure for chronic HBV infection, and novel therapeutic approaches, including gene editing and epigenome engineering, are being investigated. Although promising, the liver-specific delivery, expression and safety profile of these gene therapies requires careful consideration as off-target effects could result in genotoxicity. Recent advances in the field of synthetic mRNA therapeutics may help to overcome some of the hurdles currently associated with delivery and expression of these gene therapies for HBV. In vitro transcription (IVT) and capping can now be achieved using good manufacturing practice (GMP) grade materials. The inclusion of modified bases and sequence optimization, particularly at the 5′ and 3′ untranslated regions (UTRs), improves in situ translation by enhancing RNA stability. Non-viral vector formulations, for instance, ligand-modified nanoparticles, could be used to direct anti-HBV mRNA therapies directly to hepatocytes. Finally, the transient expression of synthetic mRNAs allows for better dose regulation and improved safety, particularly when using gene editing tools. This chapter will discuss the recent advances that could be used to expand and improve on synthetic mRNA gene therapies for chronic HBV infection.

D. Kairuz · P. Singh · T. Smith · P. Arbuthnot · A. Ely · K. Bloom (✉)
Wits/SAMRC Antiviral Gene Therapy Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Private Bag 3, WITS, Johannesburg 2050, South Africa
e-mail: kristie.bloom@wits.ac.za

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_8

Keywords Hepatitis B virus · Covalently closed circular DNA · Messenger RNA · Gene therapy · Lipid nanoparticles

1 Introduction

The hepatitis B virus (HBV) is a human pathogen that infects hepatocytes and can cause both acute and chronic infection of the liver (Seeger and Mason 2000). While most exposed individuals are able to clear infection during the acute phase, a large number develop chronic hepatitis B (hep B) and account for the estimated 296 million worldwide chronic carriers worldwide (WHO 2021). Individuals with a robust immune system can eliminate infected hepatocytes, resolving the infection before it progresses beyond the acute phase (MacLachlan and Cowie 2015). In contrast, individuals who do not have a fully developed immune system or have a compromised immune system fail to contain the initial infection which then progresses to chronicity. Chronic hep B is associated with significant morbidity and mortality as a consequence of the increased risk of developing cirrhosis and hepatocellular carcinoma (HCC) which cumulatively accounts for close to 820,000 deaths worldwide (WHO 2021). Acute infection has lower rates of morbidity and mortality but nevertheless still accounts for approximately 100 000 deaths per year (WHO 2017). Chronic HBV infection is endemic to sub-Saharan Africa, East and Southeast Asia and the Western Pacific islands (WHO 2017). Sub-Saharan Africa has the highest prevalence of chronic hep B with close to 9% of the population testing positive for viral antigenemia (Schweitzer et al. 2015). The WHO estimates that a very small proportion of HBV chronic carriers have been diagnosed (~ 9%) and that as little as 8% (1.7 million chronic carriers) receive treatment (WHO 2017). The infectious viral particle exists as a partially double-stranded DNA (relaxed circular DNA/rcDNA) (Cummings et al. 1980) that upon infection and transport to the nucleus is repaired to covalently closed circular DNA (cccDNA) (Tuttleman et al. 1986). In the nucleus, the cccDNA serves as template for the transcription of viral RNAs including the pregenomic RNA (pgRNA). The pgRNA serves as template for synthesis of new viral genomes (Nassal 2008). Once generated, cccDNA remains in the nucleus for the lifetime of the hepatocyte and it from this viral DNA intermediate that viral replication proceeds.

Licensed HBV therapeutics aim to control infection by blocking different stages of viral replication. However, complete eradication of the virus is rarely achieved as cccDNA is not easily eliminated by conventional therapy. As the template for viral transcription, this stable episomal minichromosome drives viral replication, and its persistence can lead to HBV reactivation even in patients with resolved infections (Yang and Kao 2014). A number of new anti-HBV drugs are in development, including some intended to disable the cccDNA and promote functional cure (Maepa et al. 2021). Direct elimination or degradation of cccDNA can be achieved using gene editing tools (Bloom et al. 2018) while targeted epigenetic therapy may permanently inactivate viral transcription (Singh et al. 2021). Although promising, liver-specific

delivery, expression and safety of these gene therapies requires careful consideration as off-target effects could result in genotoxicity. Recent advances in the field of synthetic mRNA therapeutics may help to overcome hurdles currently associated with delivery and expression of these gene therapies for HBV.

2 Gene and Epigenome Editing Technologies to Disable HBV

Difficulties in eradicating episomal cccDNA and the potential for reactivation represent a major obstacle in the development of a cure. The cccDNA is an ideal target for nuclease gene editing, owing to the remarkably condensed and multifunctional nature of the viral genome. Approximately two-thirds of the viral sequence codes for more than one functional element. The arrangement of these overlapping reading frames restricts sequence plasticity (McNaughton et al. 2019) and limits the development of escape mutants (Locarnini and Zoulim 2010), thus making the viral genome vulnerable to gene editing. In addition, the cccDNA forms a minichromosome-like structure in the nucleus of infected hepatocytes (Bock et al. 2001), rendering it amenable to epigenetic control (Pollicino et al. 2006). Minichromosome organization and viral transcription is controlled by an intricate network of host proteins, including cellular transcription factors and chromatin-modifying enzymes and viral proteins (Singh et al. 2021). Sequence-specific gene silencing of cccDNA using epigenetic editors offers an alternative non-mutagenic approach to traditional nuclease mediated therapy which could improve the safety of HBV gene therapy.

2.1 HBV Designer Nucleases and Base Editors

Most gene editing tools are designed to induce double-stranded breaks (DSBs) at predefined target sites. This requires a DNA binding domain (for specificity and orientation), and a nuclease domain to enable cleavage. In the absence of a donor template, repeated cleavage at the target site eventually leads to small deletions and/or insertions (indels) (Ray and Raghavan 2020). This process can be exploited to disrupt cccDNA and permanently render the virus inactive. The therapeutic potential of anti-HBV gene editors was first described using zinc finger nucleases (ZFN) (Cradick et al. 2010) and later with transcription activator-like effector nucleases (TALENs) (Bloom et al. 2013). Since then, numerous anti-HBV gene editing tools targeting the cccDNA have been described, including a variety of clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein (CRISPR/Cas) systems (Moyo et al. 2018; Maepa et al. 2021).

Adapting these editing approaches into a clinically relevant chronic HBV therapy remains a challenge. Key hurdles, including mitigating off-target effects and

achieving liver-specific delivery, need to be addressed. As both ZFNs and TALENs function as dimers, modified heterodimeric *Fok*I monomers that only cleave when both the left and right catalytic domains are correctly orientated have been used to improve specificity of the nuclease for their cognates. Recently second- and third-generation *Fok*I nuclease domains were used to generate obligate heterodimeric TALENs targeting HBV sequences (Smith et al. 2021). The antiviral efficacy of second-generation TALENs was comparable to that of first-generation (non-obligate heterodimers) with improved specificity (Smith et al. 2021). Enhancing the specificity of CRISPR/Cas systems has also been a priority as high rates of off-target cleavage have been reported (Fu et al. 2013; Zhang et al. 2015). To circumvent this, an orthologous CRISPR/Cas system based on the *Streptococcus thermophilus* Cas9 was shown to degrade HBV cccDNA while minimizing off-target cleavage (Kostyushev et al. 2019). Replacing the Cas nuclease with a nickase has also been reported to improve specificity, as heterodimerization is then required for cleavage of HBV DNA (Karimova et al. 2015; Sakuma et al. 2016). Alternatively, HBV targeted mutagenesis can be achieved in a nuclease-independent manner using CRISPR/Cas base editors to introduce nonsense and missense mutations into cccDNA (Yang et al. 2020).

Liver-specific delivery of HBV gene editors is crucial to developing a relevant drug product. To this end, hepatotropic non-replicating adenovirus (Ad) and adeno-associated virus (AAV) vectors have predominantly been used (Fig. 1). However, vector immunogenicity, limited packaging capacities in conjunction with the large size of designer nucleases, and the reliance on heterodimers in the case of ZFN, TALENs and CRISPR/Cas nickases have complicated delivery efforts. Self-complementary AAV-vectors (scAAV) encoding ZFNs achieved site-specific cleavage and mutagenesis of HBV DNA in liver-derived cells (Weber et al. 2014). However, efficacy relied on the transduction of two different scAAV vectors, each encoding one of the cognate ZFNs. Achieving equal transduction rates of both vectors in vivo may complicate the feasibility of this approach. To circumvent this, high-capacity adenoviral vectors (HCAd) have been used to deliver both TALEN cognates in a single vector but were found to be ineffective (Schiwon et al. 2018). The same study demonstrated that HCAds encoding a multiplexed CRISPR/Cas9 (*Streptococcus pyogenes* Cas9 and three HBV-targeting guide RNAs) efficiently reduced the number of cccDNA molecules in vitro (Schiwon et al. 2018). Alternatively, packaging of CRISPR/Cas into AAVs can be achieved when using the smaller *Staphylococcus aureus* (Sa)Cas9 (Scott et al. 2017; Liu et al. 2018). This approach has been shown to inactivate and degrade cccDNA in HBV-infected cells (Scott et al. 2017), and recently demonstrated antiviral efficacy in a humanized mouse model of chronic HBV infection (Stone et al. 2021). Pairing AAV delivery of CRISPR/saCas9 with liver-specific promoters may further improve specificity of HBV gene therapy (Yan et al. 2021).

Using synthetic messenger RNA (mRNA) to express nucleases and base editors may overcome some of the current limitations associated with viral vector-mediated HBV gene therapy (Ely et al. 2021). Hepatotropic delivery of mRNAs can be achieved using different non-viral vector formulations (Sect. 4, Figs. 1 and 4). In a mouse model of HBV replication, TT3 lipid-like nanoparticles (LLN) have been used to

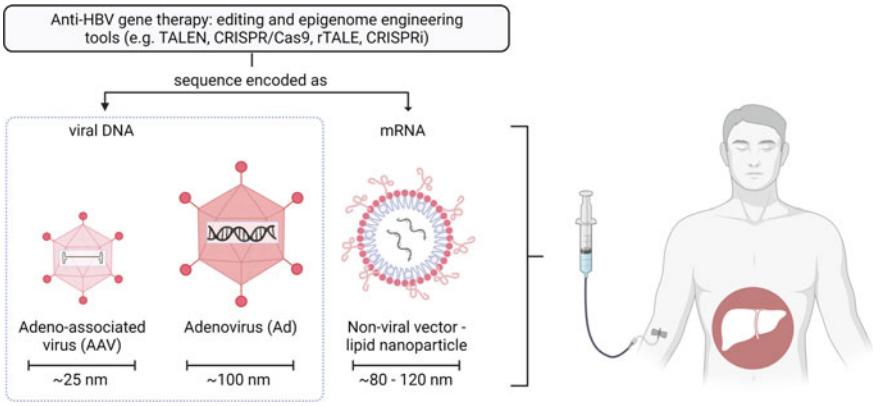

Fig. 1 In vivo HBV gene therapy concepts. Sequences encoding gene editing and epigenome engineering tools designed to disable HBV cccDNA can be incorporated into hepatotropic viral and non-viral delivery vectors. The small adeno-associated virus (AAV) and larger adenovirus (Ad) non-replicating vectors have predominantly been used to delivery HBV gene therapies in preclinical studies. The sequence encoding the therapy is incorporated into the viral DNA and packaged within the vector. Following intravenous administration, the viral vectors are naturally sequestered to the liver where they transduce hepatocytes. The viral DNA is translocated to the nucleus, and the therapeutic sequence is expressed. In the case of non-viral vector delivery, the HBV gene therapy is encoded in synthetic mRNA. These transcripts are then formulated within in a cationic lipid-based nanoparticle which can be adapted to improve liver-specific targeting. Following intravenous administration, the lipid nanoparticles deliver the mRNA to the cytoplasm of hepatocytes. Here, the mRNA is rapidly translated, and the anti-HBV gene therapy is expressed. Image created with BioRender.com

deliver pseudouridine (ψ)-modified mRNA encoding Cas9 and HBV-specific guide RNAs (Jiang et al. 2017). Interestingly, expression of guide RNAs peaked at 2 h post-injection and had completely dissipated by 8 h. To achieve targeted disruption of HBV DNA, the mRNA expressing the Cas9 was administered separately, six hours prior to the guide RNA (Jiang et al. 2017). Although CRISPR/saCas9 gene editing appears promising, immune-mediated clearance of gene edited hepatocytes was shown to occur in mice with preexisting Cas9 immunity (Li et al. 2020). As preexisting immunity to Cas9 proteins has been detected in humans (Charlesworth et al. 2019; Wagner et al. 2019), how this will affect in vivo CRISPR/Cas based gene therapy requires further validation. As chronic HBV infection is often associated with liver damage, additional immune-mediated clearance of gene edited hepatocytes could lead to liver failure.

2.2 HBV Epigenome Modifiers

Targeted epigenome editing involves the precise placement or removal of epigenetic marks. This is achieved by fusing different effector domains to a programmable

DNA binding domain, similar to those used in designer nucleases (Singh et al. 2021). As effector domains alone may cause global changes in gene expression, the addition of a DNA binding domain helps to guide epigenetic modifications to specific genes. Repressors are designed to silence gene expression or mediate recruitment of complexes that elicit heterochromatin formation and include effectors domains like the Krüppel-associated box (KRAB), DNA methyltransferases (DNMTs), histone lysine methyltransferases (KMTs), and histone deacetylases (HDACs) (Sgro and Blancafort 2020). The HBV genome contains three conventional CpG islands that are natural sites for DNA methylation (Jain et al. 2015). Furthermore, methylation of cccDNA is associated with reduced viral replication and burden of infection (Kim et al. 2011), suggesting that permanent targeted epigenome modifications could lead to a functional cure.

Successful epigenetic modulation of HBV DNA was initially achieved using zinc finger (ZF) DNA binding domains in conjunction with either the KRAB transcriptional repression domain (Zhao et al. 2013; Luo et al. 2018) or the catalytic domain of DNMT3a (Xirong et al. 2014). Importantly, the ZF/DNMT3a epigenetic modulator was shown to increase de novo methylation of HBV DNA in vitro (Xirong et al. 2014). Recent studies using transcription activator-like effector (TALE) DNA binding domains in conjunction with the KRAB repressor showed significant inhibition of HBV replication associated with increased methylation of intrahepatic HBV DNA at CpG island II (Bloom et al. 2019). Despite the widespread application of CRISPR/Cas for HBV cccDNA gene editing, studies using the associated CRISPR interference (Larson et al. 2013) or deactivated Cas9 fusions (Brezgin et al. 2019) have yet to be published. Similarly, liver-specific delivery of epigenome modifiers has not been described, although delivery is likely to mimic that of HBV gene editing approaches. As such, non-viral vector formulations encoding synthetic mRNAs are likely to provide a convenient method for intrahepatic delivery of different epigenome modifiers.

3 Developing mRNA as Therapeutics

Since the discovery of mRNA in 1961, there have been several critical breakthroughs that have led to the establishment of mRNA as a therapeutic (Sahin et al. 2014). The ability to synthesize mRNA through in vitro transcription (IVT) and the generation of cap analogs are examples of important milestones that helped initial studies demonstrate the therapeutic potential of mRNA. Despite these early successes, the stability and immunogenic nature of unmodified IVT mRNA were considered barriers to further drug and vaccine development. Researchers continued to find ways of reducing immunogenicity and improving in situ expression. This has included sequence optimization (codon usage, RNA folding, and uridine depletion), the inclusion of non-immunogenic modified nucleotides, and refining mRNA purification to remove immunostimulatory by-products of IVT (Weissman and Kariko 2015). The

merits of this ultimately facilitated the rapid development of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) mRNA vaccines BNT162b2 and mRNA-1273 (Verbeke et al. 2021), which both received emergency use authorization making them the first global mRNA drug product approved for humans.

Developing mRNA therapeutics offers several benefits over traditional DNA-based approaches particularly with regards to manufacturing and safety. IVT and formulation of mRNA is a cell-free process, which reduces biological manufacturing risks and improves production turnaround times. Large-scale current Good Manufacturing Practice (cGMP) production of mRNA is now achievable, which is likely to reduce costs associated with manufacturing these therapeutics (Kis et al. 2020). The overall safety of mRNA therapeutics is considered to be better than that of DNA-based approaches, as the mRNA is confined to the cytoplasm, reducing genotoxic risk, and expression of the therapeutic is transient, allowing better dose control. This is particularly suited to gene editing approaches as prolonged translation of nucleases from a DNA template may increase the likelihood of off-target cleavage. As such, mRNAs encoding ZFNs, TALENs, and CRISPR/Cas have been developed for the treatment of a variety of genetic and infectious diseases (Zhang et al. 2019). Although only one study has reported such an approach for HBV (Jiang et al. 2017), preclinical studies demonstrating liver-specific delivery and efficacy of mRNA as protein replacement, immunomodulatory, or gene therapy are encouraging (Kowalski et al. 2019).

3.1 Enhancing Translation and Stability of Synthetic mRNA

Large-scale production of synthetic mRNA is achieved by means of IVT (Baronti et al. 2018). This requires a suitable purified linear DNA template encoding the therapeutic sequence downstream of an appropriate phage promoter, ribonucleotide triphosphates (rNTPs), bacteriophage DNA-dependent RNA polymerase, and a buffer containing dithiothreitol (DTT), spermidine, and Mg^{2+} ions (Asrani et al. 2018; van de Berg et al. 2021). As IVT mRNA structurally resembles that of fully matured endogenous mRNA, the synthetic molecule is single stranded and comprises five cis-acting structural elements namely the 5′ cap, 5′ and 3′ untranslated regions (UTRs), a therapeutic coding sequence, and a poly (A) tail (Fig. 2) (Pardi et al. 2018). In the cytosol, synthetic mRNA is governed by the same cellular machinery that modulates the translation of endogenous mRNA (Wadhwa et al. 2020). This results in a protein that undergoes post translational modifications yielding in the desired mature product. To address inherent issues regarding mRNA stability and immunogenicity, several modifications have been applied to improve in situ translation of synthetic mRNA.

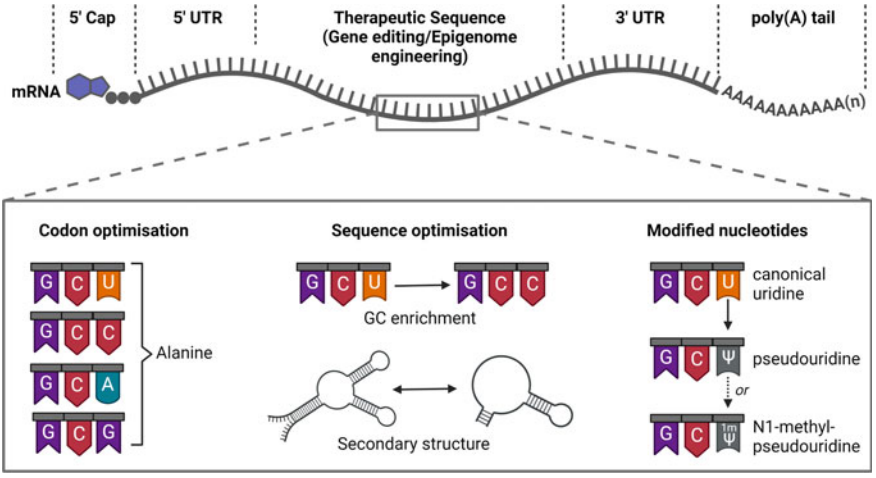

Fig. 2 Organization of synthetic mRNA transcripts and sequence optimization strategies. A typical synthetic mRNA transcript contains a therapeutic sequence flanked by 5′ and 3′ untranslated regions (UTRs). At the 5′ end, a cap is linked to the mRNA transcript through a 5′-5′ triphosphate bridge. At the 3′ end is the poly(A) tail comprising multiple adenosines. Strategies used to improve stability and translation of therapeutic mRNA includes codon optimization, sequence optimization, and incorporation of modified nucleotides. Codon optimization is based on synonymous codon usage used to improve host-specific translation. Sequence optimization strategies include guanine-cytosine (GC) enrichment, which can be achieved through codon degeneracy, or by altering the RNA secondary structure to change mRNA translation dynamics. Inclusion of modified nucleotides, in particular the substitution of canonical uridine with pseudouridine (ψ) derivatives, may improve in situ translation and reduce immunogenicity. 1mψ: N1-methyl-pseudouridine. Image created with BioRender.com

3.1.1 5′ Cap Modifications and Poly (A) Tails

Eukaryotic mRNA contains a 7-methylguanosine (m^7G) cap that is attached to the 5′ end of the mRNA through a 5′-5′-triphosphate bridge and is referred to as cap 0 (Sonenberg et al. 1980). The cap serves to stabilize the mRNA as it protects against degradation by exonucleases, plays a vital role in the initiation of translation, and protects the mRNA from immune clearance (Roers et al. 2016). In the case of synthetic mRNA, the cap is readily added to the transcript either in a co-transcriptional manner during IVT, or enzymatically post-IVT. Several different cap analogs have since been developed to improve capping efficiencies and reduce immune stimulation (Fig. 3). Antireverse cap analogs (ARCA) improve translational efficiency when compared to the original m^7G analog, by ensuring co-transcriptional capping (cap 0) in the correct orientation (Grudzien-Nogalska et al. 2007). Recently, TriLink BioTechnologies launched their CleanCap® technology which allows incorporation of various synthetic cap 1 ($m^7GpppN_{2'Ome}N$) moieties during IVT with improved capping efficiencies (Vaidyanathan et al. 2018; Henderson et al. 2021), rivaling the high efficiency normally only associated with post-IVT enzymatic

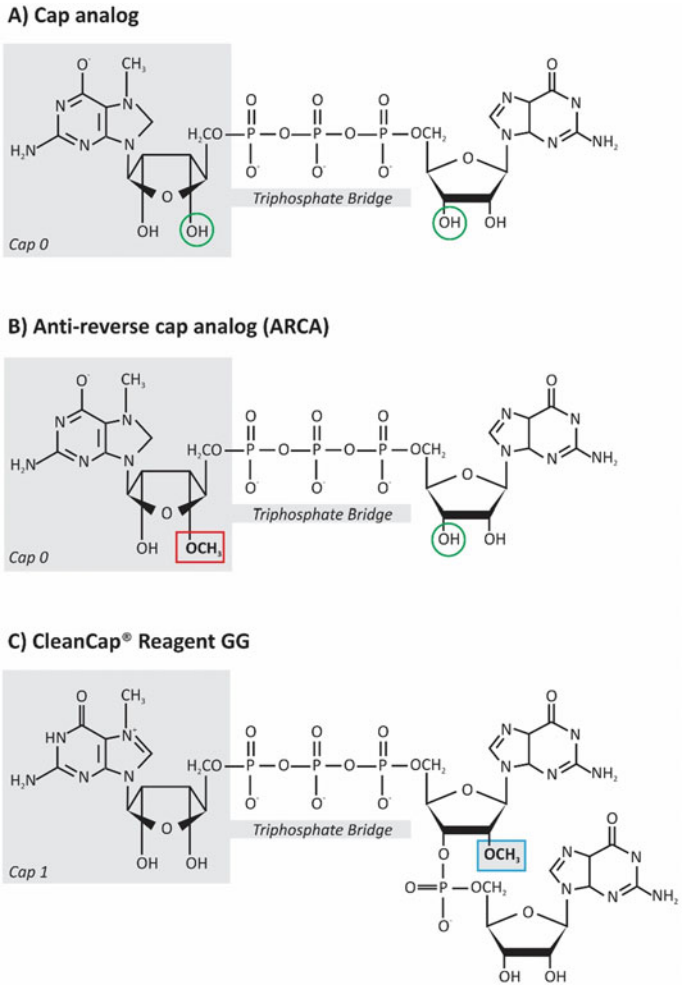

Fig. 3 Chemical structures of three common cap analogs. Cap 0 and cap 1 analogs are linked to the mRNA transcript by a standard phosphodiester bond. **a** Native cap 0 structures (m^7GpppG) are dinucleotides linked by a triphosphate bridge. Both the cap ribose and transcription initiation ribose have 3′ hydroxyl groups (green circles). **b** The anti-reverse cap analog (ARCA) is a modified dinucleotide cap 0 (m7(3′OMeG)pppG) that includes a 3′ methoxy group on the cap ribose (red rectangle), which ensures that transcription is initiated from the transcript ribose (green circle) and mRNA is capped in the correct orientation. **c** CleanCap® reagents are chemically modified trinucleotide caps that include a 2′ methoxy group (blue rectangle) on the transcription initiation ribose, generating cap 1 analogs as m7Gppp(2′OMeN)pN. There are many variations of CleanCap® with different initiation sequences. Depicted here is CleanCap® reagent GG, which has two guanines as the transcription initiation motif resulting in a m7Gppp(2′OMeG)pG cap

capping. The vaccinia virus-derived capping enzyme can post-transcriptionally cap IVT mRNA with cap 0 or cap 1 structures (Shuman 1990). Conversion to cap 1 simply requires the addition of 2′-O-methyltransferase and S-Adenosyl methionine (SAM), which acts as a methyl donor. A cap 1 structure can reduce innate immune recognition in cells (Ramanathan et al. 2016) which is important for the development of mRNA gene therapies.

The poly (A) tail is composed of multiple adenosine nucleotides on the 3′ end of mRNA and works in synergy with the 5′ cap to regulate translation and enhance overall stability of the transcript (Goss and Kleiman 2013). The poly (A) tail also prevents de-capping as well as 3′-5′ degradation (Ford et al. 1997). For in vitro transcribed mRNA, the poly (A) tail can be included within the DNA template, resulting in polyadenylated mRNA with defined lengths, or it can be added enzymatically post-IVT using recombinant poly (A) polymerase (Raynal et al. 1996). The optimal poly (A) tail length for IVT mRNA has been estimated at 120 base pairs (Holtkamp et al. 2006); however, further studies may be needed to help define optimal tail lengths. The finding that naturally abundant and highly expressed mRNAs have short poly

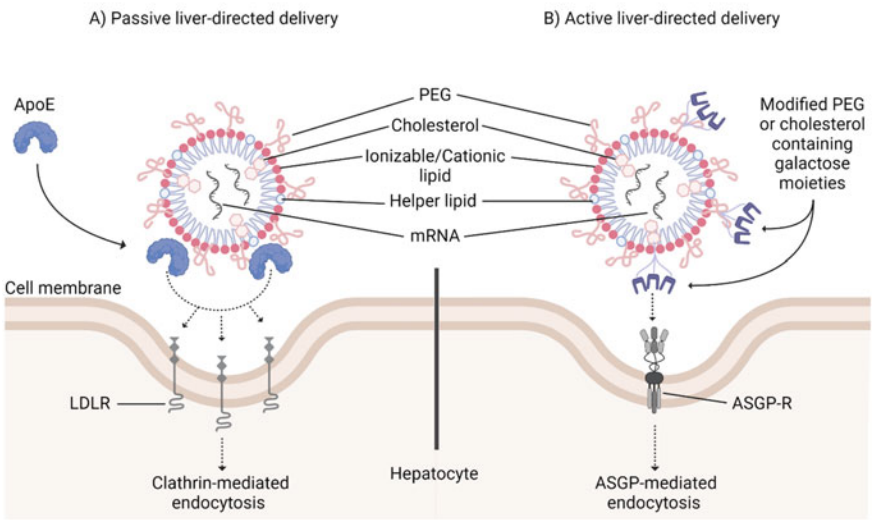

Fig. 4 Hepatotropic delivery of synthetic mRNA gene therapies. Lipid nanoparticles are comprised of a permanently cationic or ionizable lipids, helper lipids like cholesterol and phospholipids, and polyethylene glycol (PEG). Hepatotropic delivery of mRNA therapeutics can be achieved through passive delivery (**a**) or ligand-based active delivery (**b**). During passive delivery, apolipoprotein E (ApoE) is absorbed onto the surface of the nanoparticle. This facilitates binding of the nanoparticle to low-density lipoprotein receptors (LDLR) on the surface of hepatocytes, resulting in clathrin-mediated endocytosis. Active targeting is achieved by conjugating ligands to PEG or cholesterol components of the nanoparticle. Ligands containing galactose moieties can be used to target nanoparticles to the hepatocyte-specific asialoglycoprotein receptor (ASGP-R) to facilitate receptor-mediated endocytosis. Image created with BioRender.com

(A) tails (Lima et al. 2017) implies that optimal tail length may be specific to the type and amount of mRNA.

3.1.2 5′ and 3′ UTR Modifications

5′ and 3′ UTRs are non-coding elements that flank the coding sequence and play a significant role in regulating translation (Suknuntha et al. 2018), coordinating subcellular localization (Creusot et al. 2010) as well as enhancing mRNA stability (Asrani et al. 2018). Incorporation of the 5′ and 3′ UTRs from the α- and β-globin genes has been shown to enhance translation and stability of synthetic mRNA (Kariko et al. 1999). Furthermore, two human β-globin 3′-UTRs arranged in a head-to-tail orientation may increase stability and translation efficiency (Holtkamp et al. 2006; Adibzadeh et al. 2019). UTR elements were found to improve mRNA stability by reducing de-capping and inhibiting 3′-5′ exonuclease degradation (Bergman et al. 2007). Conversely, incorporating AU-rich elements into the 3′ UTR can promote rapid mRNA degradation in cases where limited protein production is required (Chen and Shyu 1995) suggesting that UTR manipulation can be used to fine-tune translation.

3.1.3 mRNA Sequence Optimization

Augmenting the mRNA sequence can improve stability and translation of RNA therapies. Common strategies include codon optimization, sequence engineering, and incorporation of modified nucleotides (Fig. 2). Codon optimization can be used to improve translation efficiency by replacing rare codons with frequent synonymous codons recognized by host-specific cognate tRNAs (Cannarozzi et al. 2010). Sequence engineering approaches such as uridine depletion (Vaidyanathan et al. 2018), enrichment of guanine-cytosine nucleotides (Kudla et al. 2006; Thess et al. 2015), and modifications that affect mRNA secondary structure (Mauger et al. 2019) can improve in situ translation. Incorporating chemically modified nucleotides during IVT has been shown to reduce immunogenicity of synthetic mRNA (Kariko et al. 2005; Anderson et al. 2010, 2011). More than 140 naturally occurring modified nucleotides have been identified, 17 of which are associated with eukaryotic mRNA (McCown et al. 2020). Substitution of canonical uridine with N1-methyl-pseudouridine (1mΨ) was shown to reduce synthetic mRNA immunogenicity (Nelson et al. 2020), induce higher protein expression than other modified nucleotides (Parr et al. 2020; Andries et al. 2015), and has been included in the BNT162b2 and mRNA-1273 SARS-CoV-2 mRNA vaccines (Verbeke et al. 2021). Uridine appears to play an important role in the design and optimization of mRNA therapeutics whether by sequence optimization or substitution with modified analogs.

3.1.4 Modulating mRNA Immunogenicity

Exogenous RNA is inherently immunogenic as it is recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) upon entry into the cell. This feature can be exploited for the development of mRNA vaccines but remains detrimental for gene therapy. While the methods already described can help reduce immunogenicity of the transcript, the IVT reaction can introduce unintended immunogenic by-products like double-stranded RNA (dsRNA) during mRNA synthesis. dsRNA is a strong pathogen-associated molecular pattern (PAMP) and is recognized by TLR3 (Alexopoulou et al. 2001), while TLR 7 and 8 recognize single-stranded RNA (ssRNA) (Diebold et al. 2004; Heil et al. 2004). In non-immune cells, retinoic acid-inducible gene I (RIG-1) recognizes short dsRNA (Schlee et al. 2009) while melanoma differentiation-associated protein-5 (MDA-5) recognizes long dsRNA lacking 2′-O-methylation (Pichlmair et al. 2009). Activation of TLRs 3/7/8, RIG-1 or MDA-5 induces secretion of type I interferons (IFNs) which leads to immune clearance of synthetic mRNA (Isaacs et al. 1963; Kariko et al. 2011). As such, preventing the synthesis of immunostimulatory by-products during IVT, or removing these post-IVT are important considerations. Purification of IVT mRNA by chromatography methods (high performance liquid chromatography (HPLC), anion-exchange, size-exclusion and affinity chromatography) or cellulose-based methods to remove dsRNA contaminants helps reduce innate immunogenicity to further enhance the stability of the transcript (Kariko et al. 2011; Baiersdorfer et al. 2019; Edelmann et al. 2014). Adapting the IVT reaction with thermostable T7 polymerase (Wu et al. 2020), co-tethered polymerase/promoter beads at high-salt conditions (Cavac et al. 2021), and 3′ DNA oligonucleotide capture probes (Gholamalipour et al. 2019) may prove useful in preventing the formation of dsRNA and other spurious by-products.

3.2 Liver-Specific Delivery of mRNA Using Non-viral Vectors

The liver consists of hepatocytes (parenchymal cells), Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs) (Ding et al. 2016). LSECs line the liver sinusoids and contain fenestrae for macromolecule transport, which are ∼ 100 nm in humans (∼ 140 nm in mice) (Wisse et al. 2008). KCs are present in the sinusoids, while hepatocytes are found on the inner side of the LSECs separated by the space of Disse. Hepatocytes are the main cell type found in the liver and the primary target for anti-HBV mRNA therapeutics. On their basolateral membrane, receptors such as the low-density lipoprotein receptor (LDLR) and asialoglycoprotein receptor (ASGP-R) are found, facing the sinusoids (Akinc et al. 2010). When developing liver-specific gene therapies for chronic HBV infection, several important factors need to be considered including pathophysiology, differential expression of liver receptors, and immune cell activation upon onset of disease (Witzigmann et al. 2020). Toxicity caused by the vector, or its payload could further exacerbate liver damage and cause decompensation (Lv et al. 2006; Tousignant et al.

2000). Many of the non-viral vectors currently used to deliver mRNAs were initially designed to deliver small interfering RNA (siRNA) or plasmid DNA to liver cells and have since been adapted.

3.2.1 Lipid Nanoparticles for Passive Liver-Directed Delivery

Lipid nanoparticles (LNPs) consist of monolayer or bilayer vesicles encapsulating an electron-dense or aqueous core. They are comprised of a permanently cationic or ionizable lipid, helper lipids (cholesterol and phospholipids), and a polyethylene glycol (PEG)-conjugated (PEGylated) lipid (Fig. 4) (Hou et al. 2021). The cationic nature of the main lipid facilitates a charge-based interaction with anionic RNA and assists in endosomal escape. Helper lipids provide structural integrity and may also be essential to RNA encapsulation within the LNP (Kulkarni et al. 2019). Phospholipids assist in endosomal escape and cytoplasmic release of RNA, for example, 1,2-dioleoyl-sn-3-phosphoethanolamine (DOPE) forms an inverse hexagonal phase which assists in destabilizing the endosome (Heyes et al. 2005). PEGylated lipids improve the stability of LNPs by reducing serum protein adsorption (Lundqvist et al. 2008; Caracciolo et al. 2010), aggregation of LNPs (Kulkarni et al. 2018), size (Belliveau et al. 2012), and opsonin binding to facilitate immune escape (Goswami et al. 2019; Kumar et al. 2014).

Ionizable LNPs (iLNPs) have predominantly been used to target hepatocytes due to their intrinsic tropism and neutral charge at physiological pH which prevents immune stimulation (Semple et al. 2010; Guo et al. 2019). Following intravenous injection, apolipoprotein E (ApoE) adsorbs onto the surface of LNPs which facilitates delivery to liver cells through interactions with LDLRs, resulting in clathrin-mediated endocytosis (Fig. 4a) (Akinc et al. 2010). Moderna has led a wide range of studies using their optimized lipid formulations for the delivery of mRNA therapeutics designed to treat liver diseases (Jiang et al. 2018, 2020; Wei et al. 2021; Cao et al. 2019, 2021; Balakrishnan et al. 2020). The first FDA approved RNA interference (RNAi) drug Onpattro® (patisiran) uses the ionizable lipid DLin-MC3-DMA to endogenously target the RNA silencer to the liver (Adams et al. 2018; Gonzalez-Duarte et al. 2020). This emphasizes the safety and feasibility of these formulations for future liver-targeted iLNP HBV gene therapy.

Lipidoid nanoparticles, which consist of lipid-like compounds, and lipoprotein nanoparticles (LPNs) have also shown promise as delivery vehicles for mRNA. Systemic administration of an mRNA formulated with the ionizable lipidoid C12-200 resulted in the specific delivery of the mRNA to sinusoidal cells and hepatocytes (DeRosa et al. 2016). The original LNP formulation was optimized by reducing lipidoid content, doubling the lipidoid to mRNA weight ratio, and replacing distearoylphosphatidylcholine (DPSC) with DOPE to adjust interaction properties and release mechanisms (Kauffman et al. 2015). This resulted in a threefold increase in liver-directed mRNA delivery. A similar lipidoid C12(2-3-2) has also shown potential for delivering mRNA to the liver (Schrom et al. 2017). cKK-E12 mRNA-LPNs mainly targeted hepatocytes but some leaky delivery was detected

in the spleen. This was a result of albumin-mediated, as opposed to an ApoE-mediated, uptake by the cells. When combined with an MC3-derived helper lipid (A6, Syn-3 LPNs), the authors observed improved membrane fusion and endosomal escape as well as reduced in vivo toxicity (Miao et al. 2020). Modifications to the cationic lipid may further improve liver tropism. Yu et al. (2020) recently described cationic lipid-modified aminoglycosides (CLAs) to improve delivery of mRNA to the liver. Aminoglycosides are commonly used as antimicrobials and are thought to disrupt cell membranes, which may be useful to improve endosomal disruption of LNPs. Gentamicin-CLAs improved LNP-mRNA delivery and expression in the liver when compared to MC3, while outperforming the previously described MC3 (Yu et al. 2020). These studies emphasize the importance of careful rational design and empirical optimization to improve targeted delivery.

3.2.2 Polymer-Based Delivery

Polymers have been used less frequently in liver-directed mRNA delivery compared to LNPs. Crowley and colleagues achieved liver-directed delivery; however, this study used HDI as the route of administration and therefore is not feasible in humans (Crowley et al. 2015). As HDI alone is a well-characterized method of delivering gene therapies to the liver (Zhang et al. 1999), the polyplex itself may not be liver-specific. Dendrimer-based LNPs have been used for liver-specific delivery of mRNA encoding fumarylacetoacetate hydrolase for the treatment of Hepatorenal Tyrosinemia Type I (Cheng et al. 2018). Liver-specific delivery was achieved which resulted in a therapeutic effect, albeit some leaky expression occurred in the spleen.

3.2.3 Modified Lipids for Active Targeting of the Liver

Nanoparticles, apart from their intrinsic targeting abilities, can be modified using ligands or antibodies to target different liver cells. The ligand or antibody is mainly conjugated to the PEG-lipid or cholesterol and can therefore be easily adapted to a broad range of nanoparticles (Akinc et al. 2010). Ionizable LNPs have been shown to deliver nanoparticles to hepatocytes, yet incorporating a mannose ligand conjugated to the PEG-lipid was used to target LSECs (Kim et al. 2021). These mannose moieties also increase the size of the nanoparticles, further improving LSEC targeting. While most of the cells transduced were LSECs, some leaky (15%) delivery to hepatocytes and KCs was detected. Increasing PEG content reduced ApoE binding (Kumar et al. 2014), which may improve LSEC targeting efficacy. Recently, three different ligands conjugated to Chol-PEG400 in a single lipidoid nanoparticle have been investigated. A self-peptide (SP) prevented macrophage clearance of nanoparticles in circulation, while mannose targeted LSECs and W5R4K, an amphipathic homochiral L-cyclic peptide, was used to target hepatocytes. After optimization of the ratio of Chol-PEG400-conjugated ligands using central composite design, efficient delivery of mRNA to the liver was shown, albeit with slight leaky expression

in the spleen and lungs. Protein expression was also markedly increased compared to the original formulation and MC3-iLNPs. However, delivery occurred mainly to hepatocytes (94%) suggesting cell culture experiments showing mannose-specific delivery of LSECs did not translate to in vivo efficacy (Zheng et al. 2021). Phospholipid modifications may help with cell-specific targeting without the use of ligands and have been used to generate novel iLNPs. By incorporating adamantyl groups to generate "constrained phospholipids," Gan and colleagues identified adamantyl-phospholipids iLNP that specifically targeted KCs in the liver as opposed to other liver cells (Gan et al. 2020). This suggests that iterative design may be key to achieving cell-specific delivery.

The asialoglycoprotein receptor (ASGP-R) is by far the most commonly used target for hepatocytes (Fig. 4b). This receptor is exclusively and abundantly expressed on hepatocytes, which can be exploited to enable efficient uptake of nanoparticles upon recognition by the targeting ligand (Zijderhand-Bleekemolen et al. 1987). As such, several different ligands have been used for targeting. Liposomes with cholesteryl-β-D- galactopyranoside (Chol-β-Gal) (Naicker et al. 2014) and galactose conjugated Polyethylenimine (Xia et al. 2012; Hayashi et al. 2012) have been developed for ASGP-R mediated delivery. Recently, Prieve and colleagues delivered human ornithine transcarbamylase encoding mRNA replacement therapy in a murine model using *N*-acetylgalactosamine (GalNAc) to target the polymer micelle portion of their dual delivery system to hepatocytes (Prieve et al. 2018). A similar approach had previously been used to deliver siRNAs by conjugating GalNAc to the PEGylated lipid of iLNPs (Akinc et al. 2010). Efficient ASGP-R targeting of Poly-L-lysine pDNA polyplexes was achieved with asialoorosomucoid (AsOR), which showed high levels of expression in the liver (99%), mainly hepatocytes (Kwoh et al. 1999). Although the polyplexes aggregated in the presence of serum, the addition of PEG substantially reduced aggregation, and therefore, the polyplexes, as well as the targeting modality, still hold potential for mRNA delivery. Asialofetuin (AF)-conjugated DOTAP/PGLA (1,2-dioleoyl-3-trimethylammonium-propane/Poly Lactic-co-Glycolic Acid) lipopolymers have been used to target delivery of pDNA encoding IL-12 as a potential immunotherapy treatment for HCC (Diez et al. 2009). Therapeutic AF-conjugated lipopolymers showed complete tumor regression in 75% of mice compared to 37.5% when using un-targeted lipopolymers (Diez et al. 2009). However biodistribution studies showed reporter gene (*luciferase*) expression was still detected at higher levels in the lungs than the liver following intravenous injection. The lipopolymers were large (370 nm in diameter) and may therefore not be suitable for mRNA delivery to hepatocytes; however, AF could still be used for receptor-mediated targeting of mRNA gene therapies in the future.

3.2.4 The Impact of Nanoparticle Size for Liver Delivery

The size of the delivery vector plays a significant role in achieving cell-targeted delivery of mRNA therapies within the liver. LNPs smaller than 100 nm can move through the fenestrae and deliver the mRNA to hepatocytes while larger particles

cannot, resulting in delivery to the LSECs and Kupffer cells (Chen et al. 2016; Khan et al. 2014). For siRNA delivery to hepatocytes, nanoparticles of 78 and 42 nm showed the highest efficacy, while the smaller 32 nm particles were found to be inefficient, probably a result of reduced stability and endosomal escape (Chen et al. 2016). The percentage of PEG-lipid used during lipid formulation can influence the size in an inversely proportionate manner (higher percentage of PEG reduces the LNP size). This could prove beneficial, by reducing vector size when targeting hepatocytes. However, too much PEG can also be detrimental by blocking targeted interactions. Microfluidics devices can be used to control lipid nanoparticle size by altering the flow rate of the lipid and buffer solutions and the flow rate ratio (FRR) of the two (Tsui et al. 2013; Ran et al. 2017; Ozcelikkale et al. 2017). When creating liposomes, the choice of organic solvent may control nanoparticle size. The polarity of the solvent (lower polarity induced larger particle size) can be manipulated by using combinations of organic solvents (Webb et al. 2019). However, the components of the liposomal formulation also need to be considered when choosing a solvent. Kimura and colleagues have described a "baffle mixing" device (invasive lipid nanoparticle production device, iLiNP) which provided accurate fine tuning of particle size in 10 nm intervals, from 20 to 100 nm (Kimura et al. 2018). The use of pressurized filtration through polycarbonate membranes, known as extrusion, may also reduce size and improve polydispersity (Olson et al. 1979). However, reduced mRNA encapsulation efficiency and poor scalability limit the use of this method (Carstens et al. 2011; Charcosset et al. 2015).

4 The Future of HBV mRNA Therapy

Recent advances in the production and formulation of mRNA and renewed interest in RNA as a drug technology bodes well for the future of HBV gene therapy. The success of the SARS-CoV-2 mRNA vaccines has led to the rapid expansion of preclinical development and commercialization of mRNA therapeutics (Damase et al. 2021). This is likely to result in an increase in the number of clinical trials which will help to determine whether preclinical studies accurately portray therapeutic effect and identify which formulations and modifications are most valuable. For chronic diseases like HBV, the concept of an mRNA-based therapy is appealing. Hepatotropic delivery can be achieved with ligand-modified LNPs targeting ASGP-R, and recent improvements in mRNA design and purification have helped to reduce immunostimulatory effects that would otherwise hamper therapeutic activity.

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Preparation of Synthetic mRNAs—Overview and Considerations

Siu-Hong Chan and Bijoyita Roy

Contents

Abstract Synthetic messenger RNAs (mRNAs) are a novel modality for vaccines and therapeutics. mRNA vaccines have been proven to be clinically efficacious and safe. The central advantage of the successful use of synthetic mRNA as a vaccine hinge on being able to rapidly manufacture the mRNA molecules. The synthetic mRNA is prepared in vitro via a cell-free enzymatic process. The aim of this chapter is to provide an overview of the enzymatic mRNA synthesis process and discuss the enzymatic components involved in the workflow. Furthermore, critical parameters that need to be considered for characterization of the synthetic mRNAs are also discussed.

S.-H. Chan · B. Roy (✉)
RNA and Genome Editing, New England Biolabs Inc., Ipswich, MA 01938, USA
e-mail: broy@neb.com

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_9

Keywords Synthetic mRNA · In vitro transcription · DNA-dependent RNA polymerase · mRNA capping

1 Introduction

1.1 Synthetic mRNAs

mRNA-based therapy is an innovative approach that involves synthesis of an mRNA in vitro, packing the mRNA molecules in a delivery vehicle, delivery of the mRNA to target cells, and expression of the desired protein using the cells' translation machinery. The idea of using in vitro synthesized mRNAs to produce a protein of interest for therapeutic purposes was first described over three decades ago when synthetic mRNAs packaged within a liposomal nanoparticle was transfected in a variety of cell types and expression of the protein of interest was observed Wolff et al. (1990). Synthetic mRNA-based vaccines and therapeutics have numerous advantages over DNA- or antibody-based therapeutics in terms of safety and efficacy (Pardi et al. 2018). mRNA does not integrate into the host genome and therefore there is no potential risk of insertional mutagenesis. mRNA expression is transient in that the synthetic mRNA is subjected to cellular degradation machinery, and therefore, the in vivo half-life of the mRNA can be regulated. Additionally, the use of synthetic nanolipid particles (LNP) as packaging and delivery vehicle opens up avenues such as multiplexing (multiple mRNA species per nanoparticle) (Hajj et al. 2020) and inclusion of carrier molecules that can increase the uptake of the mRNA and increase the expression levels in the cells. From a manufacturing standpoint, production of mRNA is rapid, inexpensive, and scalable due to high yield in vitro transcription reactions. The generality of the approach where any protein of interest can be encoded and expressed by altering the sequence of the RNA molecule further allows for diverse products to be manufactured using the same established production process.

Despite its numerous advantages and huge success in manufacturing and deployment of mRNA vaccines against COVID-19 in record time during a pandemic, there are still on-going challenges. The critical factors that have impeded the advancement of synthetic mRNA-based vaccines/therapeutics as a novel modality are the reduced stability of synthetic mRNAs in vivo and the inherent immunogenicity of the synthetic molecule. Furthermore, pharmacology of mRNA drugs is complex because the synthetic mRNA is not the final pharmacologically active agent. Once delivered in vivo, the protein encoded by the mRNA needs to be expressed and the expression needs to be controlled under clinical conditions for consistent dosing across patients. The mRNA also needs to be delivered to the correct tissue, as there could be cell-type-specific differences in mRNA regulation and expression. In recent years, innovations have centered around addressing the challenges of increased immunogenicity, reduced stability, and the inefficient delivery of the mRNA biomolecules in vivo.

Synthetic mRNAs can trigger the cell's antiviral defense mechanism with undesirable outcomes in therapeutic applications where an immune response is detrimental or unnecessary (i.e., protein-replacement therapies). The immune response from synthetic mRNAs is imparted by more than one mechanism. Impurities in the in vitro transcription reaction (discussed in details in Sect. 2) and recognition of GU-rich sequences in ssRNA have been demonstrated to activate cellular immune receptors by independent mechanisms (Kariko et al. 2005). Dodging the host immune system while ramping up protein expression has been a major bottleneck in the successful implementation of this class of biologics. Advances have been made to address the immunogenicity of in vitro transcribed mRNA, including structural and chemical engineering of the mRNA. For instance, U-depleted mRNA sequences have been observed to be poor ligands for cellular immune receptors (Vaidyanathan et al. 2018). One of the major breakthroughs for the success of synthetic mRNA came from the observation that incorporation of naturally occurring chemical base modifications (such as pseudouridine and N1-methylpseudouridine) in the mRNA can alleviate part of the immunogenicity from synthetic mRNAs (Anderson et al. 2010; Andries et al. 2015; Kariko et al. 2011, 2012, 2008; Svitkin et al. 2017).

1.2 Kinds of Synthetic mRNAs

The use of mRNA-based vaccines has been described previously in preclinical and clinical settings, and three main classes of mRNAs have been evaluated: conventional unmodified non-replicating mRNAs, chemically modified non-replicating mRNAs, and self-amplifying mRNAs (saRNAs or replicons). Self-amplifying mRNAs maintain the auto-replicative activity derived from an RNA virus and require a lower dose of RNA due to the self-replicative properties (Blakney et al. 2021). The structural features of these three kinds of synthetic mRNAs are quite similar, and all three are manufactured using a similar cell-free enzymatic transcription reaction.

1.3 Key Structural Attributes for a Functional Synthetic mRNA

The core principle of synthetic mRNA-based therapeutics relies on the delivery of the transcript encoding the protein of interest into the cytosol of the host cell where the mRNA is translated by the cellular translation machinery. Hence, the design of the synthetic molecule is aimed toward maximizing the interaction of the synthetic molecule with the cellular translation machinery once it is released in the cytosol while avoiding the immune receptors and premature encounter with the RNA degradation machinery. All classes of mRNAs have a few structural elements that are common—the 5′ m^7GpppN cap, 5′- and 3′-UTRs, the open reading frame (ORF) and

the poly-A tail. In addition to these structural elements, the self-amplifying mRNA constructs contain the genetic replication machinery (the viral RNA-dependent RNA polymerase and accessory proteins) derived from positive stranded mRNA viruses to direct amplification of the saRNA construct in the cytosol of the host cell. The current technology for synthesis of all three kinds of mRNAs relies heavily on in vitro enzymatic synthesis; chemical synthesis of long mRNA is challenging with a size limit of 100–150 nucleotides. The 5′ cap and the poly-A tail, required for efficient expression in the cell, are added during in vitro transcription or added enzymatically post transcription. The 5′ cap and poly-A tail work synergistically—protein factors that bind to the cap and poly-A tail interact and form a closed-loop complex which improves binding of translation factors and protects the mRNA from degradation (Roy and Jacobson 2013). Poly-A tails are added to synthetic mRNAs in vitro to recapitulate the stable closed-loop mRNP conformation in vivo. Furthermore, the addition of at least 150 nucleotides during polyadenylation has also been shown to reduce mRNA immunogenicity (Koski et al. 2004).

The expression from the synthetic mRNA can be optimized further by optimization of the UTR sequences to increase mRNA stability and translation efficiency. Replacing unstable regions in the untranslated areas with stable structures, such as replacing AU-rich regions in the 3′ UTR or complete replacement of 3′-UTRs with those from α- and β-globin, can increase mRNA half-life (Aviv et al. 1976; Ross and Sullivan 1985). Protein expression can also be optimized by duplicating 3′ UTR sequences in tandem (Ferizi et al. 2016). UTRs can also be deliberately modified to encode regulatory elements (i.e., microRNA binding sites) as a way to control RNA expression in a cell-specific manner (Jain et al. 2018). Recently, large-scale selections have been performed to identify naturally occurring 3′ UTRs that stabilize specific mRNAs and improve expression over globin UTRs, leading to synthetic mRNA molecules that induce a stronger antigen-specific immune responses in vaccinated mice (Orlandini von Niessen et al. 2019). Finally, several approaches can be used to optimize the ORF sequence to enhance translation efficiency. Codon optimization (the replacement of rare codons for synonymous codons) can be implemented to increase translation efficiency. However, this method should be applied carefully since some proteins require slow translation, accomplished through rare codons, for correct folding (Spencer et al. 2012). The GC content can also impact protein expression and incorporation of certain chemical modifications into the ORF have also been shown to increase translation efficiency.

1.4 Chemical Modifications of Synthetic mRNA

Chemical modifications are almost ubiquitously used for short RNA therapeutics, such as siRNA and ASO. In contrast, mRNAs must be efficiently recognized by a multitude of cellular factors in the endosome and the cytoplasm and are therefore more sensitive to the effect of modifications. Most applications of synthetic mRNAs have focused on naturally occurring chemical modifications to alleviate

any detrimental effect. However, there are significant differences in the occupancy of modification in a synthetic mRNA as compared to a natural mRNA. Typically, modifications are incorporated in synthetic mRNAs during the in vitro transcription reaction where a modified nucleotide partially supplements or completely replaces the standard unmodified nucleotide. Partial substitution generates a heterogeneous mRNA population because the incorporation location of the modified nucleotide cannot be controlled by the process. For therapeutic purposes, it has become a standard practice to completely replace a standard nucleotide with a modified version (i.e., 100% replacement). This allows for consistency and reproducibility in the synthesis of the mRNA and results in mRNA molecules that are identical with respect to the location of the modified nucleotides. Given that modifications such as pseudouridine is known for altering synthetic mRNA folding (Mauger et al. 2019) and translation decoding (Svitkin et al. 2017; Eyler et al. 2019), and that modified nucleotides are present at low frequencies in endogenous mRNA, it is therefore critical to understand how complete substitution by modified nucleotides may affect in vivo outcomes such as the accuracy of translation, stability of the synthetic mRNA molecules in addition to the well-studied reduction in innate immunogenicity (Roy 2020).

1.5 The mRNA Cap

All eukaryotic mRNAs contain a cap structure—an N7-methylated guanosine linked to the first nucleotide of the RNA via a reverse 5′–5′ triphosphate linkage (Cap-0). The 5′ cap is essential for cap-dependent initiation of protein synthesis. It is a focal point where proteins bind and perform biological functions such as mRNA export, splicing and miRNA synthesis in the nucleus, and efficient protein synthesis in the cytoplasm (Ramanathan et al. 2016; Gonatopoulos-Pournatzis and Cowling 2014). In the cytoplasm, the mRNA cap interacts with eIF4E, which goes on to assemble other protein factors and the ribosome to carry out protein translation (Ramanathan et al. 2016). The cap structure also play an important role in regulating 5′ mRNA degradation. The cap prevents cellular nucleases from degrading the mRNA molecule at 5′ end. To initiate mRNA degradation from the 5′ end, cells adopt sophisticated systems to decap and then degrade the mRNA in response to specific signals (Grudzien-Nogalska and Kiledjian 2017).

In metazoans, the Cap-0 structure is further modified to Cap-1 where a methyl group is added to the 2′-O position of the ribose of the first nucleotide. Recent studies have revealed that 2′-O-methylation of + 1 nucleotide is central to the non-self-discrimination of innate immune response against foreign RNA (Daffis et al. 2010). Structural studies have shed light on the structural basis of such discrimination (Devarkar et al. 2016). In mammalian cells, 5′ capping takes place as soon as the first 12–25 nucleotides are transcribed by the RNA polymerase II complex. Three enzymes and four enzymatic activities act in tandem to convert a 5′ triphosphate group of a nascent transcript to the Cap-1 structure. First, a bifunctional enzyme RNA guanylyltransferase and 5′ phosphatase (RNGTT) interacts with the phosphorylated

C-terminal Domain (CTD) of RNA PolII and converts the 5′ triphosphate group to a diphosphate. It then adds a GMP to the diphosphate to form the unmethyl-G cap. The second enzyme RNMT then adds a methyl group to the N7 position of the G cap to form Cap-0. A third enzyme CTMR1 further adds a methyl group to the 2′-O position of the ribose of the first nucleotide to form the Cap-1 structure. For therapeutic applications, the installation of the Cap-1 structure on synthetic mRNA is crucial for efficient protein translation and evasion of innate immune response.

2 Platforms for mRNA Synthesis

Synthetic mRNAs are produced in vitro in cell-free enzymatic transcription reactions. In vitro transcription of synthetic mRNAs was first demonstrated in the 1980s using phage RNA polymerases (Krieg and Melton 1984; Melton et al. 1984). Over the years, it has become a well-established platform for large-scale production of synthetic RNAs-kits that can synthesize milligram quantities of RNA are readily available commercially. It is also a relatively simple manufacturing process and is adaptable to many categories of synthetic (m)RNAs. In addition, the cell-free nature of in vitro transcription means that the mRNA vaccines produced are not expected to have the safety concerns associated with vaccines produced using cell-based methods, such as live or attenuated viral vaccines, recombinant subunit proteins, and viral vectors.

A typical synthetic mRNA workflow consists of a few distinct steps: template generation, followed by in vitro transcription and then post-transcriptional modification of the RNA to modify the 5′ and 3′ ends of the RNA to generate a functional mRNA (Fig. 1). Briefly, an expression plasmid that encodes a DNA-dependent RNA polymerase promoter (from T7, T3, or SP6 bacteriophages) and the RNA vaccine candidate is first designed as a template for in vitro transcription. The template DNA contains the mRNA sequence (including UTRs, ORF) and can also contain the 3′ poly-T sequence for attaining the 3′ poly-A tail of the mRNA. The choice of the UTRs and ORF sequences are critical determinant of the efficacy from the synthetic

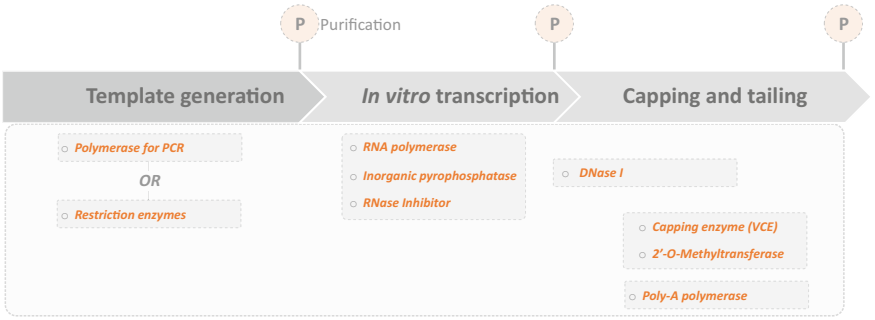

Fig. 1 mRNA synthesis workflow

mRNA and can be rationally designed for optimal expression from the synthetic mRNA. Furthermore, for saRNA vaccines, the pDNA templates contain additional viral replicon genes and other conserved sequence elements. The linearized template DNA is used for in vitro transcription with a single-subunit DNA-dependent RNA polymerase (such as T7 RNA polymerase) resulting in multiple copies of the RNA transcript that is then capped and tailed post-transcriptionally. There are variations of this standard workflow where RNA capping with a synthetic cap analog can be performed co-transcriptionally (Kore and Charles 2010; Kowalska et al. 2008; Strenkowska et al. 2016). Apart from the enzymatic reactions, there are intermediate purification steps that are also critical for the mRNA synthesis workflow. In this section, the different enzymatic components that are involved are discussed with emphasis on the transcription and capping steps.

2.1 Restriction Enzymes for Template Linearization

In vitro transcription using phage RNA polymerases requires a linear DNA template that contains an appropriate promoter sequence. While cellular transcription terminates at highly structured terminator sites, in vitro transcriptions are terminated by allowing the RNA polymerase to run off a linear DNA template. In principle, PCR-amplified DNA or linearized plasmids can be used as a template. In practice, linearized plasmids are preferred because PCR-amplified DNA can contain undesirable reaction components carried over from PCR. In addition, plasmid preparation is more amenable to up-scaling than PCR reactions PCR reactions. Propagation of a long poly-T sequence in a plasmid can be challenging. The use of engineered bacterial strains (Grier et al. 2016) and introduction of specific sequences in the poly-T region (Trepotec et al. 2019) have been demonstrated to aid in maintaining of a long stretch of poly-T sequence in a plasmid.

In transcription template design, a restriction site is usually added at the end of the transcribed region such that a simple restriction digestion can generate a linear plasmid to facilitate transcription termination at the desired position. In traditional cloning, DNA molecules are fragmented at specific sites using restriction enzymes (REases) and then ligated to plasmid DNA linearized by the same restriction enzymes. These Type IIP REases recognize palindromic sequences that span 4–8 bps and cut within the recognition sites. Using Type IIP REases to linearize a plasmid transcription template will invariably leave behind extra nucleotide at the end of the transcription template, generate a scar at the end of the transcript that may not be desirable. Whether or not the presence of a few nucleotides from the restriction enzyme recognition site at the 3′ UTR of the mRNA will have any biological relevance might depend on the mRNA sequence and needs to be taken into account. To overcome this, Type IIS REases that cut outside of recognition sites can be used (Fig. 2). Unlike Type IIP REases whose active sites combine sequence recognition and DNA cleavage activity, Type IIS REases, where S stands for "shifted cleavage," usually adopt a modular structure with separate specificity domain and a DNA cleavage domain. This unique

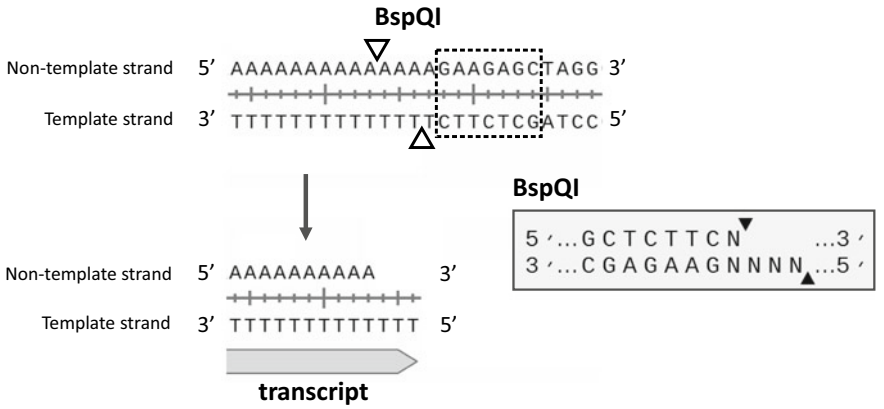

Fig. 2 Linearization of plasmid template using type IIS restriction enzyme BspQI

modular structure has been used to create strand-specific DNA nicking enzymes that have found applications such as isothermal amplification (Chan et al. 2011). The "shifted cleavage" property has also been utilized Golden Gate Assembly where large DNA pieces can be assembled seamlessly using double-stranded synthetic gene blocks (Pryor et al. 2020). It has been reported that the nature of the 3′ end of the template can result in spurious product formation during in vitro transcription (Schenborn and Mierendorf 1985). REases leaving a 3′ protruding end can result in the synthesis of transcripts longer than the expected run-off. For a synthetic RNA to be used for therapeutic applications, it is critical to have transcripts with precise ends and the template DNA linearization approach plays a key role.

2.2 RNA Polymerase

In vitro RNA synthesis is most commonly catalyzed by single-subunit DNA-dependent RNA polymerases such as T7 RNA polymerase which was first isolated from bacteriophage T7-infected *Escherichia coli* cells. Solid-phase chemical RNA synthesis is routinely used to generate RNAs up to 50–100 nucleotides in length, but synthesis of long RNA with high yield is not achievable with current solid-phase chemical synthesis technologies. T7 RNA polymerase is a 98 kDa polypeptide with unique properties: (a) single-subunit enzyme compared to prokaryotic and eukaryotic RNA polymerases that are multi-subunit, (b) has high specificity toward a DNA sequence called the promoter sequence for initiating transcription, (c) transcription does not require additional factors for activity and termination, (d) capable of robust elongation and can transcribe long RNAs.

The T7 phage genome is mainly classified into three different classes based on the temporal expression pattern during the infection cycle. Class I genes are expressed

early in the infectious cycle and their expression initiates phage transcription, inhibits host Type I restriction systems and host-catalyzed transcription. DNA replication proteins are encoded by class II genes whereas proteins responsible for DNA packing, viral assembly and cell lysis are expressed during later stages of infection from class III genes. T7 RNA polymerase is a class I gene transcribed by the host *E. coli* RNA polymerase during the early stages of infection. A Rho-dependent termination event results in the halting of the transcription by the host polymerase and the T7 RNA polymerase takes over the transcription of the downstream class II and class III genes.

2.2.1 T7 RNA Polymerase Structure

T7 RNA polymerase is structurally related to polymerases that include single-subunit DNAPs as well as reverse transcriptases where the polymerase domain is characterized by the presence of conserved thumb, fingers, and palm sub-domains. These conserved domains form a deep cleft—the binding site for the DNA template. There is extensive structural and mechanistic information available on T7 RNA polymerase and there are excellent reviews available for readers to get a better understanding of T7 RNA polymerase structure and function (Sousa and Mukherjee 2003). For simplicity, a short summary of the structural features that are relevant for synthetic mRNA workflows is highlighted here.

T7 RNA polymerase also consists of a unique N-terminal domain (residues 1–325) that is involved in promoter binding and opens up the duplex template DNA for transcription initiation. The promoter sequence in the template DNA is recognized by the the N-terminal domain and the specificity loop. During the transition from initiation to elongation phase of transcription, the N-terminal domain undergoes a major conformational change that results in the dissociation of the RNA polymerase from the promoter and formation of the RNA exit and substrate entry channels essential for elongation. Residues 93–101 and 232–242 have been reported to be important for this transition. Structural studies as well as mutational analyses of the *N*-terminal domain of T7 RNA polymerase have demonstrated that Lys172 is involved in RNA binding. The presence of a proteolytic nick at Lys172 results in reduced processivity of T7 RNA polymerase during transcription elongation and reduces the affinity of the polymerase for single-stranded DNA.

The thumb sub-domain of T7 RNA polymerase ranges from residues 326–411 and forms an a-helical projection on the template-binding cleft. Crystal structure of the promoter polymerase complex revealed that the thumb domain prevents transcription complex dissociation while still allowing the complex to slide along the DNA template.

The palm sub-domain of the T7 RNAP spans from residues 412–553 and 785–879. The palm domain harbors two catalytically critical residues: Asp537 and Asp812. The two negatively charged residues orient two metal ions (Mg^{2+}) to catalyze the extension of the transcript. The Mg^{2+} ions interact with the beta- and gamma-phosphates of the incoming nucleotide triphosphate. One of the Mg^{2+} ions is first released with

the pyrophosphate (PPi) and the second Mg^{2+} ion remains in the active site to orchestrate the nucleophilic attack of the 3′ oxygen of the RNA terminal nucleotide to the a-phosphate of the incoming nucleotide. Lys472 in the palm sub-domain aids in the PPi release.

Translocation of T7 RNAP is facilitated by conformational changes in the fingers sub-domain (spanning aa 554–784). Association with the promoter DNA results in a switch from the open conformation to a close conformation (together with the palm sub-domain). Residues Lys627 and Lys631 bind to the incoming rNTP via interactions with the phosphate groups. Met635 interacts with the ribose moiety. T7 RNA polymerase discriminates deoxyribose from ribose triphosphates via an interaction mediated by a magnesium ion located between the 2′-OH of the rNTP ribose and the Tyr639 hydroxyl group. Substitution of Tyr639 with valine or phenylalanine confers an enhanced ability to incorporate 2′-modified nucleotides compared to wild type RNA polymerase. Residues 740–769 of T7 RNAP form a specificity loop that is involved in promoter recognition.

2.2.2 T7 RNA Polymerase for in Vitro mRNA Synthesis

The enzymatic synthesis of RNA with T7 RNA polymerase has been widely adopted for a wide range of applications including the use of synthetic mRNAs for therapeutics where high yield of precise RNA of the correct sequence is critical. Even though T7-mediated in vitro transcription is robust and results in higher yield of RNA, there are certain attributes that are problematic for some of the therapeutic applications. For example, it has long been known that in addition to the full-length run-off transcript, T7 RNA polymerase also generates short abortive transcripts (2–7 nucleotides in length) (Martin et al. 1988) as well as RNAs that are longer than the expected length (Arnaud-Barbe et al. 1998; Konarska and Sharp 1989; Triana-Alonso et al. 1995). The formation of abortive transcripts can drive rNTPs away from forming the target transcript to detrimental byproducts that need to be purified away. The longer products are generated through mechanisms such as template strand switching, non-templated nucleotide additions, and cis- or trans- primed extension of the RNA. These non-promoter driven events can be exacerbated under high yield RNA synthesis conditions where the run-off transcript accumulates in solution to high concentration, prompting the RNA polymerase to rebind the RNA at its 3′ end and further extend the RNA. The resulting 3′ extended byproducts are heterogeneous in nature and results in formation of double-stranded (ds) RNAs that are longer than the run-off transcript (Wu et al. 2020; Gholamalipour et al. 2018). For therapeutic applications, presence of dsRNA contaminants in the in vitro synthesized RNA can result in an innate immune response that is detrimental for certain applications. Two main types of byproducts in the in vitro transcription reaction that result in the formation of dsRNA molecules have been recently identified. One that is formed by the 3′-extension of the run-off products which can anneal to complementary sequences in the body of the run-off transcript. Hybridization of an antisense RNA molecule to the run-off transcript can also form dsRNA byproducts (Wu et al.

2020; Gholamalipour et al. 2018; Mu et al. 2018). dsRNA byproducts from the in vitro transcription reactions can activate sensors such as retinoic acid inducible gene (RIG-I), Toll-like receptor 3 (TLR-3), and protein melanoma-differentiation-associated antigen 5 (MDA5) (Hur 2019). It is therefore desirable to either prevent formation of these byproducts or remove these byproducts after the in vitro synthesis process via extensive purification of the RNA. Several purification strategies have been reported to help remove the dsRNA byproducts but most of the purification strategies are not amenable to scale-up and are not cost-effective (Kariko et al. 2011; Baiersdorfer et al. 2019). Furthermore, each purification step also compromises the final yield of the RNA.

The other approach has been to alter in vitro transcription reaction conditions to reduce the formation of the dsRNA byproducts. Methods to prevent the rebinding of run-off transcript to the T7 RNA polymerase by either adding a competing oligonucleotide that anneals to the 3′ end of the RNA or by increasing the salt concentrations in solution to reduce protein–nucleic acid interactions have been demonstrated to reduce the formation of certain kinds of dsRNA byproduct in the in vitro transcription reactions (Gholamalipour et al. 2018, 2019; Cavac et al. 2021). Alternatively, mRNAs can be synthesized with engineered thermostable T7 RNA polymerase at temperatures greater than 48 °C to reduce 3′-extended dsRNA byproducts (Wu et al. 2020).

RNA polymerases that do not have this inherent property of forming spurious byproducts during in vitro transcription will be advantageous in streamlining the RNA synthesis workflows where some of the downstream purification steps can be omitted.

2.3 RNase Inhibitor

To prevent RNA degradation due to spurious RNase contamination, it is a common practice to include a RNase inhibitor such as the murine RNase inhibitor (RNH1) in mRNA manufacturing. RNH1 is a relatively small protein (~ 450 aa) that adopts a horseshoe shape where the target RNase A is sequestered at one of the flat horseshoe faces (Kobe and Deisenhofer 1993; Lomax et al. 2014). The physiological target nuclease of RNH1 is angiogenin (ANG), also known as RNase 5, ANG belongs to the Ribonuclease A superfamily and plays an important role in neovascularization—is a potent inducer of blood vessel growth (Fett et al. 1985). It has been shown that ANG plays an important role in translation by upregulating ribosomal RNA synthesis (Tsuji et al. 2005; Hoang and Raines 2017) and tRNA cleavage (Yamasaki et al. 2009; Ivanov et al. 2011).

2.4 Inorganic Pyrophosphatase

Inorganic pyrophosphatase is a ubiquitous enzyme that catalyzes the reversible hydrolysis of inorganic phosphate (PPi) into two inorganic phosphate molecules (Baykov et al. 1996). Inorganic pyrophosphatases are present in two sequence—and structurally diverse forms—the transmembrane proton-/Na^+-pumping pyrophosphatase that couple the energy of PPi hydrolysis to proton or Na^+ translocation across biological membranes and the soluble inorganic pyrophosphatases whose function appears to be hydrolyzing inorganic pyrophosphate (Kajander et al. 2013). During in vitro transcription, as the RNA polymerase extends the RNA transcript using ribonucleoside triphosphates, inorganic pyrophosphate (PPi) is released as a byproduct. The accumulation of PPi can trigger pyrophosphorolysis whereby the RNA polymerase catalyzes the reverse reaction, condensing the 3′ ribonucleotide with PPi, forming NTP, and effectively shortening the transcript. To mitigate this phenomenon, inorganic pyrophosphatase derived from *E. coli* or *S. cerevisiae* is usually included in the in vitro transcription reactions to remove PPi to improve the yield of transcription.

2.5 DNase I

DNase I is an endonuclease that catalyzes the hydrolysis of double-stranded DNA predominantly by a single-stranded nicking mechanism under physiological conditions in a Ca^{2+}-dependent manner to produce mono- and oligodeoxyribonucleotides with 5′-phospho and 3′-hydroxy termini (Kunitz 1950; Vanecko and Laskowski 1961; Campbell and Jackson 1980). In vivo, DNase I has been demonstrated to be responsible for internucleosomal DNA degradation during apoptosis as well as to be related to several diseases (discussed in Fujihara et al. (2012)). The catalytic activity of DNase I is dependent on four aa residues (Glu78, His134, Asp212, and His252) and structural stability is imparted by two Cys residues (position 173 and 209) forming a disulfide bond. Two well-conserved *N*-glycosylation sites have been reported in Mammalian DNase I. In vertebrates, DNase I activity is maintained by imparting thermal stability and proteolysis resistance of the enzyme. DNase I is used in vitro for multiple applications. For in vitro transcription reactions, DNase I is used to remove the template DNA following the transcription reaction. Hyperactive variants of DNase I with over 35-fold activity have been rationally designed by replacing residues close to the DNA phosphate backbone with positively charged amino acids that favor the interaction with negatively charged phosphate groups in the DNA (Pan and Lazarus 1997). Another interesting feature of the hyperactive variants that make them better suited for their use in synthetic mRNA workflows is that they are not inhibited by physiological salt concentrations. Higher affinity of the DNase I variants for DNA and greater catalytic efficiencies can help remove trace amounts DNA

template from the in vitro transcription reactions, and salt-tolerance of the DNase I variants makes the workflow compatible for scale-up.

2.6 Capping Enzyme

The conversion of the 5′ triphosphate-RNA to the Cap-1 structure requires multiple enzymes. A total of four enzyme activities are required: the RNA triphosphatase activity (TPase) removes the γ-phosphate of the 5′ triphosphate and generates a 5′ diphosphate group; the RNA guanylyltransferase activity (GTase) transfers a GMP group to the 5′ diphosphate to form a Gppp-cap structure (incomplete); the RNA cap guanine-N7 methyltransferase activity (MTase) adds a methyl group to the N7 amine of the guanosine cap to form the Cap-0 structure. Finally, mRNA cap 2′-O-methyltransferase activity finishes the Cap-1 structure by adding a methyl group to the 2′-O position of the ribose of the first nucleotide of the RNA (Fig. 3). In mammalian cells, the RNA TPase and GTase activities are invariably carried by the bifunctional enzyme RNGTT (known as MCE1 in mice). The guanine-N7 MTase activity is carried by RNMT, and the cap 2′-O-MTase activity is carried by Cap methyltransferase 1 (CMTR1). In lower eukaryotes such as *S. cerevisiae* where mRNA contains the Cap-0 structure, the three activities are carried by separate enzymes (Cet1, Ceg1 and Abd1). In large DNA viruses such as Vaccinia virus and Africa Swine Fever Virus, a single protein carries for all three enzyme activities, although in Vaccinia virus (and all poxviruses), a small subunit is required to facilitate efficient guanine-N7-MTase activity. Much like their mammalian hosts, a separate enzyme carries the cap 2′-O-MTase activity. Notably, Orbiviruses have evolved a quadruple-functional protein that carries all four enzyme activities required to convert the nascent 5′ triphosphate group to the Cap-1 structure.

2.6.1 RNA Triphosphatase Activity

RNA triphosphatase is a polynucleotide 5′-phosphatase that converts the terminal triphosphate of polyribonucleotides to diphosphate (Reaction 1, Fig. 3) and hydrolyzes ribonucleoside triphosphate to diphosphate in vitro. In metazoans, the RNA triphosphatase is independent of divalent metal ions and physically linked to the GTase activity in a bifunctional protein (Mce1 in mice, or RNGTT in mammals in general). These metal-independent RNA TPases contain the conserved HCXXXXXR (S/T) motif of the cysteine phosphatase superfamily that includes protein tyrosine phosphatases and phosphoinositide phosphatases (Changela et al. 2001). The RNA TPase of lower eukaryote and most DNA virus capping enzymes belong to the triphosphate tunnel metallozymes (TTMs) family that adopts a β-barrel tunnel structure and require divalent metal ions for catalysis (Gong and Shuman 2002; Bisaillon and Shuman 2001). Crystal structures show that these TPases share a β-barrel tunnel structure of the triphosphate tunnel metallozymes (TTMs). These TTMs share the

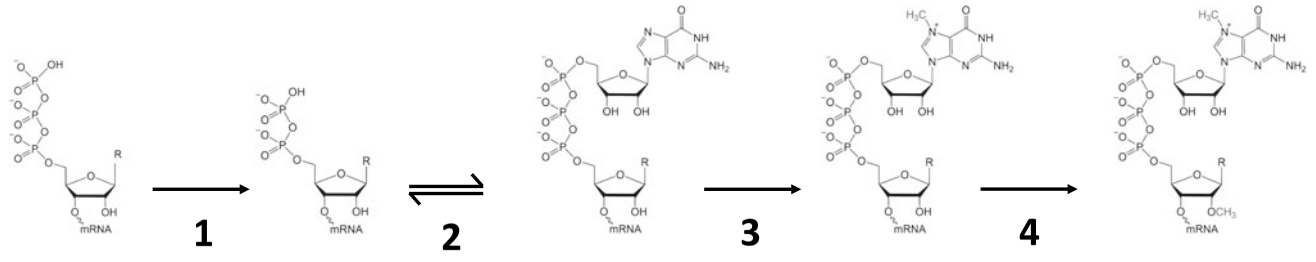

Fig. 3 Enzyme activities involved in RNA capping

conserved feature of coordinating the metal ions by negatively charged amino acid residues and the positioning and stabilizing the γ-phosphate by positively charged residues (Martinez et al. 2015). Mutation of the charged residues lining the tunnel of the prototypic RNA TPase Cet1 from *S. cerevisiae* leads to the loss of in vitro TPase activity and a lethal phenotype (Bisaillon and Shuman 2001; Lima et al. 1999).

2.6.2 RNA Guanylyltransferase Activity

RNA guanylyltransferase, formally known as GTP-RNA guanylyltransferase, transfers a GMP moiety from GTP to the 5′ diphosphate of TPase-processed RNA, forming a Gppp-capped 5′ end. RNA GTases belong to a group of nucleotidyl transferases that includes ATP- and NAD^+-dependent DNA and RNA ligases (Shuman and Lima 2004). This class of nucleotidyl transferases catalyzes nucleic acid ligation through a reversible two-step mechanism that involves a lysyl-Nz-linked covalent intermediate and the formation of a 5′-5′ phospho(deoxy)ribose product. A detailed review of the enzymatic properties of RNA GTase can be found here (Ramanathan et al. 2016). An interesting characteristic of RNA GTases is their reversibility. A detailed kinetic and thermodynamic study of Chlorella virus GTase showed that in the absence of a cap-accepting RNA, the reverse reaction of the first step of the reaction (enzyme self-guanylylation; Reaction 2.1, Fig. 3) can proceed with a low GTP concentration and at a much higher rate than the forward reaction (Souliere et al. 2008). On the other hand, the rate constant of the forward reaction of the second half of the reaction (the transfer of GMP to ppRNA) is tenfold higher than that of the reverse reaction, suggesting that the presence of a cap-accepting RNA drives the GTase reaction forward. In mammalian cells, the bifunctional protein RNGTT (RNA TPase activity and GTase) is recruited to the Ser5-phosphorylated C-terminal domain (CTD) of RNA Pol II during transcription initiation. That positions RNGTT to the transcript exit tunnel where it acts on the nascent transcript as it emerges from the polymerase (Martinez-Rucobo et al. 2015). Interaction with CTD has been shown to increase the activity of the GTase (Ghosh et al. 2011).

2.6.3 mRNA Cap Guanine-N7 Methyltransferase Activity

mRNA cap guanine-N7 methyltransferase RNMT catalyzes the transfer of a methyl group from S-adenosyl methionine (SAM) to GpppRNA to form m^7GpppRNA and S-adenosyl homocysteine (SAH) (Reaction 3, Fig. 3). RNA guanine-N7 MTases adopt the Rossmann fold commonly found in nucleic acids methyltransferase (Schubert et al. 2003; Byszewska et al. 2014). Unlike N6 deoxyadenosine MTases, where the N6 amine of the deoxyadenosine is invariably in close proximity to a basic amino acid residue that deprotonates the nitrogen as it attacks the electrophilic methyl carbonation (Bheemanaik et al. 2006), crystal structures of Ecm1 of *Encephalitozoon cuniculi* and Bluetongue virus capping enzyme VP4 suggest that the cap-specific MTases do not make direct contacts with the N7 atom or the methyl carbon of SAM.

It is therefore suggested that RNA guanine-N7 MTases catalyze by coordinating the reacting parties in the correct position instead of stabilizing the transition state or activating the nucleophile (Fabrega et al. 2004; Sutton et al. 2007).

As the last step of Cap-0 formation, the cap methylation process is regulated by numerous mechanisms. Previously thought to act as a monomer, RNMT has been shown to interact with a previously uncharacterized protein, RAM/Fam103a1 in mammalian cells. RAM increases the binding affinity of RNMT to SAM and RNA, activates its MTase activity and recruits the RNMT-RAM complex to transcription initiation sites {Gonatopoulos-Pournatzis, 2011 #2500;Aregger, 2013 #2499} (108,109). RAM is also found to be required for the maintenance mRNA levels, translation and cell viability {Gonatopoulos-Pournatzis, 2011 #2500}(108). More recently, the cap methylation process is found to be further regulated by the phosphorylation of RNMT in a cell cycle dependent manner {Aregger, 2016 #2501}(110). The presence of multiple control mechanisms for RNMT activities suggests that the methylation status of the cap is potentially an important regulation point in mammalian gene expression.

2.6.4 Cap 2′-O-Methyltransferase Activity

In mammals, Cap Methyltransferase 1 (CMTR1) modifies the first transcribed nucleotide by transferring a methyl group to the 2′-O position of the ribose (Belanger et al. 2010) (Reaction 4, Fig. 3) such that the mRNA is tolerated by the cellular innate immune system. CMTR1 is a multi-domain protein consisting of a G-patch domain, a RrmJ/FtsJ methyltransferase domain, a non-functional cap guanylyltransferase-like domain and a WW domain (Pichlmair et al. 2011; Balagopal and Parker 2009). Like the rest of the RNA capping apparatus, CMTR1 is recruited to the RNA exit tunnel of RNA Pol II at the initiation of transcription by the interaction between the WW domain and the Ser5-phosphorylated CTD of RNA Pol II (Inesta-Vaquera et al. 2018).

2.7 Poly-A Polymerase

In eukaryotes, polyadenylation is an essential step in mRNA maturation. Addition of the poly-A tails at the 3′ end of mRNA facilitates transport of the mRNA from the nucleus to the cytosol and alters the translation efficiency and half-life of the mRNA. In vitro transcribed RNAs are also modified at the 3′ end to resemble a endogenous mRNAs. The addition of the poly-A tail to an in vitro transcribed mRNA confers stability to the mRNA and allows it to form a translation-competent ribonucleoprotein (RNP), together with the 5′ cap of the mRNA. The poly-A tail can be added to the synthetic RNA during transcription where the poly-A sequence is encoded by the DNA template. Alternatively, poly-A tail can be added enzymatically with *E. coli* PAP I after the in vitro transcription reaction. In *E. coli*, two poly-A polymerases (PAPs)

have been identified (PAP I and PAP II) (Cao et al. 1996; Cao and Sarkar 1992; Raynal and Carpousis 1999). PAP I, encoded by the pcnB gene, was initially perceived to control ColE1 plasmid copy number (Lopilato et al. 1986; March et al. 1989). The role of PAP II in mRNA metabolism is not well understood. In contrast to eukaryotic PAPs that require additional protein factors for activity, *E. coli* PAP I is highly active by itself in vitro and therefore a great choice for biotechnology applications. Furthermore, PAP I does not require any specific recognition sequence and can polyadenylate at a variety of sites. PAP I belongs to the nucleotidyltransferase (Ntr) superfamily that also includes tRNA CCA-adding enzymes and eukaryotic PAPs (Raynal et al. 1998). The polymerase activity is conferred by five highly conserved aspartic acids in the putative catalytic site of PAP I. Recombinant PAP I has been used for different biotechnology applications including adding a poly-A tail to synthetic RNAs. The tail length can be adjusted by titrating PAP I in the reaction. However, in contrast to template-encoded poly-A tailing where the tail length is expected to be uniform, PAP-generated poly-A tails are heterogeneous in length.

3 Purification of the Synthetic mRNA

The synthetic mRNA needs to be purified from the impurities after in vitro transcription and capping. The impurities consist of materials used in the enzymatic steps such as residual DNA template, the RNA polymerase, capping enzymes, and nucleotides among others. As discussed in the previous sections, byproducts from in vitro transcription reactions such as dsRNA and abortive transcripts also need to be eliminated from the mRNA preparation. The negatively charged mRNA can be purified via implementing approaches such as reversed-phase chromatography (RPC), size-exclusion chromatography (SEC), anion-exchange chromatography (AIEX), hydrophobic interaction (HIC), and thiophilic adsorption chromatography (TOC). For research use, mRNA can also be precipitated by ethanol in the presence of a positively charged ion such as Na+ or Li+. The positively charged ion neutralizes the negative charge of the RNA backbone, whereas ethanol shields the neutralized RNA molecules from water. Together the positively charged ion and ethanol decreases the solubility of the RNA to a point that it precipitates. Precipitation approaches, however, are not scalable and therefore not suitable for the preclinical and clinical scale manufacturing. Furthermore, precipitation approaches do not purify the RNA byproducts away from the run-off transcript of choice. Tangential flow filtration (TFF)-based strategies can allow efficient separation of mRNA from smaller impurities as well as adjusting the mRNA concentration for final formulation. Other chromatographic approaches including reverse-phase ion pair, anion exchange and affinity chromatography using poly(dT) capture can be implemented in tandem with TFF for purification of synthetic mRNAs. Reversed-phase ion pairing is not amenable for scale-up but is an efficient and rapid RNA purification approach for separating single-stranded RNA from the DNA template as well as the dsRNA byproducts and abortive transcripts.

Poly(dT)-based affinity capture can specifically capture the poly-A tail of the full-length mRNA. However, dsRNA byproducts that contain a poly-A stretch cannot be separated from poly-A tailed run-off products using this approach. If enzymatic poly-A tailing is performed on in vitro transcribed RNA, truncated RNA products that get poly-A tailed will also be purified by poly(dT) resins. Improved RNA purification approaches that are scalable, capable of removing the various impurities in a cost-efficient manner are highly desirable.

4 Analyses of Synthetic mRNAs

mRNA manufacturing processes that can deliver high quality and consistent products are crucial for producing synthetic mRNAs as therapeutics and vaccines. In addition to establishing robust enzymatic manufacturing workflows, it is also pivotal to establish specifications for critical process steps and criteria for the drug substance and drug product. Analytical technologies that can support mRNA (as well as in vitro transcription process) characterization need to be established and defined. The potency of a synthetic mRNA is dictated by critical quality attributes (CQAs) including: sequence of the mRNA, presence of regulatory sequences that affect the translation and stability of the mRNA, purity of the mRNA preparation, 5′ capping efficiency, extent of modifications present in the mRNA, poly-A tail length. The mRNA quality is typically assessed using analytical techniques such as gel electrophoresis and high-performance liquid chromatography (HPLC). The identity of the synthetic mRNA can be analyzed using sequencing techniques. Furthermore, the presence of enzymatic components, residual template DNA, as well as dsRNA and truncated RNA byproducts need to be determined. In the following sections, we will focus on the capping efficiency at the 5′ end and poly-A tail length at the 3′ end of the synthetic RNA.

4.1 Capping Efficiency

RNA capping is one of the critical quality attributes in mRNA manufacturing. The capability to assess the capping efficiency is also crucial in developing a robust process. Verifying the identity of the cap structure and estimating the extent of capping requires the quantitation of a single additional nucleotide and 2′-O-methyl modification within the context of kilobase or longer synthetic mRNA. One approach to simplify mRNA cap analysis is to specifically cleave a small fragment from the 5′ end of the RNA and subject it to liquid chromatography-mass spectrometry (LC–MS) analysis. Two approaches had been used successfully to achieve such targeted cleavage.

4.1.1 Deoxyribozymes (DNAzymes)

Much like ribozymes, DNAzymes or deoxyribozymes are single-stranded DNA molecules that have a strong propensity to fold into complex three-dimensional structures and perform catalysis. DNAzymes are not found in nature but are evolved and selected in vitro. The first active DNAzyme that cleaves RNA in a sequence dependent manner was reported in 1994 (Breaker and Joyce 1994). Since then, DNAzymes that catalyze DNA self-adenylation (Li et al. 2000), DNA self-phosphorylation (Li and Breaker 1999), DNA cleavage in cis (Carmi et al. 1998) and in trans (Drachenberg et al. 1998), RNA ligation (Flynn-Charlebois et al. 2003; Hoadley et al. 2005; Purtha et al. 2005), RNA lariats formation (Wang and Silverman 2003a, b) and nucleopeptide ligation between tyrosine residues and the 5′ end of an RNA oligo (Pradeepkumar et al. 2008) have been reported. Sequence-specific RNA-cleaving DNAzymes generally consist of a catalytic domain and sequence specificity elements that bind target sequences by sequence complementarity (Cairns et al. 2003). For example, the 10–23 DNAzyme has two variable binding domains, designated arm I and arm II, that flank a conserved 15 nucleotide catalytic domain. Sequence-specific cleavage takes place between a purine-pyrimidine (RY) dinucleotide where the purine is unpaired to the DNAzyme (Fig. 4a) (Cairns et al. 2003).

4.1.2 RNA–DNA Chimera-Guided RNase H Cleavage

In the cell, RNase H (RNase H1) is an endonuclease that removes the RNA primers from Okazaki fragments of the replicating DNA and processes R-Loops to modulate R-Loop-mediated biological processes such as gene expression, DNA replication and DNA and histone modifications (Huang et al. 1994; Broccoli et al. 2004; Parajuli et al. 2017). It has been shown that *E. coli* RNase H can be guided to cleave ssRNA at a specific site using an RNA–DNA chimera in vitro (Lapham and Crothers 1996; Lapham et al. 1997; Yu et al. 1997). To guide RNase H to a specific site for cleavage, one can design a DNA-RNA chimera such that the first 4–6 nucleotides of the 5′ end are deoxynucleotides. The next 10–20 nucleotides can be ribonucleotides or 2′-O-ribonucleotides. The advantage of using 2′-O-ribonucleotide instead of ribonucleotides was high resistance to hydrolysis during subsequence manipulations, even though the 2′-O-methylation does not appear to improve cleavage specificity or efficiency. When hybridized to the target RNA, the DNA-RNA hybrid segment generated presents an approximation to the physiological substrate for RNase H which cleaves the RNA strand at a site opposite to the 5′ end of the DNA-RNA chimera.

To facilitate the recovery of the 5′ cleavage fragment, one can attached a biotin group to the 3′ end of the DNA-RNA chimera such that the cleaved 5′ RNA fragment, which stays hybridized to the DNA-RNA chimera at ambient temperatures, can be enriched by methods such as affinity purification using streptavidin magnetic beads (Beverly et al. 2016). The purified 5′ cleavage fragments are normally analyzed by intact LC/MS analysis (Beverly et al. 2016) (Fig. 4b).

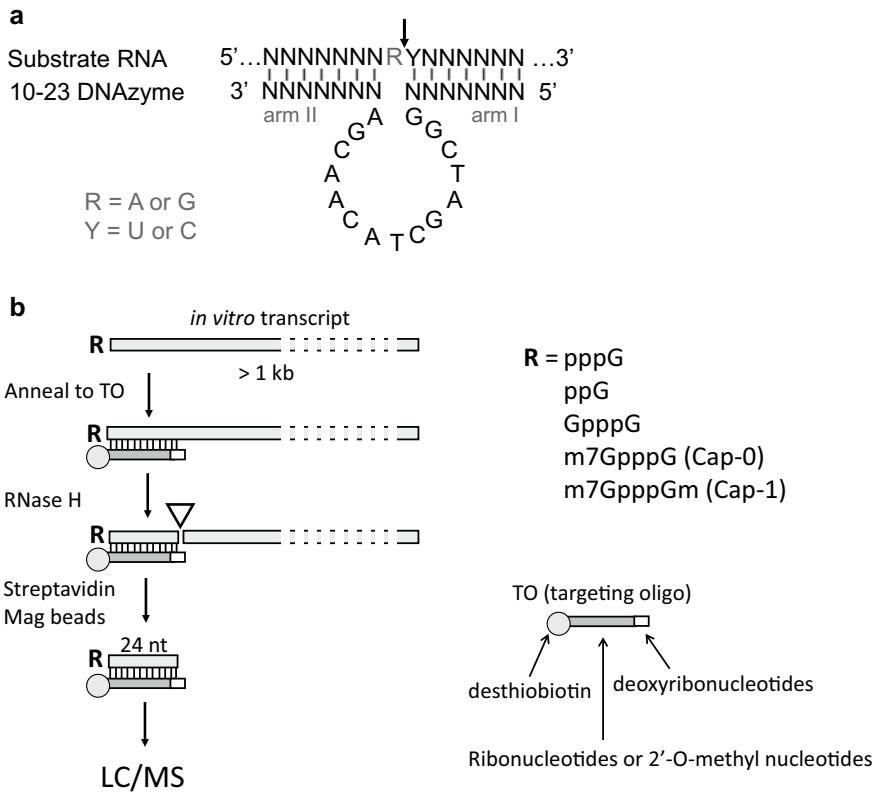

Fig. 4 **a** DNAzyme general design. **b** RNase H-based synthetic mRNA cap analysis

4.2 Poly-A Tail Length

Enzymatic poly-A tailing with poly-A polymerase results in a heterogenous population. Therefore, an accurate characterization of the poly-A tail length is critical to gauge the efficacy from the synthetic mRNA. Poly-A tail length can be measured with a variety of techniques including PCR-based assays, sequencing-based approaches, RNase H cleavage, and chromatographic approaches using poly-dT-mediated enrichment (Chang et al. 2014; Janicke et al. 2012; Murray and Schoenberg 2008; Salles and Strickland 1995; Subtelny et al. 2014; Minasaki et al. 2014). Sequencing can distinguish single base differences, but it involves conversion of the RNA to cDNA as well as ligation steps that are not efficient and can introduce bias. Mass spectrometry-based approaches does not only resolve polynucleotides with single nucleotides resolution based on mass differences but can also analyze multiple polynucleotides simultaneously. This can be applied to study the poly-A tail length of synthetic mRNAs by first performing a Ribonuclease T1 digestion of the synthetic mRNA to cleave the poly-A tail followed by isolation of the poly-A tail fragment with oligo dT beads

(Beverly et al. 2018). The poly-A tail fragments can then be analyzed by LC–MS to gain single-nucleotide resolution information of the poly-A tail length.

5 Perspective and Future Directions

Rapid manufacturing response, manufacturing versatility and flexibility are some of the potential benefits of synthetic mRNA-based vaccines. That said, the current in vitro transcription workflow consists of multiple unit operation and the manufacturing process can be further streamlined. The process yields and the manufacturing scale impact manufacturing costs and improvement to both will help lower the costs and help accelerate global adaptation of this novel class of drug. With the extremely high standard for consistency and purity at a global supply scale, innovations around the enzymatic components, purification methodologies, and workflow simplification can help accelerate the adaptation of synthetic mRNAs as an important component of the global health defense arsenal.

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In Vitro-Transcribed mRNAs as a New Generation of Therapeutics in the Dawn of Twenty-First Century: Exploitation of Peptides as Carriers for Their Intracellular Delivery

A. N. Miliotou, I. S. Pappas, I. S. Vizirianakis, and L. C. Papadopoulou

Contents

Abstract In vitro-transcribed mRNAs (IVT-mRNAs) are easily and rapidly designed in vitro synthesized RNA molecules. After their intracellular delivery through an efficient delivery system, the host cell ribosomes will be recruited to translate and produce the corresponding desired proteins. Nowadays, about 20 years after the first report as for the use of IVT-mRNA, this technology has all the spotlight on it, due to the recently produced vaccines against SARS-CoV-2. All this enthusiasm around IVT-mRNA has pushed a wave of biotech companies to leverage this technology, raising significant investments annually. Thus, IVT-mRNA technology has gained an impressive dynamic, with multidimensional applications [as protein replacement therapy (PRT), cancer immunotherapy, vaccine production, production

A. N. Miliotou · I. S. Vizirianakis · L. C. Papadopoulou (✉)
Laboratory of Pharmacology, School of Pharmacy, Faculty of Health Sciences, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Macedonia, Greece
e-mail: lefkotea@pharm.auth.gr

A. N. Miliotou
Department of Health Sciences, KES College, 1055 Nicosia, Cyprus

I. S. Pappas
Laboratory of Pharmacology and Toxicology, Faculty of Veterinary Science, University of Thessaly, 431 00 Karditsa, Thessaly, Greece

I. S. Vizirianakis
Department of Life and Health Sciences, University of Nicosia, 1700 Nicosia, Cyprus

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_10

of antibodies, cytokines and growth factors, gene silencing, cellular reprogramming, and gene editing] with remarkable results. This chapter will emphasize the recent advances in IVT-mRNA delivery. A wide range of in vitro and in vivo transfection reagents have been shown to protect IVT-mRNA from degradation, to escape immunosurveillance and facilitate its intracellular delivery. IVT-mRNA delivery systems can be classified into two broad categories: (i) physical transfection methods, like electroporation, that temporarily disrupt cell membrane barrier function and (ii) chemically formulated nanocarriers, like polymer-based, lipid-based nanovectors, lipid–polymer hybrid nanoparticles, and peptide vectors. As the peptide-based delivery systems are gaining ground due to the flexibility that peptides can offer, this chapter will present this very interesting aspect that combines IVT-mRNA technology with protein transduction domain (PTD) technology. Compared to cationic polymers, the peptides are of low-molecular weight, with degradable amino acid sequences and distinct biological properties, such as cell permeability efficiency and cell and nuclear surface targeting. Either by non-covalent or covalent binding, peptide-based carriers and hybrids are suggested as interesting alternatives to the various existing non-viral vectors for IVT-mRNA delivery.

Keywords IVT-mRNA · Gene therapy · Delivery · Non-viral systems · Peptides

Abbreviations

ASO	Antisense oligonucleotide
CPPs	Cell-penetrating peptides
DCs	Dendritic cells
DLinDMA	1,2-Dilinoleyloxy-3-dimethyl-amino-propane
DOPE	Dioleoyl-phosphatidyl-ethanol-amine
DOTAP	1,2-dioleoyl-3-trimethyl-ammonium-propane
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride
dsRNA	Double-stranded RNA
HIV	Human immunodeficiency virus
IVT-mRNA	In vitro-transcribed mRNA
LNPs	Lipid nanoparticles
NAs	Nucleic acids
PACE	Poly-amine-co-ester
PBAE	Poly-beta-amino-ester
PBNs	Peptide-based nanoparticles
PEG	Polyethylene glycol
PeI	Polyethyleneimine
PF14	Pepfect14
PFVYLI-IVT-mRNA	PTD-IVT-mRNA
PLGA	Polylactic-co-glycolic acid

PNAs	Peptide nucleic acids
PRT	Protein replacement therapy
PTD	Protein transduction domain
saRNA	Self-amplifying mRNA
UTRs	Untranslated regions

1 In Vitro-Transcribed mRNA as a Powerful Gene Therapy Tool

The continuously growing field of gene therapy has been emerged over the past three decades after the first intracellular delivery of nucleic acid drugs for therapeutic or vaccination purposes. In vitro-transcribed mRNA (IVT-mRNA) made its appearance in the early 1990s (Wolff et al. 1990). It is a synthetic molecule that resembles the endogenous mRNA and recruits the host cell's own ribosomes to be translated and leads to the transient expression of the corresponding desired protein. With the development of in vitro transcription methods and modifications in the IVT-mRNA structure, as well as the recent pandemic, where IVT-mRNA vaccines were recruited, the IVT-mRNA has become one of the most popular gene therapies (Dolgin 2021).

Back to the end of the 1990s, the first, successful intradermal injection of IVT-mRNA was demonstrated (Hoerr et al. 2000), with the skin cells being able to express the corresponding proteins encoded by the injected IVT-mRNA, while the IVT-mRNA was also proved to be stable, after incubation in serum. However, for many years it was generally accepted that IVT-mRNA nature was too unstable to be used effectively in vivo. Since 2005, several research teams faced this challenge via several modifications in IVT-mRNA structure to overcome the obstacles for its wider in vivo exploitation, leading to astonishing results on the efficacy of transfection and the duration of protein expression. Katalin Karikó and Drew Weissman have worked for decades with IVT-mRNA technology, despite opposing scientific opinions that making IVT-mRNA vaccines would be impossible, due to the unwanted stimulation of the recipient's immune system, which would immediately degrade and destroy the imported mRNA (Kariko et al. 2005). In the mid-2000s, Karikó and Weissman replaced uridine in the IVT-mRNA molecule with pseudo-uridine (Ψ), bypassing the immune system response (Pardi et al. 2013). Recently, in 2015, the involvement of methylpseudouridine (m1ψ) in combination with 5-methylcytidine (5mC) was supported to improve cell viability and protein synthesis (Andries et al. 2015), but also to reduce the immunogenicity of the IVT-mRNA (Kauffman et al. 2016).

Furthermore, the mRNA 5′ cap structure plays a very important role in many cellular processes, including translation, splicing, intracellular delivery, and degradation. During the transcription of the IVT-mRNA (using a bacteriophage promoter), it is observed that more than half of the caps are placed inverted, which made them unrecognizable by the mRNA stabilizing cap-binding proteins. Anti-reverse cap analogs (ARCAs), structured 3'–O–Me–m7G(5)ppp(5)G (Pardi et al. 2013),

were developed to overcome this obstacle, by using $–OCH_3$ to replace or remove the normal 3′–OH of the natural cap to avoid the misalignment (Kocmik et al. 2018; Jemielity et al. 2003).

The important signals of mRNA degradation, the adenylate–uridylate-rich elements (AREs), located in the 3′-untranslated regions (UTRs) of the most eukaryotic mRNAs, can control the mRNA output from nucleus and the translation efficiency, orchestrating mRNA subcellular localization and stability (Asrani et al. 2018). Furthermore, the elongated poly-(A) tail converts the IVT-mRNA into a very stable molecule, since its removal enhances its degradation (Goss and Kleiman 2013). Another procedure for optimizing IVT-mRNA technology, named codon optimization, uses the more frequently occurring codons in the open reading frame of the IVT-mRNA, without altering the resulting protein sequence, to facilitate the translation process. Finally, the IVT-mRNA stability can be increased by using 3′-UTRs of more stable mRNAs, like those derived from α- or β-globin mRNAs (Wadhwa et al. 2020), as well as by adding a strong Kozak sequence (Kozak 1987, 1984) into 5′-UTR of the IVT-mRNA to initiate translation and increase translation efficiency.

The use of IVT-mRNA is a directed process of protein production, which is orchestrated by the host itself with the appropriate posttranslational modifications, which are difficult or impossible to be carried out in the widely used heterologous systems. The use of IVT-mRNAs as therapeutic molecules is beneficial due to their biological origin. Thus, natural, transient, and cytoplasmic active IVT-mRNAs are considered safer and more potent alternatives than most viral DNA/RNA vectors for use in clinical applications (Guan and Rosenecker 2017). In fact, the strongest advantage of IVT-mRNA technology is that mRNA molecules do not enter the nucleus, have no intervention in human DNA, and all the action is done inside the cytoplasm. IVT-mRNA is degraded after 2–3 days to 2 weeks (depending on its complexation to a nanocarrier) in the cytoplasm by the normal mechanisms of the cell itself (Plews et al. 2010), and therefore, neither its inactivation (by using suicide genes) nor its removal is needed. Furthermore, IVT-mRNA, complexed to the nucleotide-binding peptide protamine (RNActive® technology), was proved to be stable even for at least two years, in room temperature, for storage purposes, without significant degradation (Alberer et al. 2017).

IVT-mRNA has an advantage over other therapeutic molecules, as in the case of recombinant therapeutic proteins, where complex purification procedures are required. In addition, the cost of producing IVT-mRNA for clinical trials, in Good Manufacturing Practice (GMP) grade, is about 5–10 times lower than that of therapeutic proteins, as it is a cell-free system (Sahin et al. 2014). Finally, because the IVT-mRNA is simply a genetic sequence, it can be easily modified to correspond to any new hypothesis or circumstance, e.g., a mutation, like the newly identified Omicron SARS-CoV-2 variant that may be resistant to the current IVT-mRNA vaccines.

Recently, a new category of IVT-mRNA technology has been arisen, the self-amplifying mRNA (saRNA), which is a type of a much larger mRNA (saRNA ~ 10,000 nts *vs.* IVT-mRNA ~ 2000 nts), that except of the gene of interest (e.g., a gene encoding a vaccine antigen), it carries four sequences for non-structural proteins,

derived from an alphavirus, encoding a replicase (Blakney 2021). Thus, the amplification of the mRNA and a higher efficiency in protein expression are facilitated. This technology shows tremendous advantages, as saRNA has the unique characteristic of self-replication, post-intracellular delivery, resulting in high translation rates and minimizing the required dose of the IVT-mRNA transduced (Chahal et al. 2016; Geall et al. 2012; Bogers et al. 2015). However, the large size and the excess of negative charge of the saRNA require an appropriate delivery method (Fuller and Berglund 2020).

IVT-mRNA technology has all the spotlight on it, as it is evaluated in preclinical and clinical trials to treat a wide variety of diseases. All this excitement around IVT-mRNA has pushed a wave of biotechnology companies to leverage this technology, amassing significant investments, and an explosion of innovative approaches is expected in the next decade. IVT-mRNA technology can potentially be applied to a wide range of diseases, acute or chronic, from various fields, including vaccines, PRT for the treatment of monogenic diseases (Miliotou and Papadopoulou 2020), infectious diseases [SARS-CoV-2, human immunodeficiency virus (HIV), Zika virus, Ebola virus, influenza virus, rabies virus, and malaria parasite (Chaudhary et al. 2021)], gene editing, cell reprogramming applications, cancer immunotherapy, and chimeric antigen receptor (CAR) T-cell therapy as well regenerative medicine for the replacement and repair of cells, tissues, and organs. Overall, the market for IVT-mRNA therapeutic products and vaccines was estimated at $15 billion in 2019, increased more than $300 billion by 2021, and estimated to be ~ $800 million globally (for products other than COVID-19 vaccines) by 2035 (Xie et al. 2021), while the global market for transfection reagents is projected to reach $1.02 billion in 2021, up from $715.4 million in 2016 (Wood 2017). At the time of writing, there are ~ 380 clinical trials using IVT-mRNAs, 54 of which are being tested on children under 17 years of age. Things seem to be brighter thanks to IVT-mRNA technology, as due to its alternative approach, it has the potential to revolutionize medicine, enabling us to tackle several deadly diseases that have hitherto resisted traditional drugs and vaccines, from malaria to HIV and cancer.

2 Advances in the Delivery of IVT-mRNA

To produce enough of the desired protein intracellularly, the safe delivery of the synthetic IVT-mRNA into the cytoplasm of targeted cells must be conducted. Since IVT-mRNAs are negatively charged hydrophilic molecules, passive diffusion through the cell membrane is prevented; therefore, a delivery system is required (Chaudhary et al. 2021). A wide range of in vitro and in vivo transfection reagents have been shown to facilitate its intracellular uptake and endosomal escape, as well as its protection from degradation.

Delivery systems are usually integrated into endosomes through cell membrane adhesions. Endosomes (especially, via the clathrin-mediated endocytosis) mature and fuse with lysosomes, where the acidic environment and the presence of hydrolytic

enzymes can degrade the delivery system and, of course, the nucleic acid (Nguyen and Szoka 2012). Therefore, endosomal escape prior to degradation is considered essential for the delivery capacity of the carrier, as well as for the efficiency of the IVT-mRNAs as therapeutics. The main proposed mechanisms of endosomal escape include endosomal disruption, active transport, or fusion of the delivery system with the endosomal membrane. It is worth mentioning that saRNAs consist a special category of therapeutic IVT-mRNAs, with distinct requirements for their intracellular delivery, mainly because of their chain length and charge density (Blakney et al. 2018).

For the effective transfection, the delivery system must meet certain conditions: (i) binding with the IVT-mRNA to form complexes; (ii) interaction with the cell membrane—promotion of cell uptake; (iii) protection of the IVT-mRNA from intracellular and extracellular degradation, due to the presence of nucleases; and (iv) release of IVT-mRNA into the cytoplasm (Guan and Rosenecker 2017).

In addition, the ideal delivery system, regarding the in vivo application of IVT-mRNA, is expected to protect its cargo from ubiquitous endonucleases, to avoid detection by the immune system and non-specific interactions with proteins or non-target cells, to allow its targeted delivery to the tissues of interest, and to induce efficient cell uptake. IVT-mRNA delivery systems can be classified into two broad categories: (i) physical methods that temporarily disrupt cell membrane barrier function and (ii) chemically formed nanocarriers (Guan and Rosenecker 2017).

Physical methods for IVT-mRNA delivery have been extensively investigated and are quite effective. The direct injection of naked IVT-mRNA into the target cells by using microneedles (Golombek et al. 2018; Moody 2018), as well the «gene gun» method, in which the naked IVT-mRNA is «shot» inside the target cell, has also been proposed as natural methods of transfection. In in vivo applications, when administered intravenously, naked IVT-mRNA is rapidly degraded by RNases and the innate immune system can be activated. In fact, the half-life of naked IVT-mRNA has been estimated at less than 5 min after IV, with a marked decrease in serum levels, and residual levels of approximately 10% after 5 min and approximately 1% after 60 min. For this reason, additional natural methods have been developed to overcome this barrier, including microporation (variation of electroporation) (Lefebvre et al. 2010; Marucci et al. 2011; Ahlemeyer et al. 2014), electroporation, iontophoresis (Kigasawa et al. 2010), sonophoresis (Ryu et al. 2018), as well as nucleofection (Hamm et al. 2002; Kraus et al. 2010) and magnetoporation.

During electroporation, cells are exposed to an external, high-tension, electrical field for a short time that causes disruption in cell membranes, creating nanoscale pores, where IVT-mRNA can bind to membrane rupture and enter the cytoplasm or nucleus. Electroporation has now been established as a proposed method for transfecting hematopoietic cells with IVT-mRNA (McLenachan et al. 2013). New developed electroporation devices, including miniaturized and on-chip integrated microsystems, enabled in situ electroporation of various subpopulation of cells (Maschietto et al. 2021) and thus the development of rapid clinical protocols. However, electroporation may cause cell death, due to unreversible cell membrane

ruptures and homeostasis loss. Furthermore, the in vivo application of electroporation has a limited potential and only in skin applications, however, causing edemas. The electroporation efficiency is variable and strongly depends on the cell type and electrical characteristics, the cell size, and conductance (Bolhassani et al. 2014). From the other hand, many studies suggest that IVT-mRNA ex vivo electroporation is more effective, with faster and more homogeneous protein expression (Heiser et al. 2002; Geurts et al. 2009; Wang et al. 2013; Warren et al. 2010, 2012; Kogut et al. 2018).

Nowadays, the use of nanocarriers is at the forefront of research on IVT-mRNA technology. A physical nanocarrier category is the exosomes, which are small intracellular membrane-based vesicles, nano-sized (30–150 nm in diameter), with different compositions, involved in several biological and pathological processes. Exosomes have been widely exploited as drug delivery systems with the advantage of their non-immunogenic nature due to similar composition as body's own cells. Exosomes have been also proposed as another alternative delivery system for IVT-mRNAs (Zhang et al. 2019; Ha et al. 2016), mainly because of the increased extracellular stability that they offer. There are many studies using synthetic IVT-mRNA loaded exosomes for gene delivery (Mittelbrunn et al. 2011; Golombek et al. 2018; Yang et al. 2019). However, several challenges characterize this approach, like the yield of isolation of the exosomes, the component characterization, the targeting efficiency, the sufficient drug loading capacity, the standardize exosome dosing, the exosome cell type origin, the large-scale exosome production, and the safety issues (Aslan et al. 2021).

Chemical nanocarriers form complexes with the IVT-mRNA and vary in composition, size, shape, and physicochemical characteristics. The components of these nanocarriers are mainly natural biocompatible or synthetic. Each type of carrier should provide protection of the nucleic acid from degradation, facilitating the transfection process, but also should be minimally toxic and should not activate immunological reactions. Another desirable characteristic of carriers would be the programming of the IVT-mRNA releasing profile, thus offering an improved pharmacokinetic profile, reduced toxicity in healthy organs/tissues, and increased circulation time of IVT-mRNA in the blood (Gomez-Aguado et al. 2020). Among the most famous and widely used nanocarrier systems are lipidic, polymeric and polypeptidic systems, dendrimers, gold nanoparticles, and hybrid systems.

However, the most widespread system is the transfection using cationic, lipid vesicles, complexed with negatively charged IVT-mRNAs via electrostatic forces, comprising the lipoplexes (Granot and Peer 2017). Positively charged lipoplexes provide protection to the IVT-mRNA from extracellular degradation by RNases and, of course, provide binding to the negatively charged cell membrane to promote cellular uptake by natural endocytosis. Carrier and nucleic acid degradation may be caused by endosome fusion to lysosomes; thus, the endosomal escape prior to the possible degradation is considered a basic prerequisite for the success of a therapeutic IVT-mRNA (Gomez-Aguado et al. 2020).

The first synthetic, cationic lipid used for complexing with the IVT-mRNA was DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride)

(Felgner et al. 1994). The synthetic lipid DOTAP (1,2-dioleoyl-3-trimethyl-ammonium-propane) was then used (Brito et al. 2014), alone or in combination with the DOPE (dioleoyl-phosphatidyl-ethanol-amine), and applied successfully for the transfection of the IVT-mRNA (Malone et al. 1989) (Fig. 1a). In addition, the incorporation of hydrophilic, non-charged polymers to the surface of the nanoparticles, such as polyethylene glycol (PEG), has been proposed to stabilize the lipoplexes (Lutz et al. 2017). Other cationic carriers are polyethyleneimine (PeI), poly-L-lysine, dendrimers, DEAE-dextran, poly(amino esters) (PBAE), and chitosan (Lallana et al. 2017; Soliman et al. 2020) (Fig. 1b).

Lately, with respect to the in vivo application of the IVT-mRNA technology, lipid nanoparticles (LNPs) have taken attention. LNPs are usually formed using cationic lipids having—in the structure—tertiary or quaternary amines to encapsulate the IVT-mRNA polyanionate. More specifically, LNPs consist of cholesterol (helping stability), natural phospholipids (supporting the structure of lipid bilayer), a PEG derivative (for solubility plus reduction of accumulation and non-specific intake), and an ionizable lipid, complexing with the negatively charged IVT-mRNA. The cationic lipids of the LNPs are spontaneously encapsulating the negatively charged IVT-mRNA, by mediating a combination of attractive electrostatic interactions. Although the mechanism is not completely clarified, the LNPs-IVT-mRNA complexes enter the cell via endocytosis, electrostatically connected and fused to the cell membrane, through inverted non-duplicate lipid phases (Kowalski et al. 2019).

Cationic polymers are non-covalent carriers that interact with nucleic acids, providing efficient in vivo transfection rates. They offer tremendous flexibility in terms of structure modifications and are beneficial in determining the exact structure/activity ratio. Cationic polymers not only bind but also condense IVT-mRNA into nanostructures, which can improve IVT-mRNA uptake by endocytosis, protecting IVT-mRNA from nuclease degradation and facilitating escape from endosomes (Hou et al. 2021).

A very interesting example is the formation of nanomicelles with self-assembled PEGylated poly (amino acid) block copolymer, called PAsp (DET) (Uchida et al. 2014) (Fig. 1b). PAsp(DET) has enhanced and improved the endosomal escape due to the destabilization of the pH-sensitive membrane, as well as the unique feature of rapid degradation in non-toxic forms under normal conditions. These nanomicelles have been shown to allow in vivo IVT-mRNA transfection in the central nervous system, providing a sustained protein expression (Chan et al. 2019).

An alternative approach, used for the IVT-mRNA intracellular delivery, consists of anionic polymers. Polylactic-co-glycolic acid (PLGA), which is anionic at physiological pH, is used solely with the addition of cationic lipid materials to encapsulate the negatively charged IVT-mRNA, creating a lipid–polymer hybrid formulation (Rosenkranz and Sobolev 2015).

PBAEs (Poly-beta-amino-esters) have been also combined with PEG-lipids in order to be efficiently complexed with the IVT-mRNA, for its delivery in the lungs, after intravenous administration in mice (Kaczmarek et al. 2016). Another cationic

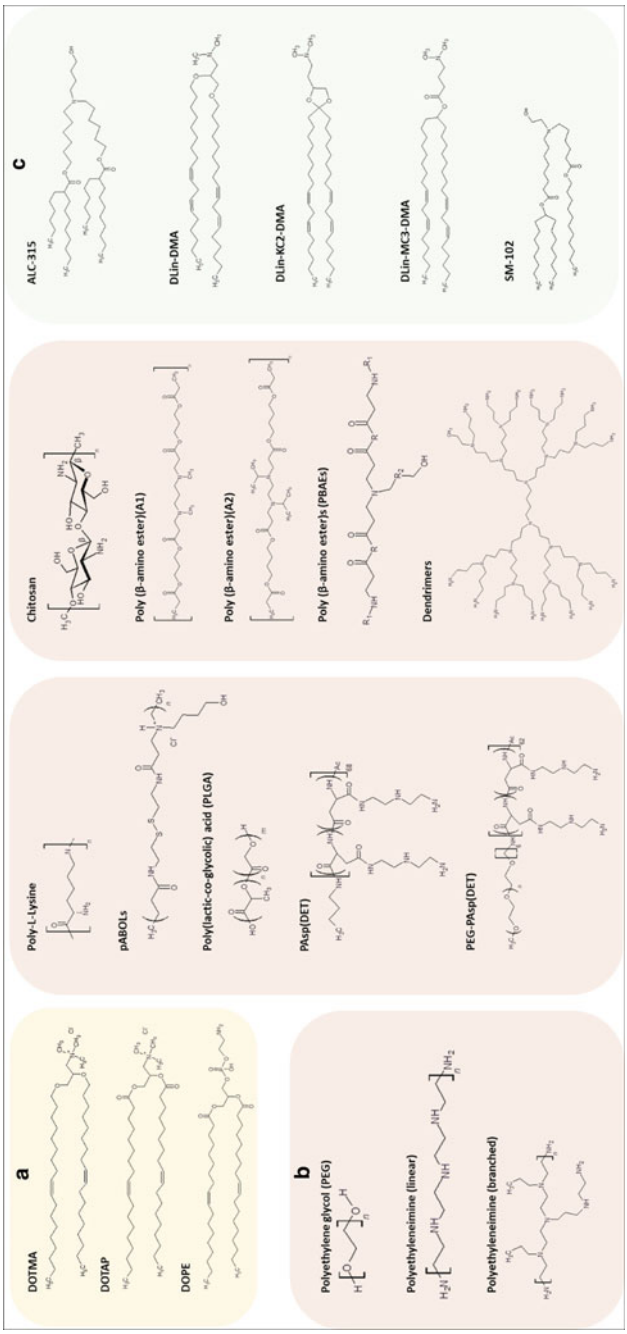

Fig. 1 Chemical structure of IVT-mRNA delivery nanocarriers: **a** cationic lipids, **b** polymers, and **c** ionizing lipids

polymer is the poly(amine-co-ester) (PACE) terpolymer, which has efficiently delivered the IVT-mRNA that codes for the human erythropoietin, through IV administration (Jiang et al. 2018). Inhaled hyperbranched poly (beta amino esters) (hPBAEs) were also developed to deliver IVT-mRNA in mice with incredibly increased rates (ten folds), compared with PEI (Patel et al. 2019; Buschmann et al. 2021).

saRNA delivery vehicles, in general, must be of higher molecular weights; however, this may lead to higher cytotoxicity. An interesting study reported the development of polydisperse nanocomplexes containing pABOL, a disulfide-linked poly (amido amine), and a saRNA, encoding an influenza hemagglutinin immunogen. pABOL also exceeded PEI efficiency in vivo and was partly protective against a lethal influenza challenge (Blakney et al. 2020; Buschmann et al. 2021). pABOL was also considered for the delivery of a saRNA for immunization against SARS-CoV-2; however, the Acuitas LNP found to be more efficient (Buschmann et al. 2021).

A very interesting approach is the use of LNPs, consisting of ionizing lipids (Fig. 1c), overcoming some safety issues that characterize the cationic lipids, like toxic and pro-inflammatory responses, mentioned above. An acidic buffer is used as the background, to positively charge the ionizing lipid in order to attract the negatively charged IVT-mRNA. DLinDMA (1,2-dilinoleyloxy-3-dimethyl-aminopropane) (Brito et al. 2014; Magini et al. 2016; Geall et al. 2012) is quite effective in the systemic delivery of siRNAs (Jayaraman et al. 2012; Semple et al. 2010) and its optimized form DLin-MC3-DMA (by the addition of specific ratios of MC3/DSPC/cholesterol/PEG–lipid) were evaluated in many preclinical and clinical studies (Bahl et al. 2017; Liang et al. 2017; Richner et al. 2017; Feldman et al. 2019). DLin-MC3-DMA was the first ever FDA-approved LNP formulation [ONPATTRO® (Partisiran)], and it has been also exploited in IVT-mRNA delivery. For the Pfizer-BioNTech mRNA vaccine, BNT162b2, the lipid ALC-0315 [(4-hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis(2-hexyldecanoate)] was used. For the Moderna mRNA vaccine, mRNA-1273, the lipid used was the SM-102 [heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino) octanoate]. Finally, Curevac, for its mRNA vaccine candidate, CVnCoV, used a cationic lipid (Acuitas Therapeutics), a phospholipid, cholesterol and PEG-lipid conjugate (Schoenmaker et al. 2021).

3 Peptide-Based Systems for IVT-mRNA Delivery

Cationic, lipid carriers, by forming lipoplexes or polymers, provided many advantages to IVT-mRNA technology, leading in its successful in vivo exploitation. However, there are several, well-documented disadvantages for their effect on transfection rates. Factors that lead to a reduction in transfection efficiency are the presence of serum, the size of the lipoplex, the density of the surface charge, the colloidal stability, the endosomal escape, the various uptake mechanisms, the easy degradation, and the absence of cellular and nuclear targeting, which have also been suggested to

play an important role in the efficacy of transfection through cationic, lipid carriers (Cui et al. 2018; Lonez et al. 2012).

Peptide-based systems for mRNA delivery are gaining momentum due to their versatility easy automated synthesis, single-step formulation, and biocompatible properties. Compared to cationic polymers, peptides are of low-molecular weight, with degradable amino acid sequences and distinct biological properties, such as cell permeability efficiency and cell and nuclear surface targeting. In addition, it is interesting that carriers, such as polymers or LNPs, regardless of the inclusion of targeting segments, tend to accumulate in liver and spleen (Shahzad et al. 2011).

The most preferable characteristic for a peptide, to be recruited as a carrier for IVT-mRNA, is to be positively charged, usually containing lysine and arginine residues, to facilitate complexing with the negatively charged IVT-mRNA, via electrostatic interactions (Qiu et al. 2019). The encapsulation efficiency strongly depends on the ratio of the charged positive amino groups of the peptide on the negative phosphate groups of the IVT-mRNA (Udhayakumar et al. 2017).

Finally, despite the significant advances in IVT-mRNA technology and delivery systems, another major challenge that prevented their fast clinical exploitation before 2020 is organ- and tissue-specific delivery difficulties. Except of the chemical modifications of the IVT-mRNA, mentioned previously, several strategies have been employed to overcome this obstacle, such as the use of cell targeting or cell-penetrating moieties, either via non-covalent, nanoparticle formulation or covalent conjugation (Boisguerin et al. 2021).

4 Cell-Penetrating Peptides or Protein Transduction Domains

The beginning of the 1990s was marked by the identification of several peptides, which have been attributed to the ability of «transduction», as they were observed to mediate the intracellular delivery of any cargo associated with them, through the cell membrane. In particular, these peptides were short in length (from 5 to 30 amino acids), mainly cationic (and/or amphipathic) and had the ability to permeate almost all biological membranes, in vivo and in vitro, without the intervention of cell membrane receptors, and without causing significant membrane damage, hereinafter referred to as protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) (Langel 2015; Guidotti et al. 2017; Vives 2005).

The first observation, which led to the discovery of these peptides, was made in 1988, by two different groups at the same time (Green et al. 1989; Frankel and Pabo 1988). The discovery referred to the HIV type 1 (HIV-1) TAT protein, which acted as a trans-activating factor of transcription and was observed to be secreted by infected cells in which the virus was active and had the ability to penetrate the cell membrane and be somehow absorbed by neighboring cells, and to stimulate in them the transcription of viral genes and the expression of heterologous proteins.

TAT peptide corresponds to the basic domain of the TAT protein and is an 11 aa (aminoacid) sequence, enriched in arginine and lysine. In 1999, the first in vivo protein transduction in mice via TAT, as PTD, was reported (Schwarze and Dowdy 1999). Furthermore, combination of the TAT peptide with proteins or fluorescent markers allowed these molecules to enter the cell.

Since then, several transduction peptides have been identified, each based on small domains of naturally occurring proteins (like the TAT peptide), synthetic peptides (like the R9) or even chimeric peptides (like the transportan) or designed de novo, forming a large, constantly expanding family of molecular carriers. PTDs can mediate the intracellular delivery of a wide range of covalent or non-covalently linked cargoes, such as small molecules (cyclosporine, doxorubicin, iron nanoparticles) as well as plasmid DNA, antisense nucleic acids, peptide nucleic acids (PNAs), and other macromolecules, such as proteins, but also viruses, liposomes, nanoparticles, and imaging agents. The intracellular delivery of various types of cargoes (including large, recombinant proteins) has been successfully performed in in vitro and in vivo experiments (Brasseur and Divita 2010). Our group has proposed PRT, via the production and successful delivery of human recombinant proteins, like mitochondrial Sco2 protein as well as α- and β-globin (Foltopoulou et al. 2010; Papadopoulou et al. 2018; Kaiafas et al. 2020; Miliotou et al. 2021a, b).

PTDs appear to be very effective carriers for the intracellular delivery even for nucleic acids. And although, for over 20 years, PTDs have been studied as tools for gene therapy, by enhancing the effectiveness of targeted cell transduction, only few PTDs were exploited for the delivery of the IVT-mRNA. The first successful binding of PTDs to nucleic acids (NAs) was based on the chemistry of PNAs (Aldrian-Herrada et al. 1998).

Peptide-based nanoparticles (PBNs) are defined by combining a PTD or a fused PTD (e.g., PEGylated) with a NA such as pDNA, IVT-mRNA, siRNA, or antisense oligonucleotide (ASO) at a given molar or charge proportion. By combining peptides and NAs, the nanoparticle is shaped by self-assembling into PBNs, a multigrafted PBNs, or imminent micelle-like PBNs. In all cases, the PBNs, being estimated between 60 and 150 nm, encapsulate NAs. From there on, cellular internalization might happen through coordinate translocation or through endocytosis-dependent pathways (Boisguerin et al. 2015).

Other PTDs used as IVT-mRNA carriers are the peptides Transportan, R7-9, pTAT, Penetratin, KFF, SynB3, and NLS. The transduction efficiency of the cationic peptide RALA was evaluated, as well as the efficiency of the PepFect14 (PF14) transduction system (van den Brand et al. 2019). Other cationic PTDs used for the IVT-mRNA delivery, through non-covalent interactions, are the LAH4-L1, the PTD Xentry, fused to protamine, and the PTD Melan-A showed efficient IVT-mRNA transduction (Haenssle et al. 2010).

Interestingly, not only cationic PTDs were used to transduce the IVT-mRNA, but anionic PTDs as well. Anionic peptides have not the ability to complex negatively charged IVT-mRNA, thus positively charged polymers or linkers are being conjugated to facilitate IVT-mRNA encapsulation. Such system is the copolymer

p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) (pHDPA) and the anionic peptide GALA (Lou et al. 2019).

Moreover, a novel PTD-mediated IVT-mRNA delivery platform as a protein therapy approach for metabolic/genetic disorders, using the amphipathic PTD, PFVYLI, was developed by our research group (Miliotou et al. 2021b). All these strategies, mentioned above, are summarized in Table 1 and are explained in detail through the next sub-chapters (chapters "Medical Use of mRNA-Based Directed Gene Delivery"–"Pulmonary Delivery of Messenger RNA (mRNA) Therapeutics for Respiratory Diseases"). These peptide-based delivery systems and hybrids are suggested as interesting alternatives to the various existing non-viral vectors for IVT-mRNA delivery, either by non-covalent or covalent binding, methods.

5 Strategies to Generate a PTD/Nucleic Acid Complex

In general, there are two strategies to generate a PTD/nucleic acid complex: non-covalent and covalent conjugation (Fig. 2). Non-covalent conjugation is based on electrostatic, stable, self-assembly between the PTD and its cargo (Bell et al. 2018; van den Brand et al. 2019), while the second strategy involves the use of chemical ligands or cross-linking that covalently bind the PTD to the cargo (Fig. 3), mainly through a cleavable disulfide (Avino et al. 2011), amide (Haralambidis et al. 1990; Kachalova et al. 2004), thiazolidine, oxime, or hydrazine bond (Zatsepin et al. 2002). The non-covalent strategy relies on cationic or amphipathic nature of peptides, which can form complexes with the negatively charged IVT-mRNA. The covalent method, although it is limited from a chemical point of view, because of the risk of altering the biological activity of the cargo, as well as the efficiency of the released cargo, does offer the advantage of less cargo loss and protection of the cargo under adverse conditions during in vivo delivery (Miliotou et al. 2021b; Meade and Dowdy 2007).

During the delivery via covalent or non-covalent strategies, the IVT-mRNA escapes and is released in the cytoplasm, where is translated by the ribosomes to the desired proteins by the cells own translational machinery. The newly synthesized proteins remain in the cytoplasm or are transported to either nucleus, organelles (like mitochondria), cell membrane, or they can be even secreted from the cell, to proceed in the corresponding function.

5.1 Non-covalent Conjugation Approach for IVT-mRNA Delivery via Peptides

Non-covalent strategies of IVT-mRNA and peptides conjugation are increasingly used, mainly through self-assembly, electrostatic, and hydrophobic interactions (Boisguerin et al. 2015).

Table 1 Protein transduction domains-based IVT-mRNA delivery systems

Protein transduction domains	Charge	Sequence	Method of complexation with the IVT-mRNA	References
R9	Cationic	CRPPR-R9	Non-covalent	Lee et al. (2015)
RALA	Cationic/amphipathic	N-WEARLARALARALARHLARALARALRACEA-C	Non-covalent	McCarthy et al. (2014)
Pepfect14 (PF14)	Cationic/amphipathic	Stearyl-AGYLLGKLLOOLAAAALOOLL	Non-covalent	van den Brand et al. (2019)
LAH4-L1	Cationic	N-KKALLAHALHLLALLALHLAHALKKA-C	Non-covalent	Coolen et al. (2019)
Xentry-protamine	Cationic	Xentry: N-LCLRPVG-C, protamine: N-RSQSRSRYYRQRQRSRRRRRRS-C	Non-covalent	Bell et al. (2018)
Melan-A	Cationic	N-ELAGIGILTV-C	Non-covalent	Haenssle et al. (2010)
GALA	Anionic	pHDPA and GALA: N-WEAALAEALAEALAEHLAEALAEALEALAA-OH-C	Click chemistry through a BCN-PEG linker	Lou et al. (2019)
PFVYLI	Neutral	PFVYLI	Covalent	Miliotou et al. (2021b)

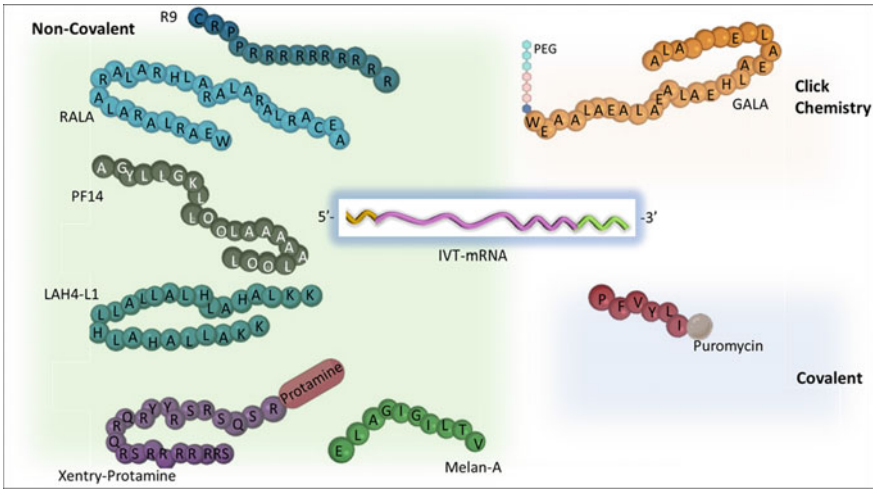

Fig. 2 Schematic illustration of protein transduction domains (PTDs)-based systems used for the intracellular delivery of the IVT-mRNA. The amino acid sequence of each PTD is illustrated, as well as the complexation strategy with the IVT-mRNA, either through non-covalent interactions, via click chemistry or through covalent conjugation

Starting from GALA/KALA/RALA family, the amphipathic RALA peptide confers as an efficient systemic delivery carrier of NAs, such as pDNA and siRNA, with increased cell and endosomal membrane permeability and reduced toxicity (Udhayakumar et al. 2017). The efficient intracellular transduction of an antigen-encoding IVT-mRNA (ovalbumin IVT-mRNA) complexed with the RALA peptide, into dendritic cells and the corresponding antigen expression, instigating T-cell immunity, was also shown (Udhayakumar et al. 2017). In that study, two more peptides were evaluated: (a) A hydrophilic motif was used to replace RALA's hydrophobic residues, named RGSG (WEGRSGRGSGRGSGRHSGRGSGRGSRG-C), and (b) peptide RRRR, where the hydrophobic alanine and leucine residues of RALA were omitted, both showing less T-cell response compared to RALA (Udhayakumar et al. 2017). All these formulations were prepared with a non-covalent approach, at amine to phosphate (N/P) ratio 10, by adding appropriate volumes of RALA peptide solution to a specific amount of IVT-mRNA.

A common cationic peptide, used as a peptide carrier for the IVT-mRNA, is protamine, which forms a complex with it through a spontaneous manner. Several modifications in protamine, for the formation of the sulfate or chloride salts, confer to reduce arginine residues, thus to decrease positive charges (low-molecular weight protamine-LMWP) or to attach PEG. LMWP was used as a PTD and as a part of drug delivery systems, like nanoparticles or liposomes. Protamine, as the self-adjuvanted RNActive® platform (Kallen et al. 2013), has also shown adjuvant activity and protection to the complexed IVT-mRNA during stability assays with RNase

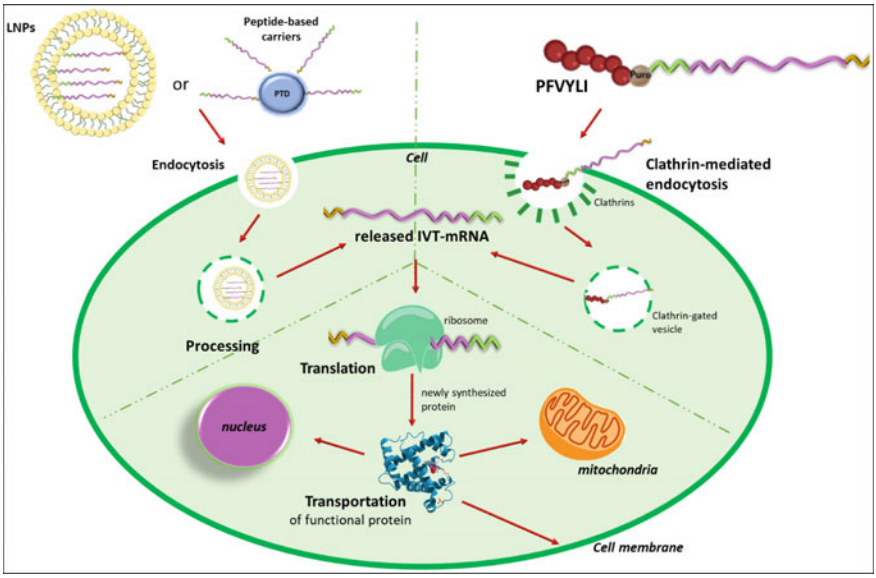

Fig. 3 Intracellular delivery of the IVT-mRNA using lipid nanoparticles (LNPs) or peptides, through non-covalent and covalent interactions. The IVT-mRNA is complexed with LNPs or peptides, through self-assembly, electrostatic, and hydrophobic interactions, and the cellular uptake is mediated through endocytosis. From the other hand, the intracellular transduction of IVT-mRNA has been proposed to be mediated through covalent conjugation with a PTD, the PFVYLI, using puromycin as a linker. The intracellular transduction is conducted via clathrin-mediated endocytosis, where clathrin-gated vesicles are formed

treatments and extreme conditions, like long-term storage in high temperature or cycles of temperature variation.

Among the PepFect/NickFect family, the amphipathic peptide PF14 (21 aa, of which five are positively charged, AGYLLGKLLOOLAAAALOOLL) is also a potential candidate for delivering NAs, such as antisense oligonucleotides ASO, siRNA, and plasmid DNA (pDNA), in vitro and in vivo (van Asbeck et al. 2013; Ezzat et al. 2011, 2012). PF14 amino acid sequence was also suggested for the intracellular transduction of the IVT-mRNA of reporter genes (GFP or mCherry), and the corresponding reporter proteins were observed in fibroblasts, tumor cells, and immune cells of xenografted mice. The formulation of PF14 and the IVT-mRNA was achieved in N/P ratio of 3, as a non-covalent strategy (van den Brand et al. 2019). The eGFP expression, after transfection, was lower in two-dimensional tissue cultures but higher in three-dimensional tumor spheroids, compared to the commercial transfection reagent, Lipofectamine MessengerMax.

The peptide p5RHH is composed of 21 aa and has an hydrophobic segment and a cationic C terminus that contains five arginine and two histidine residues (VLTTGLPALISWIRRRHRRHC). p5RHH is a modified version of the membrane lytic protein melittin with reduced cytolytic activity, and it was successfully employed

for the intracellular transduction of siRNA, to reduce the JNK2 expression in the plaques of an atherosclerotic mouse model (Pan et al. 2018). p5RHH was also used for the transduction of near-infrared fluorescent protein (niRFP)-encoding mRNA in a femoral artery wire injury mouse model, leading to the efficient expression of the corresponding protein (Lockhart et al. 2021). The formation of the p5RHH-IVT-mRNA complex was mediated by a non-covalent, self-assembly manner (350 ng of IVT-mRNA: 2.0 nmol p5RHH).

In addition, a study published in 2018 proposed the use of the truncated (D-amino acid-based) protamine, fused to the peptide Xentry, and the nanocarrier was named Xentry-protamine (Bell et al. 2018). Xentry-protamine allowed the cystic fibrosis transmembrane regulator (CFTR) IVT-mRNA to be transfected into epithelial cells in the presence of a transfection enhancer, the Toll-like receptor antagonist (Toll-like receptor E66).

Effective transfection of the IVT-mRNA-induced cardiomyocyte-like gene, fused to peptide R9 (CRPPR-R9) and Lipofectamine, was achieved into mouse cardiac fibroblasts. The results showed partial immediate reprogramming of cardiac fibroblasts into cardiomyocytes, due to the effectiveness of transfection with also low toxicity (Lee et al. 2015).

An alternative strategy was followed to develop a delivery system for the intracellular delivery of the IVT-mRNA, using poly(lactic acid) nanoparticles (PLA-NPs) and cationic PTDs. PLA is a biodegradable and biocompatible polymer, and PLA-NPs are widely used for their efficiency of transfecting dendritic cells (DCs) in vitro and in vivo, inducing desired immune responses. PLA-NPs are negatively charged, as also the IVT-mRNA, thus complexation of these compounds is facilitated by reducing the molecular weight of the cationic polymers (Coolen et al. 2019; Bettinger et al. 2001). Furthermore, a penetrating moiety is needed for the endosomal escape and for the delivery of the IVT-mRNA into the cytosol. In addition, PLA-NPs and PTDs are used to facilitate the intracellular transduction of the IVT-mRNA. The cationic PTDs used were RALA, LAH4, and LAH4-L1, with the highest protein expression in DCs observed with LAH4-L1 and PLA-NP/LAH4-L1 formulations. These formulations were produced by mixing equal volumes of the IVT-mRNA with the peptides and then again mixing with equal volumes of the PLA/NPs (Coolen et al. 2019).

Melan-A, a cationic HIV TAT domain, and an MHC class I-binding antigenic domain consist of cationic R-rich peptides that can interact via their guanidino head groups through hydrogen bonds with the negatively charged phosphate backbone of double-strand RNA (dsRNA) molecules. Through this non-covalent conjugation, complexes of poly(I:C) and cationic fusion peptides are formed (Haenssle et al. 2010). Poly(I:C) dsRNA was incubated with the cationic Melan-A TAT fusion peptide, to form peptide/poly(I:C)/dsRNA complexes that successfully transduced monocyte-derived immature DCs (iDCs), without cytotoxicity, providing a strong expansion/activation of antigen-specific T cells in the context of an IL-12p70 secretion.

5.2 *Covalent Conjugation Approach for IVT-mRNA Delivery via Peptides*

Regarding the covalent conjugation approaches of peptides with the IVT-mRNAs, only the library screening, named «mRNA display» method, has been reported. The «mRNA display» is an in vitro selection technique, which allows the identification of polypeptide sequences with the desired properties, both from a natural proteomic library and from a synthetic peptide combination library (Roberts and Szostak 1997; Nemoto et al. 1997; Roberts 1999; Liu et al. 2000). The central feature of this method is that the respective polypeptide chain is covalently linked (via a peptide bond, formed by the incorporation of puromycin) to the 3′-terminus of its own mRNA. Since the genotypic, coding sequence and phenotypic, polypeptide sequence are covalently combined within the same molecule, the selected polypeptide chain can be identified by PCR from the cDNA—of the covalently linked mRNA—as a template and finally by DNA sequencing. Thus, visualizing through mRNA provides a powerful means of reading and amplifying a peptide sequence or protein, having been functionally isolated from a wide variety of libraries. Multiple selection and amplification rounds can be performed, allowing the enrichment of rare sequences with the desired properties.

Compared to previous peptide or protein selection methods, the «mRNA display» application has many important advantages. The «genotype» is covalently linked and always exists in conjunction with the «phenotype». This stable conjugation makes it possible to use any strict conditions in the operating selection. The reaction scale is tuned, usually from microliters to milliliters. Peptide or protein libraries, containing up to 10^{12}–10^{14} unique sequences, can be easily created and selected, in a much more quantity, using the phage display method or other peptide/protein selection platforms. Therefore, both the probability of isolating rare sequences and the diversity of sequences isolated in a given selection are significantly increased (Wang et al. 2012).

Regarding the implementation of the covalent conjugation of peptides with NAs, the main feature offering advantages for NA complexation and membrane interaction abilities is the amphipathic nature of the peptides, having both hydrophilic and hydrophobic domains. The primary category of amphipathic PTDs consists of two opposite domains, distributed in each amino acid position, as MPG peptide (27 aa) (Morris et al. 1997) or Mgpe-1 peptide (derived from human protein phosphatase 1E) (Sharma et al. 2013). The second category of amphipathic peptides results from the formation of these opposite domains, hydrophilic and hydrophobic, during the secondary structure folding (Konate et al. 2010; Boisguerin et al. 2021). Amphipathic PTDs that can form PBNs, through non-covalent conjugation with NAs, are PepFect (Andaloussi et al. 2011), RICK (Vaissiere et al. 2017), or WRAP (Konate et al. 2019).

The 30 amino acid long, amphipathic, pH-sensitive fusogenic and α-helical GALA peptide (Hatakeyama et al. 2009; Li et al. 2004) was used to form GALA-modified IVT-mRNA polyplexes (PPx-GALA) and efficiently transduced the IVT-mRNA of

EGFP to macrophages and dendritic cells and achieved higher expression of GFP compared to the commercial reagent Lipofectamine, without any cytotoxicity. GALA also successfully delivered IVT-mRNA encoding ovalbumin into T cells and achieved their subsequent activation in vitro. The conjugation of the peptide to the IVT-mRNA was achieved in three consecutive steps: complexation, post-PEG-peptide modification, and cross-linking. The complexation step consisted of mixing the polymer p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) (pHDPA) and the IVT-mRNA at an N/P ratio of 4. Then, low or high degree of PEG-BCN_{6000}-peptide is conjugated to the pHDPA/IVT-mRNA polyplexes by click chemistry. Finally, the third step consisted of the particle stabilization by cross-linking the polymer chains of the core with DTT (Lou et al. 2019).

The peptide PFVYLI is a shorter version of the peptide C105Y, a synthetic PTD, based on the amino acid sequence corresponding to residues 359–374 of α1-antitrypsin, and it was exploited for the intracellular transduction of DNA nanoparticles, larger proteins, siRNAs, etc. (Rhee and Davis 2006; Jones et al. 2013; Barkalina et al. 2015; Fang et al. 2013). PFVYLI is a hydrophobic peptide, with neutral surface charge, thus a linker is needed for the covalent conjugation with the negatively charged IVT-mRNA. Our group, Miliotou, et al., developed a universal platform, using this hydrophobic PTD peptide covalently conjugated to any IVT-mRNA, through a novel covalent chemical reaction (Fig. 4). The methodology covering the novel chemical reaction for generating PTD (stands for the PTD peptide PFVYLI)-IVT-mRNAs is in international patent-pending status, published under No. WO2021/094792A1 (Miliotou et al. 2021b).

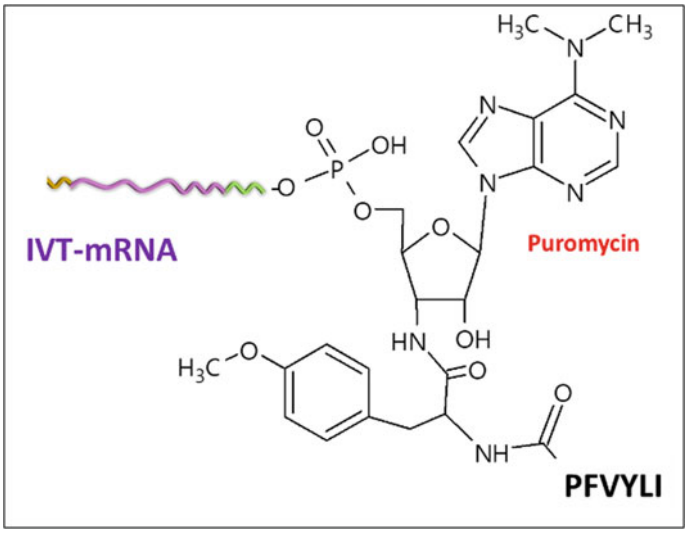

Fig. 4 Chemical structure of the novel PFVYLI-IVT-mRNA, which is produced through the innovative, patent-pending, chemical reaction, designed by our research team

In this novel chemical reaction, puromycin serves as a linker to the IVT-mRNA, as puromycin is conjugated via an amide bond to the PFVYLI (Miliotou et al. 2021b). PFVYLI was successfully conjugated to IVT-mRNAs, generating the PTD-IVT-mRNA of *SCO2* and the PTD-IVT-mRNA of *β-globin*, confirmed by NMR data analysis and a band shift assay compared to the naked IVT-mRNA. The proposed PTD-IVT-mRNA delivery platform showed significant stability of the studied IVT-mRNAs in various conditions, even in environments rich in harmful RNases (FBS and RNase A), indicating that the PTD protects the IVT-mRNA from immediate nuclease digestion. Both conjugates were successfully transduced (via clathrin-mediated endocytosis) into three types of human cells, leading to increased production of Sco2 and β-globin proteins, in two different disease models of monogenic/metabolic disorders. PTD-IVT-mRNA of *SCO2* even reversed the monogenic disease phenotype *in culture* and led to the recovery of reduced COX activity, in the case of COX deficiency caused by *SCO2* mutations, all compared to the naked IVT-mRNA and IVT-mRNA conjugated to a non-PTD, control peptide. These findings suggest that this novel PTD-IVT-mRNA delivery strategy could be a promising platform for effective gene expression, with the potential to be used in clinical trials as a protein replacement therapy for metabolic/genetic disorders (Miliotou et al. 2021b).

6 Future Perspectives

Peptides are excellent IVT-mRNA delivery systems and could be used in a variety of biomedical applications. PTDs have little cell cytotoxicity and can be quickly digested into amino acids, making them ideal for preclinical and clinical investigations. PTD-mediated techniques are still not exploited in clinical applications, despite their extraordinary efficiency in delivering therapeutic cargoes into cells. Several factors must be considered in order to move the clinical translation ahead.

First, the stability, size, and monodispersity of the peptide-IVT-mRNA complexes are crucial characteristics that must be controlled and might be improved by grafting PEG motifs or fatty acids to the PTDs (Freimann et al. 2016; Boisguerin et al. 2021). Grafted shielding groups, such as polysaccharides and PEGs, are also used to limit the amount of surface charges, resulting in neutrally charged particles with a significantly lower charge. PEGylation blocks the electrostatic and hydrophobic interactions of nanoparticles and hence diminishes their opsonization and clearance by the reticuloendothelial system, giving advantages in pharmacokinetic characteristics and longer blood circulation times (Nie 2010; Qiu et al. 2019). In general, there is a need for modulating peptide carriers' stereochemistry, such as altering their L/D-amino acid sequence pattern, in order to have fine-tuning peptide carrier properties/functions (Holjencin et al. 2021).

Furthermore, the specificity of peptide carriers internalization might be boosted by grafting targeting (or homing) sequences that recognize certain proteins or a particular receptor overexpressed in cancer cells or on cellular organelles.

Through passive targeting, nanoparticles may accumulate in the liver and lungs. In contrast, active targeting moieties and homing peptides use particular ligand molecules to direct the nanoparticles to the desired organ or tumor site. Finding the right ligand to fuse to the peptide carrier is therefore crucial for their clinical exploitation.

Finally, groundbreaking work has been done on the construction of stimulus responsive «smart» CPP (PTD)-based systems that may be activated by pH or enzymes (Ye et al. 2016). Specifically, developed «smart» peptide nanocomplexes should be able to pass through a variety of physiological obstacles, without inducing undesired immunological reactions or decreasing colloidal stability after IV injection and achieving the most efficient transduction into targeted cells.

The IVT-mRNA technology dynamic unfolded only a little in relation to the innumerable applications it may have. In addition, the formidable future of IVT-mRNA is strongly correlated to the development of alternative delivery systems. There is a constant need of researching the optimum delivery platform, and there is a hard work being conducted with peptide-based delivery platforms. Peptides may confer an alternative—and maybe an advantageous—solution to difficulties in IVT-mRNA delivery, during future clinical developments and the challenges that lie ahead in this vibrant field of therapeutic approach.

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Lipid Nanoparticle-Mediated Delivery of Therapeutic and Prophylactic mRNA: Immune Activation by Ionizable Cationic Lipids

Melike Ongun, Abhijeet Girish Lokras, Camilla Foged, and Aneesh Thakur

Contents

Abstract Messenger RNA (mRNA) can be harnessed as vaccines and therapeutic drugs via transient in situ expression of protein antigens and therapeutic proteins, respectively. Currently, mRNA-based vaccines are used worldwide in mass vaccination programs to induce protective immunity against COVID-19, and a number of prophylactic vaccines, therapeutic vaccines, and therapeutic drugs based on mRNA are now tested in clinical trials. Although chemical modification of the mRNA components has considerably ameliorated mRNA stability and immunogenicity, further improvements in formulation and delivery systems, which are used to transport mRNA to the cytosol of target cells, are still required to enhance the efficacy and safety of mRNA therapeutics. However, our knowledge about the mechanisms by which mRNA therapeutics activate the immune system is still very limited, partly because the activation of immune cells by ionizable lipids commonly used in mRNA delivery systems is poorly understood. Lipid-mediated induction of innate immune pathways can be exploited in mRNA vaccines by providing an adjuvant effect, whereas innate immune activation is undesired for the therapeutic use of mRNA. Here, we review recent studies focusing on the hurdles that challenge in vivo delivery of mRNA. We subsequently discuss the state of the art in formulation design approaches, which are used to overcome these challenges, with focus on the marketed COVID-19 mRNA vaccines. Finally, we present research centered on how ionizable and cationic lipids used for delivery of mRNA therapeutics activate immune cells

M. Ongun · A. G. Lokras · C. Foged · A. Thakur (✉)
Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen Ø, Denmark
e-mail: aneesh.thakur@sund.ku.dk

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_11

and engage innate immune pathways, including future challenges and opportunities in formulation and delivery to optimize the safe and efficacious use of mRNA therapeutics.

Keywords Messenger RNA (mRNA) · Lipid nanoparticles · Ionizable lipids · Immune activation · Toll-like receptors

1 Introduction

Messenger RNA (mRNA) has emerged as a potent and promising drug for the prevention and treatment of infection diseases, cancer, and genetic abnormalities (Chaudhary et al. 2021; Pardi et al. 2018b; Sahin et al. 2014). In addition, it is expected that mRNA-based therapeutics will represent a major breakthrough for personalized therapy, because they enable in situ expression of protein inside the patient's own cells (Weng et al. 2020). Vaccines based on mRNA offer several advantages with respect to safety, efficacy, and specificity, as compared to traditional vaccines based on live attenuated pathogens, which have safety concerns due to the risk of reversion to virulence (Hanley 2011). On the other hand, subunit vaccines, which are based on synthetic peptides or highly purified recombinant protein antigens, represent safer alternatives to the traditional live attenuated or inactivated vaccines, but they display lower immunogenicity, and co-administration of adjuvant(s) is required to enhance vaccine efficacy (Reichmuth et al. 2016).

By using carefully designed and finely tuned delivery systems, mRNA drug molecules can get access to the ribosomes present in the cell cytosol, which translate exogenous mRNA into protein (Pardi et al. 2018b; Sahin et al. 2014). Unlike plasmid DNA and viral vectors, mRNA does not become integrated into the host genome, which excludes the risk of insertional mutagenesis (Sahin et al. 2014). Manufacturing of in vitro transcribed (IVT) mRNA takes place via an enzymatic process in a cell-free system, which is faster and more cost-effective than the expression and purification of recombinant protein antigens (Chaudhary et al. 2021; Iavarone et al. 2017). Despite these advantages, it is challenging to translate mRNA-based therapeutics or vaccines to the clinic, because mRNA is a very large molecule (300–5000 kDa, 1–15 kb) that displays a polyanionic phosphate backbone, which cannot diffuse across the plasma membrane into the cytosol. In addition, IVT mRNA is unstable and is highly susceptible to enzymatic degradation by nucleases, and it activates the innate immune system (Kariko et al. 2005; Sorrentino 1998).

To overcome these challenges, different strategies have been adopted to improve mRNA delivery, e.g., (i) chemical modification of the mRNA structure (Kariko et al. 2005, 2008), (ii) physical methods, including microinjection (Golombek et al. 2018), administration via a gene gun (Tavernier et al. 2011), electroporation (Cu et al. 2013), and microneedles (Koh et al. 2018), and (iii) incorporation of mRNA into delivery systems (Kowalski et al. 2019). Delivery systems for nucleic acids are categorized into viral and non-viral systems. Non-viral delivery systems, e.g., lipid-based

systems, polymer-based systems, lipid-polymer hybrid systems, and protein/peptide-based systems, have been widely investigated for mRNA delivery, as compared to viral vectors, due to ease of manufacturing and reduced risk of inducing host immunogenicity against the delivery system (Hou et al. 2021; Reichmuth et al. 2016; Weng et al. 2020).

Lipid nanoparticles (LNPs) containing, among others, an ionizable lipid, represents the clinically most advanced mRNA delivery system with two marketed products, i.e., the COVID-19 mRNA vaccines Comirnaty® and Spikevax® from Pfizer/BioNTech and Moderna, respectively. Cationic lipids enable efficient encapsulation of negatively charged mRNA and interaction with the endosomal membrane during the fusion stage in the phagolysosomes, but their poor tolerability and rapid elimination from the blood circulation have limited their use (Lv et al. 2006). On the other hand, ionizable lipids, which are neutral at physiological pH and positively charged at the slightly acidic pH in the endosomes, are promising for mRNA delivery, as they possess the advantages of cationic lipids without their drawbacks. Although the mechanism(s) by which cationic and ionizable lipids activate the immune system is not fully understood, it is suggested that the physicochemical properties of the individual lipid components of the LNPs, including the molecular shape (Gruner et al. 1985), the acid dissociation constant (pKa) (Alabi et al. 2013), the lipid saturation degree (Heyes et al. 2005; Lee et al. 2021), and the molar ratio between the individual components (Roces et al. 2020) play critical roles for the efficiency and safety of mRNA delivery.

2 Cytosolic Delivery of mRNA

Two major types of mRNA have been evaluated as vaccines, i.e., non-replicating mRNA and self-amplifying mRNA. Non-replicating or conventional mRNA encodes only the protein of interest, whereas self-amplifying RNA, in addition to a protein, also encodes the RNA replication machinery, which increases the protein expression levels (Vogel et al. 2018). Therefore, self-amplifying RNA has shown enhanced antigen expression at lower doses, as compared to conventional mRNA (Vogel et al. 2018). During manufacturing, mRNA is transcribed from a linear DNA template in a cell-free system (Van Hoecke and Roose 2019). Two approaches are generally used to transfect IVT mRNA into cells, i.e., ex vivo transfection and direct in situ transfection.

The cytosolic bioavailability of IVT mRNA is mainly determined by two crucial factors: (i) the rate at which the mRNA is degraded by nucleases, and (ii) the ability of mRNA to cross the cell membrane and enter the cytosol (Steinle et al. 2017; Wadhwa et al. 2020). Naked mRNA only traverses to a very limited extent through the lipophilic and slightly negatively charged cellular membrane by passive diffusion, which is due to the large molecular size, the hydrophilicity, and the high negative charge density of the polyanionic phosphate backbone (Hajj and Whitehead 2017; Wadhwa et al. 2020). Therefore, naked mRNA enters cells via caveolae/lipid

raft-rich membrane domains, but following endocytosis, mRNA accumulates in the lysosomes, which results in rapid degradation, and only a very small fraction of the administered dose escapes into the cytosol (Wu and Li 2021).

Transfection of cells can be improved using delivery systems, which function by (i) protecting the mRNA from degradation by ribonucleases (RNases) and (ii) enhancing the cellular uptake (Sahin et al. 2014). IVT mRNA loaded into a nanoparticulate delivery system is taken up into the endosomes of cells via endocytosis, where it undergoes endosomal escape and is subsequently released into the cytosol (Jiang et al. 2020; Wu and Li 2021) (Fig. 1). In the cytosol, the mRNA is translated into protein by the cellular translational machinery of the ribosomes. The synthesized protein undergoes post-translational modification and is bioactive. Subsequently, the protein is either degraded into peptide fragments by the proteasomes (Gaczynska et al. 1993), or it is excreted from the cell (Reichmuth et al. 2016). After degradation by the

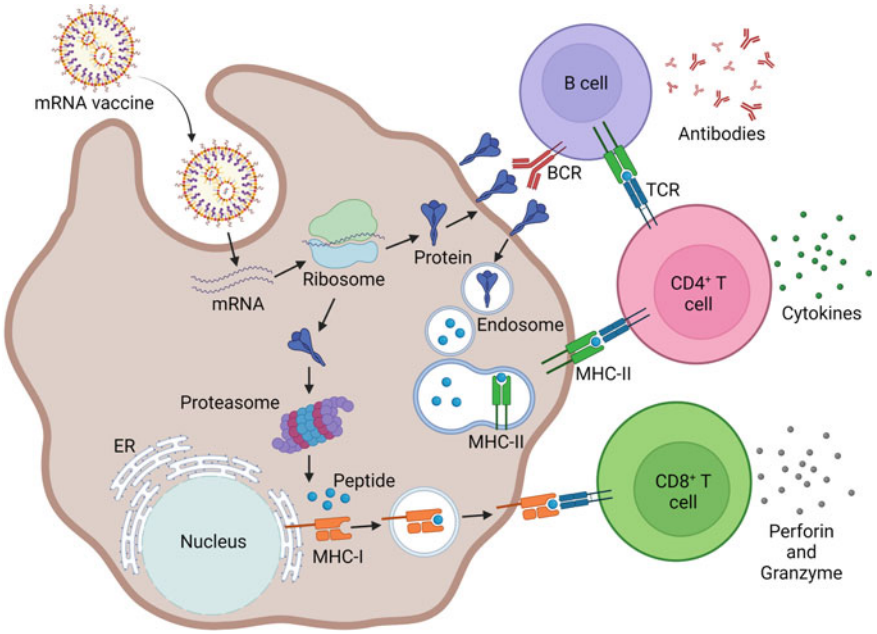

Fig. 1 Messenger RNA (mRNA) vaccine activation of antigen-presenting cells (APCs) and induction of immunity. Following mRNA vaccine uptake by APCs, mRNA escapes the endosome and is translated into protein by the ribosome. The translated secreted protein can be recognized by B cells through BCR, or it can be endocytosed by cells, degraded inside endosomes, and antigenic peptides are presented on the cell surface to helper ($CD4^+$) T cells by major histocompatibility complex (MHC) class II proteins. $CD4^+$ T cells stimulate B cells to produce neutralizing antibodies and activate phagocytes, e.g., macrophages, by secreting cytokines. Intracellularly produced protein is broken down into peptide fragments by the proteasome complex, and antigenic peptides are displayed on the cell surface to cytotoxic ($CD8^+$) T cells by MHC class I proteins. Activated $CD8^+$ T cells secrete perforin and granzyme and kill infected cells. BCR, B-cell receptor; ER, endoplasmic reticulum; TCR, T-cell receptor. Created with BioRender.com

proteasomes, antigenic peptides are presented on major histocompatibility complex (MHC) class I molecules (MHC I) to $CD8^+$ T cells (Leone et al. 2013), and in the presence of appropriate co-stimulatory signals, this leads to induction of antigen-specific $CD8^+$ T-cell responses after recognition by cognate T-cell receptors (Heath and Carbone 2001). In contrast, excreted protein is cleaved into peptide fragments, which are presented on MHC II molecules, eventually leading to the induction of antigen-specific $CD4^+$ T-cell responses (Sahin et al. 2014).

3 Lipid Nanoparticles (LNPs) as Delivery Systems for mRNA

LNPs have been widely investigated, and they are currently used in three marketed drug products for delivery of nucleic acids, including one drug product based on small interfering RNA (siRNA) and two products based on mRNA (Akinc et al. 2019; Polack et al. 2020). Onpattro® (patisiran) represents the first systemically administered RNA interference therapy drug product based on siRNA-loaded LNPs, which was approved by the US Food and Drug Administration in 2018 for the treatment of polyneuropathy caused by hereditary transthyretin amyloidosis (Akinc et al. 2019). The LNP technology is also exploited in Comirnaty® and Spikevax® to deliver mRNA encoding the SARS CoV2 spike protein antigen, and these two vaccines are currently used worldwide in mass vaccination programs.

In general, LNPs used for mRNA delivery typically include four components, i.e., (i) an ionizable lipid, (ii) cholesterol, (iii) a helper phospholipid, and (iv) a PEGylated lipid (Hou et al. 2021; Witzigmann et al. 2020). Cationic lipids consist of a cationic headgroup, one or several hydrophobic tails, and a linker group. The positively charged headgroups of cationic lipids interact with the polyanionic phosphate backbone of nucleic acids via attractive electrostatic interactions, and condensation and encapsulation of nucleic acids into LNPs occurs spontaneously by nanoprecipitation (Wolff and Rozema 2008). Using cationic lipids in formulations increases the encapsulation efficiency of nucleic acids owing to these attractive electrostatic interactions (Granot and Peer 2017). Examples of cationic lipids used in lipid-based formulations are 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), which is a biodegradable analogue of DOTMA, and *N*-decyl-*N*,*N*- dimethyldecan-1-aminium bromide (DDAB) (Blakney et al. 2019b; Lu et al. 2007; Zohra et al. 2007).

Cationic lipids, which are used in mRNA vaccines, do not only enhance the encapsulation of mRNA, but may also act as adjuvants (Lonez et al. 2014). For example, the quaternary ammonium lipid DDAB serves as an immune adjuvant by stimulating an innate immune response, and in combination with the zwitterionic helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), it is commercialized as TransfectACE (Blakney et al. 2019a; Hilgers and Snippe 1992). However, lipid-based formulations with a high cationic charge density are rapidly eliminated

from the blood circulation after intravenous administration due to embolization in the narrow capillaries of the lungs (Landesman-Milo and Peer 2014) because they interact with anionic serum proteins, which causes aggregation, toxicity, and reduced efficacy (Lv et al. 2006).

Ionizable lipids, e.g., 1,2-dioleoyl-3-dimethylammoniumpropane (DODAP) and 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), were introduced to overcome the unfavorable pharmacokinetics and toxicity of cationic lipids (Heyes et al. 2005; Semple et al. 2010) (Table 1 and Fig. 2). Ionizable lipids are positively charged at pH values below their pKa value, but are neutral at pH values above their pKa value (Bailey and Cullis 1994). Hence, they are formulated with nucleic acids by complexation at a pH value below their pKa (Cullis and Hope 2017). At physiological pH, the complexes display a neutral surface charge, and they become re-charged by protonation in the slightly acidic environment of the endosomes after cellular uptake (Semple et al. 2010). In the endosomes, the positively charged lipids promote membrane destabilization via interaction with the negatively charged lipids in the endosomal membrane, which facilitates endosomal escape of the LNPs and release of mRNA into the cytosol (Hou et al. 2021). Hence, the pKa value of ionizable lipids influences the delivery efficiency and eventually the efficacy. In addition to the endosomal escape efficiency, the pKa value of ionizable lipids is also suggested to influence cellular uptake and opsonization of LNPs (Sabnis et al. 2018). Although the mechanism(s) is not fully understood, the pKa value of the lipids may also influence the interaction between LNPs and the immune system (Hassett et al. 2019). Therefore, the pH sensitivity of ionizable lipids plays an important role for in vivo mRNA delivery by preventing the unfavorable cationic surface charge of the LNPs in the blood circulation, hence enhancing the biocompatibility of LNPs.

Based on the structure of DODMA, the ionizable lipid 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3- aminopropane (DLin-DMA) was designed specifically for siRNA delivery (Heyes et al. 2005), and modification of the linker and the hydrophobic regions of Dlin-DMA resulted in 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), which displays increased delivery efficiency (Semple et al. 2010). Further modification of the amine headgroup of DLin-KC2-DMA resulted in (6*Z*,9*Z*,28*Z*,31*Z*)- heptatriaconta-6,9,28,31- tetraen-19- yl 4-(dimethylamino) butanoate (DLin-MC3-DMA; MC3), which is a main constituent of Onpattro® (patisiran) (Akinc et al. 2019). Despite these advantages, a major impediment for the use of lipid-based nanocarriers for therapeutic RNA delivery is their undesired activation of the innate immune system (de Groot et al. 2018; Kedmi et al. 2010). For example, patients treated with lipid nanoparticle-containing patisiran have to be pre-dosed with a strong cocktail of anti-inflammatory drugs to reduce inflammation (Adams et al. 2018). Other approaches for inhibiting dose-limiting undesired immune activation include co-encapsulation of anti-inflammatory drugs in the LNPs, e.g., lipophilic dexamethasone prodrugs (Chen et al. 2018) and aliphatic ester prodrugs of anti-inflammatory steroids (rofleponide or budesonide) (Davies et al. 2021).

MC3 has been used in several preclinical and clinical studies for the delivery of nucleoside-modified mRNA (Bahl et al. 2017; Richner et al. 2017). However, a

Table 1 Examples of ionizable lipid components of lipid nanoparticles tested for prophylactic mRNA vaccines and therapeutics

Ionizable lipid	mRNA type	Disease/target tissue	Administration route	Phase	Clinical trial identifier	Sponsor/manufacturer	References
Clinical studies							
ALC-0315 (BNT162b2)	Nucleoside-modified mRNA	SARS-CoV-2	i.m.	Phase 3	NCT04368728	Pfizer/BioNTech	Sahin et al. (2020) and W.H.O. (2021)
SM-102 (Lipid H) (mRNA-1273)	Nucleoside-modified mRNA	SARS-CoV-2	i.m.	Phase 3	NCT04470427	Moderna Inc.	Baden et al. (2021)
ALC-0315 (CVnCoV)	Unmodified mRNA	SARS-CoV-2	i.m.	Phase 3	NCT04652102	CureVac AG	Chaudhary et al. (2021)
Acuitas A9 (LNP-nCoVsaRNA)	Self-amplifiying mRNA	SARS-CoV-2	i.m.	Phase 1	ISRCTN17072692	Imperial College London	McKay et al. (2020)
C12-200 (MRT5500)	Unmodified mRNA	SARS-CoV-2	i.m.	Phase 1/2	NCT04798027	TranslateBio/Sanofi	Kalnin et al. (2021)
Lipid 10a or Lipid 2,2 (8,8) 4C CH3 (ARCT-021)	Self-amplifiying mRNA	SARS-CoV-2	i.m.	Phase 2	NCT04728347	Arcturus Therapeutics	Buschmann et al. (2021)
DMAP-BLP mRNA-1325/Moderna	Nucleoside-modified mRNA	Zika	i.m.	Phase 1	NCT03014089	Moderna Inc.	Richner et al. (2017)
Preclinical studies							
Lipid 14	Nucleoside-modified mRNA	SARS-CoV-2	i.m.	–	–	Tel Aviv University	Elia et al. (2021)

(continued)

Table 1 (continued)

Ionizable lipid	mRNA type	Disease/target tissue	Administration route	Phase	Clinical trial identifier	Sponsor/manufacturer	References
Genevant (CL1 lipid-like)	Nucleoside-modified mRNA	SARS-CoV-2	i.m.	–	–	Chulalongkorn University, University of Pennsylvania	Buschmann et al. (2021) ara>
DLin-MC3-DMA (MC3)	Unmodified mRNA, sequence-optimized mRNA	Influenza (HA), Rabies (RABV-G)	i.m.	-	-	CureVac AG	Jayaraman et al. (2012) and Lutz et al. (2017)
	Nucleoside-modified mRNA	HIV	i.d.	–	–	University of Pennsylvania, BioNTech	Pardi et al. (2018a)
	Nucleoside-modified mRNA	Zika virus	i.d., i.m.	–	–	University of Pennsylvania, BioNTech	Pardi et al. (2017)
DMAP-BLP	Nucleoside-modified mRNA	HCMV	i.m.	–	–	Moderna Inc.	John et al. (2018)
	Nucleoside-modified mRNA	Ebola virus	i.m.	–	–	Moderna Inc.	Meyer et al. (2018)

(continued)

Table 1 (continued)

Ionizable lipid	mRNA type	Disease/target tissue	Administration route	Phase	Clinical trial identifier	Sponsor/manufacturer	References
DLin-DMA	Self-amplifiying mRNA	İnfluenza virus (NP,M1)	i.m.	–	–	Novartis Vaccines	Lazzaro et al. (2015) and Magini et al. (2016)
Lipid-5	Modified mRNA	Anemia (hEPO)	i.m.	–	–	Moderna Inc.	Sabnis et al. (2018)
LNP-INT01/LP-01	Cas9 mRNA	TRR amyloidosis	i.v.	–	–	Intellia	Finn et al. (2018)
ATX-100	Nucleoside-modified mRNA	Hemophilia B	i.v.	–	–	Arcturus Therapeutics	Ramaswamy et al. (2017)
OF-Deg-Lin	Luciferase	Spleen targeting	i.v., i.p.	–	–	Massachusetts Institute of Technology	Fenton et al. (2017)

ALC-0315, ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate); Cas9, clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9; C12-200, ((1,1′-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)n(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol)); DLin-DMA, 1,2-dilinoleyloxy-n,n-dimethyl-3-aminopropane; DLin-MC3-DMA,([6Z,9Z,28Z,31Z]-heptatriacont-6,9,28,31-tetraene-19-yl-4-(dimethylamino)butanoate; DMAP-BLP, 3-(dimethylamino)propyl(12Z,15Z)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl]henicosa-12,15-dienoate; HCMV, human cytomegalovirus; hEPO, human erythropoietin; HIV, human immunodeficiency virus; i.m., intramuscular; i.d., intradermal; i.p., intraperitoneal; i.v., intravenous; Lipid H (SM-102) heptadecan-9- yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy) hexyl)amino) octanoate; Lipid 5, heptadecan-9-yl 8-((2-hydroxyethyl)(8-(nonyloxy)-8-oxooctyl)amino)octanoate; OF-Deg-Lin, (((3,6-dioxopiperazine-2,5-diyl)bis(butane-4,1-diyl))bis(azanetriyl))tetrakis(ethane-2,1-diyl) (9Z,9′Z,9″Z,9‴Z,12Z,12′Z,12″Z,12‴Z)-tetrakis (octadeca-9,12-dienoate)

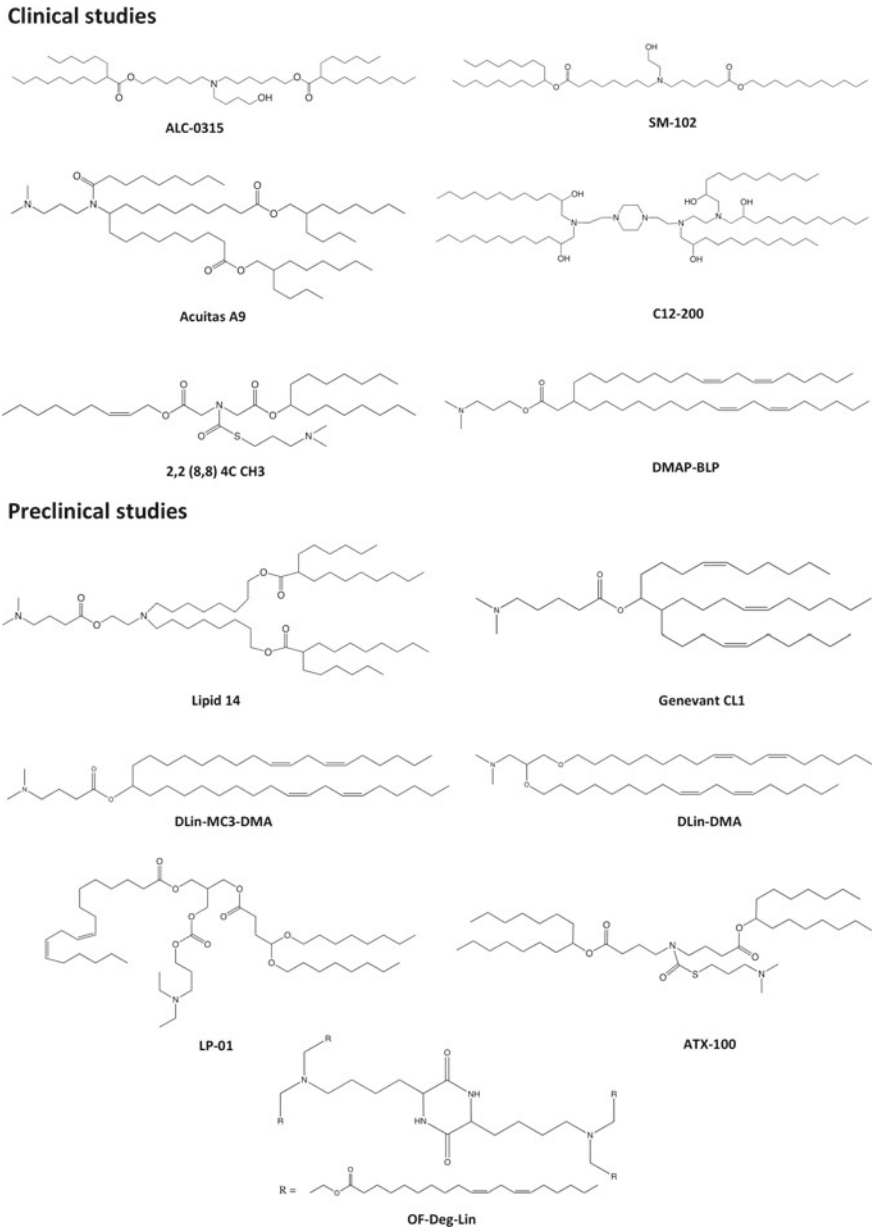

Fig. 2 Representative structures of ionizable lipids in lipid nanoparticles tested for prophylactic mRNA vaccines and therapeutics

concern is that MC3 may accumulate in the liver and spleen and cause toxicity upon repeated dosing due to the slow degradation of the dilinoleic alkyl chain (Sabnis et al. 2018). Therefore, a biodegradable analogue of MC3 was designed, i.e., Lipid 319, by introducing an ester group in each alkyl chain, which is degraded by esterases in vivo, and the potency was enhanced by increasing the branching of the lipid tail, as compared to the dilinoleic lipid tail of MC3 (Maier et al. 2013). Lipid 319 represents the Acuitas Therapeutics (ALC-0315) LNP class, which is also used in Comirnaty® and CureVac's mRNA vaccine CVnCoV against SARS-CoV-2 (Buschmann et al. 2021; W.H.O. 2021). A novel class of biodegradable ionizable lipids, including Lipid H (SM-102, Spikevax®), Lipid 5, OF-Deg-Lin and ATX (Arcturus), has subsequently been developed (Baden et al. 2021; Fenton et al. 2017; Ramaswamy et al. 2017; Sabnis et al. 2018).

The structure of lipids affects the delivery efficiency of mRNA (Li et al. 2016). A cone-shaped structure in the acidic environment of the endosomes is hypothesized to be essential for interaction with anionic lipids, which are present in the endosomal membrane, e.g., phosphatidylserine (Maier et al. 2013). Increased branching has been suggested to result in ionizable lipids, which are cone-shaped, and this feature has been shown to provide enhanced membrane disrupting ability (Gruner et al. 1985). MC3 displays two C18 linoleic acid tails, while Lipid H and Lipid 5 have three tails, Acuitas ALC-0315 has four tails, and A9 has five alkyl tails (Hassett et al. 2019; Sabnis et al. 2018). Lipid H and Lipid 5 display similar pKa values, but they have been shown to promote fourfold increased endosomal escape of mRNA as compared to MC3 (Hassett et al. 2019; Sabnis et al. 2018). The gene silencing efficiency of siRNA-loaded, ALC-0315-based LNPs has been shown to be tenfold higher in hepatocytes, as compared to the gene silencing efficiency mediated by MC3 (Steven and Xinyao 2017). This suggests that although endosomal release has not been reported for Acuitas ALC-0315, its more cone-shaped four-branched structure promotes higher endosomal release. Another important feature of lipids is the degree of lipid saturation, which affects the pKa value, the fusogenic properties, and the cellular uptake of nucleic acid cargoes (Heyes et al. 2005; Lee et al. 2021). Increasing the degree of unsaturation in the hydrophobic domain significantly enhances the potency of ionizable lipids, decreases the phase transition temperature, and increases the fluidity of the cell membrane, which may improve the fusion process and endosomal escape (Heyes et al. 2005; Weng et al. 2020).

In addition, the ratio of component lipids and selection of phospholipids are important factors that determine mRNA delivery efficiency and safety (Weng et al. 2020). The presence of helper lipids like phospholipids and cholesterol in LNPs generally enhances the colloidal stability of the formulation by increasing the membrane rigidity and reducing the membrane permeability (Campbell et al. 2001; Monteiro et al. 2014). Although data regarding the role of helper lipids in LNPs is limited, it has been demonstrated that the presence of 40–60 mol% helper lipid in LNPs is optimal for the encapsulation efficiency of siRNA (Kulkarni et al. 2019). Another study has shown that incorporation of DOPE and increasing the weight ratio of ionizable lipid to mRNA in LNPs enhance the mRNA delivery efficiency in vivo (Kauffman et al. 2015). The PEG lipids control particle size and prevent particle

aggregation in formulation by forming a stabilizing hydrophilic shell that hinders vesicle fusion (Ryals et al. 2020). Moreover, polyethylene glycol (PEG) lipids reduce opsonization of nanoparticles by serum proteins and consequently prolong the blood circulation time (Guevara et al. 2019). However, the PEG lipids on the LNP surface may promote immunogenicity and strong antibody responses. It has been reported that the anti-PEG antibody response following repeated intravenous (i.v.) administration of PEGylated LNPs may significantly accelerate the blood clearance of LNPs and initiate acute hypersensitivity. Therefore, the circulation time of LNPs and the amount of PEG lipids in the formulation are critical (Judge et al. 2006).

4 Immune Activation by Lipids Used for mRNA Delivery

To overcome the safety issues of cationic lipids used for mRNA delivery, a new generation of lipidoids was engineered (Akinc et al. 2008; Love et al. 2010; Whitehead et al. 2014). At a dose of 3 mg/kg siRNA, C57BL/6 mice immunized with LNPs formulated using C12-200 as the new generation ionizable lipid displayed elevated levels of the cytokine IL-6, and the chemokines keratinocyte chemoattractant (KC) and monocyte chemoattractant protein-1 (MCP-1), while the lipidoid 304O13 did not induce any activation of these cytokines and chemokines (Whitehead et al. 2014). Incorporation of immunostimulatory mRNA in LNPs can facilitate interaction with endosomal toll-like receptors (TLRs), e.g., TLR3, TLR7, and TLR8, and activation of these TLRs can inhibit antigen expression and result in a reduced antigen-specific immune response (Pardi et al. 2018b). However, mRNAs containing chemically modified nucleotides or optimized codon sequences have been reported to display reduced mRNA-related immunogenicity triggered by activation of pattern-recognition receptors (PPRs) (Kariko et al. 2008; Whitehead et al. 2011). It should also be noted that mRNA might be responsible for immune activation, depending on whether the bases are modified or not (Cabanillas et al. 2021; Reichmuth et al. 2016). LNPs formulated using the cKK-E12 lipid, but without mRNA, were injected once at an equivalent lipid dose of 1.5 mg/kg mRNA in C57BL/6 mice, and was found induce elevated levels of liver enzymes, and multiple cytokines, specifically IFN, IL-6, and TNF-α, while one of the most recent-generation alkyne lipidoid A6 did not induce upregulation of proinflammatory cytokines (Miao et al. 2020). A recent study has shown that the LNP formulation has intrinsic adjuvant properties, which depend on the ionizable lipid component, and promotes the secretion of IL-6, using influenza virus and SARS-CoV-2 mRNA and protein subunit vaccines. Comparison of LNP formulations containing either nucleoside-modified mRNA [firefly luciferase (FLuc)-encoding mRNA] or no payload (empty LNPs, eLNPs) showed that the mRNA displayed no significant effect on the adjuvant properties of LNPs loaded with FLuc mRNA. To demonstrate that the adjuvant activity is caused by the ionizable lipid, eLNP formulations with and without ionizable lipids were prepared. Increasing the molar ratio of ionizable lipid in LNPs slightly increased

their adjuvant activity, while adjuvant activity was not observed for the formulation that did not contain ionizable lipid. The adjuvanticity of ionizable lipid was compared to the adjuvanticity of the frequently used cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) formulated at the same ratios of components used for the eLNPs. Unlike LNPs containing ionizable lipid, LNPs containing DOTAP did not induce increased hemagglutination inhibition titers. The LNP formulation was more potent than Addavax™, which is an MF59-like adjuvant, by significantly inducing IgG (IgG, IgG1, IgG2a, IgG2b) and IgA titers. The LNP formulation containing ionizable lipids induced high levels of IL-6, which is a cytokine critical for early follicular helper T cell differentiation in mice. Furthermore, data indicated that the immunostimulatory activity of the LNP component did not rely on MyD88- (adaptor protein for TLRs) or MAVS- (mitochondrial anti-viral signaling)-dependent sensing of LNPs (Alameh et al. 2021). In another study, LNP comprising an asymmetric ionizable amino lipid enhanced B-cell responses to subunit vaccine viral antigens in BALB/c and C57BL/6 mice (Swaminathan et al. 2016a) and elicited strong antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. These responses were comparable to responses induced by known vaccine adjuvants, including aluminum-based adjuvants, the immunomodulatory oligonucleotide IMO-2125, and the TLR4 agonist 3-*O*-deacylated monophosphoryl lipid A (MPL) (Swaminathan et al. 2016a). In a follow-up study, the content of ionizable lipid was reported to be important for the ability of LNPs to boost immune responses against a Dengue antigen. LNPs containing ionizable cationic lipid significantly enhanced the induction of neutralizing antibodies to levels, which were comparable to the levels induced by the strong ISCOMATRIX™ adjuvant (Swaminathan et al. 2016b). A recent study showed preclinical evidence of the highly immunogenic nature of LNPs containing DLin-MC3-DMA as the ionizable lipid (Ndeupen et al. 2021). Intradermal injection of both non-loaded LNPs and LNPs loaded with non-coding poly-cytosine mRNA caused upregulation of several genes, particularly genes involved in activation of inflammasomes and deregulation of nucleotide-binding and oligomerization domain receptor family pyrin domain containing 10 (NLRP10), which is known to inhibit inflammasomes. Intranasal administration of 5 or 10 μg of mRNA loaded into these LNPs in adult wild-type B6 mice resulted in approximately 20% or 80%, respectively, mortality during the first 2 days after administration (Ndeupen et al. 2021). Similarly, DLin-MC3-DMA was compared to four lipids (Lipids H, P, Q, and N) from Moderna for protein expression and immunogenicity (Hassett et al. 2019). The lipids were tested for tolerability in rats by grading mixed cell inflammation, muscle fiber necrosis, and degenerate neutrophils, and the MC3 lipid was the least tolerated lipid, while lipid H appeared to be the safest lipid. Non-loaded LNPs formulated with DLin-MC3-DMA exhibited mild to moderate but reversible proinflammatory responses in rats at a dose of 0.3 mg/kg, while minimal and transient complement activation was observed in female monkeys (Sedic et al. 2018). Moreover, it has been reported that some cationic/ionizable lipids may function as danger-associated molecular patterns (DAMPs), which activate PRRs, e.g., TLRs (Lonez et al. 2014). The cationic lipid component of LNPs is mainly recognized by the extracellular TLR2 and TLR4, and by the intracellular NLRP3 (Lonez et al. 2014; Wilmar et al. 2012). Activation of cell

surface-localized TLRs, e.g., TLR2 and TLR4, triggers induction of high levels of cytokines (Kawai and Akira 2006), and in some cases the so-called cytokine release syndrome (CRS) (Shimabukuro-Vornhagen et al. 2018). It has been shown that LNPs comprising of the cationic lipid CLinDMA is primarily responsible for the induction of an innate immune response and the production of both pro- and anti-inflammatory cytokines (Abrams et al. 2010). A subset of ionizable lipids, i.e., the so-called lipidoids, have been used for siRNA delivery (Akinc et al. 2008). New generations of lipidoids, which display improved potency, e.g., C12-200, cKK-E12, OF-02, and 503-O13, have subsequently been developed (Dong et al. 2014; Fenton et al. 2016; Love et al. 2010; Whitehead et al. 2014). Lipidoids based on a tetraamine backbone have been shown to be strong agonists for TLR4, and they activate murine APCs in vitro (de Groot et al. 2018). The C1 lipid-like ionizable compound, which is a constituent of an LNP-based mRNA nanovaccine for cancer immunotherapy, was also found to mainly activate TLR4 (Zhang et al. 2021). Furthermore, C1-containing LNPs induced expression of inflammatory cytokines, e.g., IL-12, by stimulating the TLR4 signal pathway in dendritic cells. Ionizable lipid-like materials designed for mRNA delivery containing a cyclic amine headgroup, an unsaturated lipid tail, and a dihydroimidazole linker, were found to have an adjuvant effect via the stimulator of interferon genes (STING) pathway, rather than through TLRs, and induced maturation of APCs. mRNA LNP formulations containing this cyclic lipid inhibited tumor growth and prolonged survival in melanoma and human papillomavirus E7 tumor mice models, compared with commercially available MC3-based LNPs (Miao et al. 2019). In another study, pH-activated lipid-like material that binds vitamin E as a hydrophobic scaffold was shown to activate the STING pathway (Kawai et al. 2018).

5 Conclusions

With the recent developments in the fields of infectious disease vaccines, cancer immunotherapies, and gene and protein replacement therapy, mRNA has without doubt become a promising new type of drug. So far, the cationic ionizable lipid component of LNPs is likely to activate the immune system through the engagement of TLRs and the NLRP3 inflammasome pathways, but the mechanism(s) has not been fully elucidated yet. In addition, further studies are needed to dissect immune activation mediated by the mRNA component from immune activation mediated by the delivery system. The gap in our understanding of causes and mechanisms of this innate immune recognition calls for new fundamental insights that can guide the rational design of new and safe cationic lipids. This knowledge will contribute significantly to elucidate immune pathways induced by ionizable cationic lipids and to improve mRNA-loaded LNP formulations.

Acknowledgements We gratefully acknowledge the financial support from the Independent Research Fund Denmark (grant number 9041-00198B). We also acknowledge the Study Abroad Program of the Ministry of National Education, The Republic of Türkiye, for the scholarship to Melike Ongun. The funders had no role in literature search, decision to publish, or preparation of the article.

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Adjuvants, the Elephant in the Room for RNA Vaccines

Sigrid D'haese, Sabine den Roover, and Joeri L. Aerts

Contents

Abstract Adjuvants are crucial components of vaccines. Nevertheless, they are frequently considered as mere "excipients", and their mode of action is often poorly understood. Although the attractiveness of mRNA as an immunogen has been recognized already more than thirty years ago, it wasn't until the current COVID-19 crisis that its full potential was shown. From a fringe approach, it has now become a leading technology in vaccine development which will no doubt result in a tremendous boost in both prophylactic and therapeutic vaccination settings. The issue of finding the right adjuvant is especially relevant for mRNA-based vaccines, as mRNA itself is a strong activator of innate immune responses which represents a double-edged sword. Moreover, given the high sensitivity of RNA to ambient RNases, and to improve delivery efficiency, in recent years, a lot of effort has been invested in developing ways to package the mRNA in so-called nanoparticle formulations. Currently approved mRNA-based vaccines are all formulated in lipid nanoparticles, but many other approaches are being explored, each of which will result in a different type of

S. D'haese · S. den Roover · J. L. Aerts (✉)
Laboratory of Neuro-Aging and Viro-Immunotherapy (NAVI), Vrije Universiteit Brussel (VUB), Laarbeeklaan 103D, 1090 Brussels, Belgium
e-mail: joeri.aerts@vub.be

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_12

immune stimulation. In this chapter, we want to provide an overview of the potential adjuvant effect of different types of nanoparticles and implications for vaccine development.

Keywords Adjuvant · RNA vaccines · Innate immunity · Lipid nanoparticles

Abbreviations

AP-1	Activator protein-1
ASC	Apoptosis-associated Speck-like protein containing caspase activation and recruitment domains
CARDS	Caspase activation and recruitment domains
CTL	Cytotoxic T lymphocyte
DCs	Dendritic cells
DDX	Dead-box helicase
DHX	DExH-box helicase
dsRNA	Double-stranded RNA
eIF2α	Eukaryote initiation factor 2α
IFN	Interferon
IFNAR	IFN-α/β receptor
IIPs	Innate inhibiting proteins
IL	Interleukin
IP-10	Interferon-induced protein-10
IRF	IFN regulatory factor
ISRE	Interferon-stimulated response element
IVT	In vitro transcribed
JAK-STAT	Janus kinase—signal transducer and activator of transcription
LGP2	Laboratory of genetics and physiology 2
LNP	Lipid nanoparticle
m1ψ	N1-methylpseudouridine
m^5C	5-Methylcytidine
m^6A	6-Methyladenosine
MAPK	Mitogen activation protein kinase
MAVS	Mitochondrial antiviral signalling protein
MCP-1	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated protein 5
MERS-CoV-ORF4a	Middle East respiratory syndrome coronavirus
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response protein 88
NF-κβ	Nuclear factor κ-light-chain enhancer of activated B cells
NLRP3	NOD-/leucine-rich repeat- and pyrin domain-containing protein 3
NOD	Nucleotide-binding oligomerization domain

NSP	Non-structural proteins
OAS	2′-5′Oligoadenylate synthase
PAMP	Pathogen-associated molecular pattern
PKR	Protein kinase R
PRR	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-I-like receptors
S2U	2-Thio-uridine
sa-RNA	Self-amplifying RNA
SARS-CoV	Severe acute respiratory syndrome coronavirus 2
ssRNA	Single-stranded RNA
STING	Stimulator of IFN genes
Tfh	Follicular helper T cells
TIR	Toll-IL-1-receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adaptor-inducing IFN-β
UTP	Uridine-5′-triphosphate
ψ	Pseudouridine

1 Introduction

The first in vivo application of mRNA dates back to 1990, when expression of in vitro transcribed (IVT) mRNA after intramuscular injection in mice has been described (Wolff et al. 1990). Shortly after, IVT mRNA was shown to induce an immune response, as an injection in the tongue muscle elicited antigen-specific antibodies (Conry et al. 1995). In the meantime, a liposome formulation of IVT mRNA injected subcutaneously was proven to induce virus-specific cytotoxic T lymphocytes (CTLs) in several mouse models (Martinon et al. 1993). Already 30 years ago, this work paved the way for the development of mRNA-based COVID-19 vaccines. Nevertheless, many hurdles still needed to be cleared for routine development of mRNA vaccines. One of the problems that arose was the fact that the innate immune system was activated after injection of mRNA, which led to reduced translational levels. In addition, mRNA is not as stable as its double-stranded nucleic acid counterpart DNA, which meant it had to be packaged in order to be delivered efficiently in vivo. In this chapter, we will discuss the improvement of mRNA vaccines, the next-generation mRNA vaccines, the self-amplifying RNA (sa-RNA) and the adjuvant mechanism of mRNA and of the packaging nanoparticles.

2 mRNA as a Natural Adjuvant

IVT mRNA is produced from a template plasmid DNA by a phage polymerase, usually T7 or SP6 (Konarska and Sharp 1989). However, this process is not without errors; during the in vitro production of mRNA, the phage polymerase generates promoter- and run-off transcript-dependent and -independent double-stranded RNA (dsRNA) contaminants (Gholamalipour et al. 2018; Mu et al. 2018) which can activate the innate immune system in several ways, conferring efficient adjuvant properties to the IVT mRNA. For nucleic acid sensing in general, we refer to a recent review (Bartok and Hartmann 2020). In this chapter, we will mainly discuss the receptors involved in the recognition of IVT mRNA and the nanoparticles it is formulated in.

In addition to classic, non-replicating mRNA, the next-generation IVT mRNA is the so-called sa-RNA and has the capacity to replicate itself via the viral replicase complex, which serves as an in situ translation machinery and enables prolonged antigen production (Ljungberg and Liljeström 2014). This type of mRNA is derived from single-stranded RNA (ssRNA) viruses, e.g. alphaviruses (Sindbis virus, Venezuelan Equine Encephalitis virus, among others) and encodes not only the antigen of interest (which substitutes for the viral structural proteins) but also four non-structural proteins (NSP1-4) and a subgenomic promoter (Maruggi et al. 2019). The main benefit of sa-RNA is that it allows for a prolonged antigen exposure. For instance, sa-RNA encoding for luciferase packaged in a lipid nanoparticle was injected intramuscularly, and bioluminescence was detected for up to 63 days in vivo (Geall et al. 2012), compared to only 8 days for non-replicating mRNA (Pardi et al. 2015). Furthermore, sa-RNA was shown to induce broad and potent immunity after delivery in a lipid nanoparticle (LNP) (Geall et al. 2012). The production process of IVT mRNA and sa-RNA is similar, leading to the same by-products which give rise to immune activation. In addition, during the self-replicating process, dsRNA is inevitably formed resulting in RNA recognition by RNA sensors (Pepini et al. 2017), making both forms of mRNA natural adjuvants.

2.1 Endosomal RNA Recognition

After engulfment of the IVT mRNA or LNPs, the mRNA ends up in the endosome where it comes into contact with several toll-like receptors (TLRs) (Fig. 1, part 1). TLRs are pattern recognition receptors (PRRs), which play a crucial role in innate immunity as they are responsible for the initial detection of the so-called pathogen-associated molecular patterns (PAMPs), in this case ssRNA and dsRNA. TLR3 recognizes dsRNA and signals through toll-interleukin(IL)-1-receptor-domain-containing adaptor-inducing interferon (IFN)-β (TRIF) (Oshiumi et al. 2003), and TLR7 and 8 are triggered by ssRNA and signal through myeloid differentiation factor 88 (MyD88) (Diebold et al. 2004; Heil et al. 2004). In addition, two other endosomal TLRs recognize RNA: TLR10 was only recently discovered to respond to dsRNA (Lee et al.

2018), and TLR13 recognizes a specific sequence within bacterial ribosomal RNA (Hidmark et al. 2012); the latter two are only detected in lower vertebrates and rodents and will not be further discussed in this chapter.

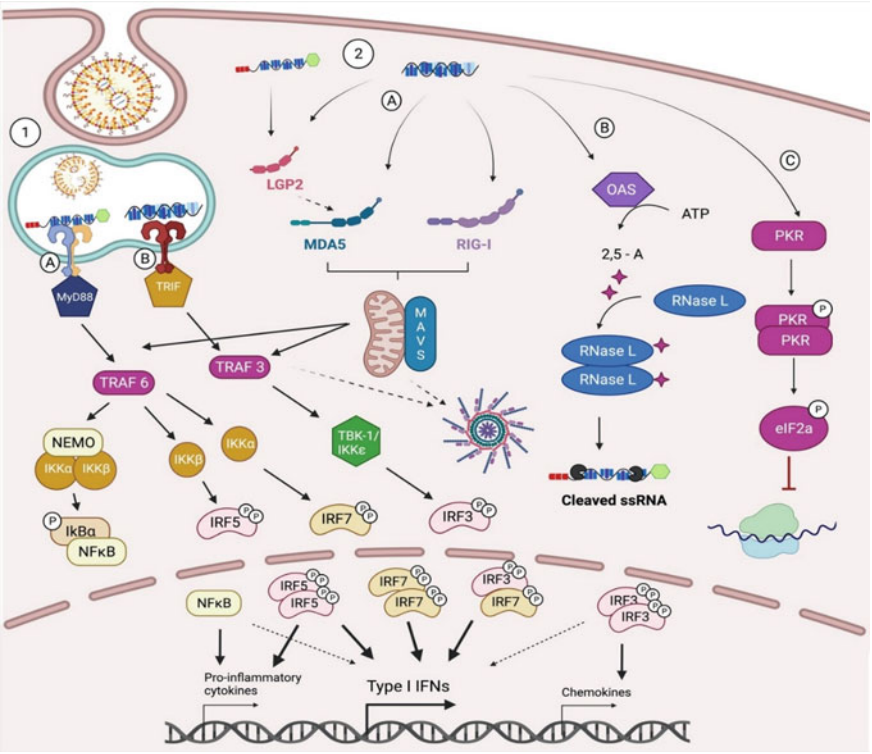

Fig. 1 Mechanisms of innate immune sensing of IVT mRNA. mRNA-containing lipid nanoparticles are taken up by bystander cells via endocytosis (1). In the endosomes, ssRNA (A) and dsRNA (B) are recognized by toll-like receptors (TLR)7/8 and TRL3, and these receptors in turn activate MyD88 and TLR3 in Toll-interleukin-1 domain-containing adapter-inducing interferon-β (TRIF) respectively. Eventually, this results in the stimulation of tumour necrosis factor receptor-associated factor (TRAF) 3 and 6 resulting in the activation of transcription factors such as nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and interferon (IFN) regulatory factors (IRF) 3, 5 and 7. Subsequently, this gives rise to the transcription of genes encoding for inflammatory cytokines, type I IFNs and chemokines. In the cytosol (2), dsRNA and ssRNA are recognized by the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) laboratory of genetics and physiology 2 (LGP2), melanoma differentiation-association protein 5 (MDA5) and RIG-I (A). LGP2 cannot by itself activate MAVS but has a role as facilitator for other RLRs. MAVS in turn results in the activation of TRAF3 and TRAF6 and may as well have an influence on inflammasome activation. Upon recognition of dsRNA by oligoadenylate synthase (OAS), OAS is phosphorylated and recruits RNase L resulting in RNA degradation (B). In addition, dsRNA is recognized by protein kinase R (PKR) (C), which leads to phosphorylation of eukaryote initiation factor 2a, leading to the abrogation of translation. (Created with Biorender.com)

Downstream TRIF signalling results in the activation of several transcription factors. Via tumour necrosis factor (TNF) receptor-associated factors (TRAF)3, it results in phosphorylation and translocation of IFN regulatory factor (IRF) 3. In addition, the mitogen activation protein kinases (MAPKs), stimulating activator protein (AP)-1 translocation and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) activation are the result of TRIF signalling. Lastly, TRAF3 can also trigger the assembly of the inflammasome by the complexation of nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeat- and pyrin domain-containing protein (NLRP)3, the adaptor protein, apoptosis-associated speck-like protein containing caspase activation and recruitment domains (CARD) (ASC) and caspase-1 (Kelley et al. 2019). MyD88 signalling mainly results in activation of NF-κB, MAPK, IRF5 and 7. TRIF and MyD88 activation therefore results in the secretion of similar inflammatory cytokines (such as TNF-α and IL-6), chemokines CXCL-8 (IL-8) and more importantly type I IFNs (Bartok and Hartmann 2020). The signalling cascades starting with TRIF or MyD88 therefore share some signalling proteins but can also work supplementary. For instance, MyD88 signalling results in the downstream production of pro-IL-1β. Simultaneously, TRAF3 will allow inflammasome assembly, resulting in active caspase-1 which can subsequently cleave the pro-IL-1β precursor in active IL-1β.

The first mechanism for IVT mRNA recognition was discovered in 2004, where it was shown that IVT mRNA could stimulate TLR7 in mice and led to the production of IFN-α among other cytokines (Diebold et al. 2004). In the same issue, TLR7 and TLR8 were revealed as the human receptors for ssRNA (Heil et al. 2004). These receptors are found on the endosomal membrane, unsurprisingly, the site where the mRNA is located after engulfment inside the cell. TLR3, which is known for binding to dsRNA, also plays an important role in the recognition of mRNA, as dsRNA is a by-product of IVT mRNA. Thus, early work showed that IVT mRNA (the whole mixture) acts as a ligand for TLR3, 7 and 8 resulting in maturation and release of cytokines by primary monocyte-derived dendritic cells (DCs) after lipofection (Karikó et al. 2005). These findings were recently confirmed when it was shown that modified mRNA gives rise to MyD88 dependent activation of the type I IFN pathway (Nelson et al. 2020).

2.2 Cytoplasmic RNA Sensors

In order for mRNA to be efficiently translated, it needs to be released from the endosome into the cytoplasm to reach the ribosomes. This release occurs passively but is much more efficient when the mRNA is packaged in LNPs and other types of packaging materials such as polymers or cell-penetrating peptides. Therefore, when mRNA is released into the cytosol, it encounters a new set of RNA sensors (Fig. 1, Part 2). One group of sensors is the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) which include three different family members: RIG-I, the melanoma

differentiation associated protein 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2) protein. All RLRs contain a central helicase domain and a carboxy-terminal regulatory domain. These two domains join forces into recognizing RNAs. Additionally, RIG-I and MDA5 contain two amino terminal CARDs and are able to interact with the IFN-β promoter stimulator protein adaptor protein, better known as mitochondrial antiviral-signalling protein (MAVS), and induce downstream signal transduction pathways (Onomoto et al. 2021). In the presence of dsRNA or ssRNA with 5′ phosphate, conformational changes lead to an exposed CARD domain. Subsequently, in the presence of ATP, the CARD domain will interact with the adaptor protein MAVS, leading to type I IFN transcription (Onomoto et al. 2021). LGP2 lacks the CARD domain, making it an atypical RLR, but although it cannot interact with MAVS, it is able to recognize and bind to dsRNA as well as ssRNA (Takahasi et al. 2009). In case of recognition of viral RNA, LGP2 acts as a facilitator for RIG-I and MDA5 (Duic et al. 2020). Nevertheless, its role in IVT mRNA sensing is uncertain and LGP2 will therefore not be further discussed.

The RLRs are part of a much larger family called the DEAD/DExH-box RNA helicases (DDX/DHX). This group entails many understudied RNA helicases which may aid in cytosolic RNA sensing by for instance acting as a co-receptor for MDA5 or RIG-I or by enhancing RIG-I signalling (Bartok and Hartmann 2020). Some members of the family have been described to even directly activate the inflammasome. However, the role of DDX/DHX proteins in IVT mRNA sensing requires thorough investigation and will not be discussed in detail in this chapter.

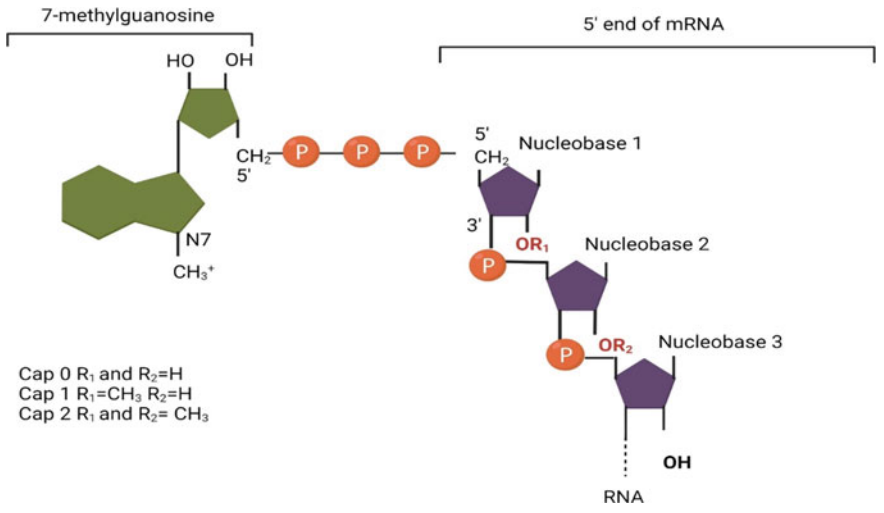

Fig. 2 Structures of cap 0, cap 1 and cap 2. The difference between these capping systems lies in the addition of a methylation group at the 2′ position of the 5′ penultimate and antepenultimate of the transcript. In some cases, the addition of a methyl group in these positions enhances the translation efficiency in vivo compared to the corresponding cap 0-mRNA (Created with Biorender.com)

Downstream signalling of RIG-I and MDA5 results in activation of MAVS (Brisse and Ly 2019). It has been shown that the ATPase activity of MDA5 and RIG-I helicases differ in a length-dependent manner: RIG-I is efficient in recognizing short poly I:C molecules, while MDA5 is only activated by long stretches of poly I:C (the cut-off being around 300 bp) (Yoneyama et al. 2005; Kato et al. 2008). RIG-I is activated by dsRNA over 18 bp but requires extra motif such as 5′ triphosphate, among others (Hornung et al. 2006). For RNA which is not poly I:C, the motifs recognized by MDA5 are still not well defined (Hartmann 2017).

To avoid recognition of RIG-I, a synthetic cap analogue was introduced to the 5′ end of IVT mRNA. To date, there are three different capping systems that can be used in IVT mRNA: cap0 (m7G(5′)pppN1pN2p), cap1 (m7G(5′)pppN1mpNp) or cap2 (m7G(5′)pppN1mpN2mp). As depicted in Fig. 2, the main difference of these capping systems lies in the methylation status of the 2′ position of the 5′ penultimate and antepenultimate nucleoside (Zhong et al. 2018). Importantly, capping mRNA with a cap 0 structure reduced RIG-I activation while cap 1 or cap 2 completely abrogated RIG-I recognition of IVT mRNA and subsequent type IFN induction (Schuberth-Wagner et al. 2015).

Downstream of MAVS, IRF3 is phosphorylated which leads to subsequent dimerization and translocation to the nucleus where they induce type I IFN genes (Brisse and Ly 2019). While the RLRs mainly give rise to the activation of the type I IFN response, other cytoplasmic RNA sensors such as 2′-5′ oligoadenylate synthase (OAS) and protein kinase R (PKR) have a more direct effect. Once OAS is activated, it in turn stimulates RNase L, which will be responsible for the cleavage of RNA. PKR on the other hand will phosphorylate eukaryote initiation factor 2α (eIF2α), resulting in an abrogation of translation in the cell. PKR and OAS where shown to be activated by IVT mRNA, leading to not only the induction of type I IFNs but also, and more importantly, to the cleavage of mRNA and the abrogation of translation (Nallagatla and Bevilacqua 2008; Anderson et al. 2010, 2011).

For vaccination purposes, this type I IFN induction is highly beneficial, stimulating B- as well as T-cell responses (McNab et al. 2015). However, type I IFNs are also association with cell death induction and due to the antiviral state of immune cells, translation is abrogated, and mRNA is actively degraded by RNases. Luckily, a solution for this conundrum came from the field of gene therapy.

3 Type I IFNs: The Double-Edged Sword

Type I IFNs play a key role in initiating and sustaining a solid T- and B-cell-mediated immune response in the context of infection or cancer (McNab et al. 2015). Type I IFNs result in maturation of DCs (Santini et al. 2000; Breckpot et al. 2005), attraction of cytotoxic $CD8^+$ T cells to the tumour environment (Fuertes et al. 2011) and increased MHCI expression (Hofbauer et al. 2001), leading to enhanced antigen presentation towards $CD8^+$ T cells. Besides maturation, type I IFNs also stimulate DCs to promote isotype switching in B cells and enhance humoral immunity and

memory response (Le Bon et al. 2001). The mechanism might be related to the fact that type I IFNs stimulate follicular helper T cell (Tfh) differentiation which can subsequently aid in the differentiation of B cells during germinal centre formation in the lymph nodes (Ray et al. 2014). Therefore, the use of type I IFNs as an adjuvant or an immune stimulator was assessed in cancer research. For instance, systemic administration of type I IFN in breast cancer mouse models resulted in a decrease in tumour progression and metastasis to the bone and prolonged metastasis free survival via NK-cell anti-tumour function (Slaney et al. 2013; Rautela et al. 2015). In the context of melanoma, type I IFNs are used in the clinic after resection as an adjuvant therapy, to prevent relapse and formation of metastasis (Mocellin et al. 2013). Therefore, in theory, the type I IFNs, driven by the natural adjuvant properties of mRNA, should be at the basis of a strong immune response. Unfortunately, the reality is not black and white.

During investigations into the potential use of IVT mRNA as a gene therapy platform, one big problem occurred: the type I IFN activity not only reduced translation of the IVT mRNA but also gave rise to substantial cytotoxicity resulting in cell death (Andries et al. 2013). The reduced translation is the result of the type I IFNs binding to their receptor, a dimer of IFN-α/β receptor (IFNAR)1 and 2 (from here onwards called IFNAR). After signalling through the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway, the IFN-stimulated genes are transcribed, including PKR and OAS. Not only the presence of nucleic acids but also type I IFNs can induce cell death (both immunologically silent and proinflammatory), with as a primary goal limiting the propagation of (RNA) viruses (Bartok and Hartmann 2020). Therefore, a crucial element is to avoid elements in the IVT mRNA-resembling viral nucleic acids. In the context of sa-RNA, type I IFN responses inhibit the amplification of the RNA replicon resulting in a loss of the efficiency of this vaccine platform. It has been reported that sa-RNA elicits an inflammatory response within a few hours by the upregulation of IFN-stimulated genes. In the absence of type I IFN signalling, RNA vaccine potency was shown to be improved (Pepini et al. 2017). Undoubtedly, different strategies need to be designed to balance out this "ying and yang" effect.

3.1 Nucleoside Modifications

By studying naturally occurring forms of RNA, the presence of several nucleoside variants in various types of mammalian and bacterial RNA were identified. Firstly, it was discovered that monocyte-derived DCs treated with bacterial RNA produced high amounts of TNF-α. This high induction of TNF-α was not seen upon transfection with mammalian RNA except for mitochondrial RNA. The induction of TNF-α was inversely correlated to the extent of nucleoside modification found in the type of RNA. Thorough investigation led to the realization that specific modification of the RNA molecules (6-methyl adenosine (m^6A), pseudouridine (ψ) and 5-methylcytidine (m^5C) reduced the production of TNF-α, IFN-α and other cytokines,

presumably through a reduced activation of the endosomal TLRs (Karikó et al. 2005). Subsequently, it was shown that nucleoside modification in combination with adding a capping structure on the IVT mRNA significantly reduced type I IFN production through a RIG-I-dependent process (Hornung et al. 2006; Karikó et al. 2008). In addition, reduced activation of PKR was observed when IVT mRNA was generated with modified nucleosides, yet whether there was reduced binding to PKR or not has so far not been clearly established (Nallagatla and Bevilacqua 2008; Anderson et al. 2010). Moreover, IVT mRNA fully substituted by ψ did not only lead to a reduced activation of OAS1 but was also resistant to cleavage by RNase L (Anderson et al. 2011). Recently, it was confirmed that 1-methyl-pseudouridine (m1ψ)-containing RNA led to a reduced activation of TLR7/8 (Nelson et al. 2020). In conclusion, the use of modified nucleosides, in particular ψ or m1ψ, results in increased levels of translation of the IVT mRNA. The chemical modifications also significantly reduce the induction of type I IFNs (Table 1). However, basal immune activation can still be detected.

3.2 Techniques for dsRNA Removal

As mentioned earlier, phage polymerases are prone to errors, resulting in dsRNA contaminants in the IVT mRNA-generated mixture. By removing these contaminants with HPLC purification, pure IVT mRNA was obtained and no type I IFN induction was observed anymore in DCs after transfection (Karikó et al. 2011). However, this technique is expensive, and approximately 50% of the mRNA is lost during the process. Therefore, other strategies were developed to further "clean-up" the IVT mRNA.

At the level of T7 polymerase transcription, there are several ways to prevent the polymerase from making these errors: reducing $MgCl_2$ concentration in the reaction mixture (Mu et al. 2018), creating an optimal dNTP ratio (Nelson et al. 2020) or using a heat-stable T7 RNA polymerase (Wu et al. 2020). Adding modified nucleosides might also have an influence on the dsRNA content, however there is still some conflicting information depending on the detection method of the dsRNA and the purification method used (phenol:chloroform and native gel electrophoresis (Mu et al. 2018) versus oligo-dT purification and dsRNA ELISA (Nelson et al. 2020). At the level of the purification process itself, progress has also been made, as it was shown that passing the IVT mRNA over a cellulose column could filter out dsRNA with a 70 to 80% recovery of the mRNA (Baiersdörfer et al. 2019). As a result, lithium chloride or oligo-dT-enriched mRNA after dsRNA removal gave rise to higher translation efficiencies and is "immunosilent", meaning that type I IFNs were no longer produced in vitro or in vivo (Baiersdörfer et al. 2019; Nelson et al. 2020).

For sa-RNA, the removal of dsRNA is considered to be less helpful since dsRNA intermediates are produced continually during the self-amplification cycle. These dsRNA intermediates may cause a translational shutdown due to their recognition

Table 1 Overview of innate immune stimulation by IVT mRNA

	Mechanism of action	Solution to reduce activation	Remarks	References
ssRNA	TLR7		Polyuridine—viral ssRNA and IVT mRNA similar IFNα response Mouse ex vivo/in vitro	Diebold et al. (2004)
Adenosine, uridine, cytidine	TLR 3	m^6A s2U		Karikó et al. (2005)
	TLR 7 and 8	m^6A, m^5C, m^5U, s2U, ψ		
5′-triphosphate RNA	RIG-I	5′-cap structure s2U, ψ, 2′-O-methylated UTP	MDA5 does not recognize this 5′-triphosphate RNA	Hornung et al. (2006)
Uridine	Not RIG-I	ψ better than m^5C, m^5U and s2U		Karikó et al. (2008)
Uridine	PKR	Ψ, m^6A, m^5C, s2U, s4U, 2′-dU, I^5U abrogated PKR activation	No significant effect on binding	Nallagatla and Bevilacqua (2008)
Cytidine, uridine and adenosine	PKR	Ψ, m^6A and m^5C decreased PKR activation	Reduced binding to PKR	Anderson et al. (2010)
Uridine	OAS1 RNase L	Ψ	Less OAS1 activation Less efficiently cleaved by RNase L	Anderson et al. (2011)
dsRNA	MDA5	m^5C, ψ, m1ψ result in reduced dsRNA formation and MDA5 stimulation Reducing $MgCl_2$ in reaction T7	MDA5 forms filaments together with dsRNA	Mu et al. (2018)
Uridine	TLR7/8 signalling	m1ψ		Nelson et al. (2020)
dsRNA impurities	Cytoplasmic sensors RIG-I/MDA5	dsRNA reduction by custom NTP ratio	Signalling via MAVS	

TLR—toll-like receptor; IVT—in vitro transcribed; IFN—interferon; MDA5—melanoma differentiation-associated protein; RIG-I—retinoic acid-inducible gene I; PKR—protein kinase R; OAS—oligoadenylate cyclase; s2/4U—2/4- thio-uridine; m^5C—5-methylcytidine; m^5U—5-methyluridine; i^5U—5-iodineuridine; ψ—pseudouridine; UTP—uridine 5′-triphosphate; m1ψ—N1-methylpseudouridine; m^6A -6-methyladenosine; dU—deoxy uridine

by the cytoplasmic RNA sensors (Pepini et al. 2017). Different strategies based on the escape mechanism of different viruses have been explored to overcome this hurdle. One of the most appealing strategies to dampen the type I IFN and to escape innate sensing is the use of innate inhibiting proteins (IIPs). For instance, the vaccinia virus IIPs E3, K3 and B18 each play a specific role in counteracting the host's antiviral response. E3 and K3 inhibit PKR and B18 disrupts the type I IFN signalling pathway by acting as a decoy receptor and thus preventing the interaction between extracellular IFN and IFNAR. As for influenza proteins, the non-structural protein 1 of the influenza A virus shows a multifunctional role as it inhibits the immune related proteins PKR, OAS, IRF3 and NF-κβ. Co-transfection of these three IIPs improved substantially the translational capacity of sa-RNA compared to the transfection of sa-RNA only (Beissert et al. 2017). As this strategy requires the administration of two different mRNA formulations (one encoding the protein of interest and one encoding the IIPs), the co-localization of both mRNAs in the same cell is an important prerequisite to overcome innate immune sensing. However, this cannot be guaranteed. More recently, a proof-of-concept study reported the use of a sa-RNA construct encoding regions for both the protein of interest and the IIPs. In this study, a plethora of IIPs derived from different viruses were screened in vitro, based on their targets in the type I IFN pathway and on their effect on protein expression and immunogenicity. One of the most promising IIPs that was identified from the screening was the accessory protein ORF 4a of MERS-CoV and the V protein of parainfluenza type 5, which showed superiority in different human cell lines. It has been reported that the ORF 4a protein of MERS-CoV has the highest potential to counteract innate immune sensing, as it is able to inhibit IFN production and IFN stimulated response element (ISRE) promoter element signalling pathways. PIV-5 on the other hand binds to MDA5 directly and inhibits its activity. These proteins could therefore dampen the IFN production (Blakney et al. 2020). Besides the use of viral IIPs, many other non-viral molecules have also been explored for their potential to reduce antiviral responses and have also been elaborately reviewed (Minnaert et al. 2021). One of the most recent attempts to quench the type I IFN response is the use of corticosteroids, a class of anti-inflammatory drugs, in combination with IIPs and cellulose-based mRNA purification. Among the corticosteroids, it was shown that clobetasol propionate, especially when applied topically, enhanced the translation of sa-RNA against Zika virus upon intradermal electroporation and reduced type I IFN responses. Although this approach might be beneficial in the context of gene therapy, it should be avoided in vaccination context as clobetasol propionate prevents the formation of antibodies against sa-RNA encoded antigens (Zhong et al. 2021).

Undoubtedly, immunosilent mRNA is of great importance in the context of gene therapy. However, for vaccination against cancer or infectious diseases, well-balanced amounts of type I IFNs could contribute to improving the vaccine. To investigate this, the role of IFNAR was assessed.

3.3 Role of Type I IFN Receptor

As stated before, systemic type I IFN activation by PRRs facilitates the adaptive immune response and induces DC activation. However, type I IFN activation is also associated with reduced translation of the IVT mRNA. To study this delicate balance in more detail, IFNAR knock-out mouse models were used in mRNA vaccination studies. After intravenous injection of mRNA-lipoplexes encoding various tumour antigens, the IFN-α induced was shown to be critical for an efficient $CD8^+$ T-cell-mediated anti-tumour response (Kranz et al. 2016). Similar results were obtained and noticed that even though translation improved in IFNAR knock-out mice, antigen-specific lysis by CTLs was decreased in these knock-out mice (Broos et al. 2016). To further complicate matters, conflicting observations were made when mRNA was injected via different routes, i.e. intradermally and subcutaneously, not only did translation improve in IFNAR knock-out mice, the antigen-specific CTL-mediated response was also higher (Pollard et al. 2013; De Beuckelaer et al. 2016; Udhayakumar et al. 2017). For sa-RNA vaccines, the limited data available shows that the type I IFN response also impeded the subsequent immune response upon intradermal electroporation (Zhong et al. 2019).

It was suggested that the discrepancy in these results might be due to differences in timing, rather than dosing. It was shown that after intravenous injection, mRNA is immediately translated and presented in the spleen by plasmacytoid DCs to T cells. The plasmacytoid DCs simultaneously produce type I IFNs, providing the "second signal", needed for efficient proliferation and activation of T cells. On the contrary, after intradermal or subcutaneous injection the translation lags behind and the DCs have to migrate from the skin to the lymph nodes to stimulate the T cells. By this time, the DCs are already producing type I IFNs, leading to the "second signal" without a first signal, resulting in T-cell apoptosis (De Beuckelaer et al. 2017). However, this hypothesis does not take into account that the type I IFNs will likely dilute systemically and may not be present at such high levels in the lymph nodes. On the other hand, the hypothesis is partly supported by the fact that the expression of IFNAR is primarily important on $CD4^+$ T cells and not $CD11c^+$ DCs to support the CTL response (Van Hoecke et al. 2020). In summary, two solutions remain to counter these effects: either decreasing the type I IFN response or speeding up the translation in the lymph nodes. It was shown that modifying the IVT mRNA but not removing the dsRNA contaminants partly reduces the type I IFN response (Nelson et al. 2020). In addition, by packing the mRNA in nanoparticles smaller than 200 nm, the bulk part was shown to migrate to the lymph nodes after injection, leading to fast and local translation (Manolova et al. 2008). For self-replicating mRNA, it has also been shown that packaging in LNPs (<100 nm) leads to expression in the lymph nodes (Huysmans et al. 2019). However, the impact on the immune reaction elicited has so far not been investigated.

When looking to the COVID-19 mRNA vaccines, there is a large difference in the efficacy of on the one hand the CureVac vaccine (CVnCoV) and on the other hand the vaccines produced by Moderna (mRNA-1273, Spikevax) and BioNTech

(BNT162b2, COMIRNATY). CVnCoV does not use modified nucleosides and therefore presumably dose reduction is required to avoid cytotoxicity due to excessive type I IFN induction, resulting in lower efficacy (Kremsner et al. 2021). mRNA-1273 and BNT162b2 use IVT mRNA fully substituted by m1ψ and obtain very high efficacies after a second dose (Baden et al. 2021; Sahin et al. 2021). While Moderna uses a purification technique of the mRNA via oligo-dT capture (Corbett et al. 2020) and dsRNA removal by cellulose chromatography (Baiersdörfer et al. 2019; Laczkó et al. 2020), BioNTech remains vague in its description and uses magnetic particle purification (Vogel et al. 2021) in addition to an undisclosed method of dsRNA removal (EMA 2021). Complete removal of dsRNA accordingly would imply that the resulting mRNA is immunologically silent, which is in contradiction to the result obtained by the clinical trials (Baden et al. 2021; Sahin et al. 2021). However, the mRNA encoding the full-length SARS-CoV-2 spike protein is over 3 kb long, meaning that there is plenty of space for the formation of secondary structures which might still be able to trigger both endosomal and cytoplasmic sensors.

4 mRNA Delivery Systems

In order to address the elephant in the room, we have to answer the following question: if the COVID-19 vaccines use immunosilent mRNA, why are they so immunogenic? The answer could perhaps in part be found in the nanoparticles used to package the mRNA. However, we only begin to understand the mechanisms behind the immune activation capacity of nanoparticles in general and lipid-based nanoparticles in particular. The current state of the art about how different intracellular pathways are activated by cationic liposomes was summarized, and they conclude that empty cationic liposomes are able to activate MAPK and result in NF-κB-dependent and -independent release of inflammatory cytokines, chemokines and co-stimulatory molecules in vitro. In addition, generation of ROS and Ca^{2+} influx also contribute to this mechanism as well as the activation of apoptotic cascade induction and inflammasome activation, leading to release of IL-1β (Lonez et al. 2012). Subsequent research with cationic lipopolyamines confirms activation of MAPK, NF-κB and NLRP3, giving rise to TNF-α, IL-6 and IL-1β in in vitro models (Li et al. 2018; Zhang et al. 2021). In most of these studies, type I IFNs were not assessed, but their induction cannot be excluded because a mild induction of type I IFNs exist for both liposomes and liposome-formulated mRNA. However, the trigger for all these inflammatory pathways still depends on the nature of the liposome including characteristics such as size, charge and composition. In addition, in these studies only one-component cationic liposomes are assessed, while in reality, the field has shifted from the cationic nanoparticles, which are often associated with systemic and cellular toxicity (Kedmi et al. 2010; Rietwyk and Peer 2017), to neutral nanoparticles by using ionizable lipids. These lipids have a neutral charge at physiological pH, reducing cell death, but become positively charged when the pH decreases (e.g. in the endo-lysosomal environment), ensuring endosomal escape of the mRNA cargo. Moreover, LNPs are

not made up of one single component but used in combination with helper lipids, PEGylated lipids and cholesterol. Only a limited amount of data is available on the immune stimulatory capacity of these lipid nanoparticles, without the presence of mRNA.

While for gene therapy purposes, LNPs were made less and less immunogenic; it was sought to identify LNPs which are safe yet provide a potent stimulus for the immune system. After screening a large number ($n = 1080$) of cationic ionizable lipids, they found that lipids containing cyclic amino head groups stimulate the stimulator of IFN genes (STING) pathway (Miao et al. 2019). In this way, type I IFNs are induced even when using modified mRNA. Another approach is adding well-defined adjuvants to the LNPs. For example, addition of monophosphoryl lipid A (MPLA) reversed the effect of immunosilent mRNA and induced higher levels of IL-6, IFN-γ and monocyte chemoattractant protein (MCP)-1/CCL-2 after 12 h compared to the LNPs with unmodified mRNA or LNPs with modified mRNA without MPLA (Verbeke et al. 2017). The combination of lipid nanoparticles with α-galactosylceramide packaging (polymer-)mRNA on the other hand led to the engagement of invariant NKT cells (Guevara et al. 2019; Verbeke et al. 2019). Improved invariant NKT cell engagement led to increased secretion of IFN-γ, IL-4, IL-12p70, IL-6 and TNF-α (Verbeke et al. 2019) and thus improved subsequent cellular immunity.

Specifically for the COVID-19 vaccines, previous work with similar formulations for other infectious diseases mainly showed the induction of Tfh and subsequent germinal centre B-cell responses (Pardi et al. 2018, 2019; Laczkó et al. 2020). However, recent work illustrates the inflammatory potential of the LNPs used in the vaccines against SARS-CoV-2. After intradermal injection in C57BL/6 mice, gene analysis in skin explants showed activation of RIG-I, NOD-like receptor and TLR signalling resulting in production of inflammatory cytokines and chemokines among which IL-1β, IL-6 and IFN-γ induced protein (IP)-10/CXCL-10 (Ndeupen et al. 2021). Even though the empty LNPs were administered via the skin and not via the muscle, it still shows the inflammatory potential of the COVID-19 vaccines. However, it still needs to be explored in more detail what type of inflammatory reaction, activating which components of the immune system, is associated with the most robust adaptive immune response.

In summary, although research into the immune stimulatory capacity of nanoparticles is incomplete, there are several indications that they might have a bigger influence than previously thought. Nevertheless, there is still insufficient information available about the immune response elicited by the different components of the LNPs themselves.

5 Conclusion

For the safe and reliable production of mRNA vaccines, it is advisable to purify the IVT and test for the absence of dsRNA contaminants (as is done for BNT162b2 and

mRNA-1273). In addition, the use of modified nucleosides for RNA synthesis has now been established as superior to the use of unmodified mRNA. Still, even when using modified, purified, dsRNA-free mRNA, secondary structures within the mRNA could still trigger the innate immune system. Especially for long IVT mRNA strands, e.g. the spike protein of SARS-CoV-2, base-pairing leads to loop formation and results in the formation of a complex secondary structure. The dsRNA components within this secondary structure or other motifs could still trigger innate immune sensors such as RLRs. Although this has not been discussed in depth in this chapter, many other RNA helicases (especially members of the DEAD/DExH-box helicase family, e.g. DDX1 or DHX9, DHX15) have been discovered and we have only started to scratch the surface with regard to their involvement in innate immune reactions. For future mRNA vaccine design, we therefore need to pinpoint which sequences or secondary structures within the IVT mRNA are related to an efficient adaptive immune response and which receptors play a role in their recognition. A more targeted modulation of either of these components should lead to more potent vaccines.

In the future, it will be interesting to develop sa-RNA, as they allow for longer antigen translation and are already effective at lower dosages. This means that with the same amount of sa-RNA, more vaccines can be made than for IVT mRNA. In addition, sa-RNA intrinsically represents a long IVT mRNA strand due to the incorporation of non-structural proteins and other sequences. Furthermore, during the replication cycle loop formation occurs, resulting in dsRNA fragments, which leads to type I IFN induction. When purified, sa-RNAs are useful tools in IVT mRNA vaccination. Interestingly, modern in silico techniques enable the design of potent sa-RNA vectors yielding both high translation and immunity by balancing type I IFN effects. For sa-RNA, self-adjuvanting activity is generally considered beneficial, but side-by-side comparisons between modified and non-modified sa-RNA are still lacking. Moreover, the clinical trials that have been performed so far, used unmodified nucleosides. The use of corticosteroids, especially when topically administered, has been suggested to improve translation efficiency for sa-RNA. However, this approach is less applicable in the context of vaccination as it completely abrogates both cellular and humoral responses (Zhong et al. 2021; Minnaert et al. 2021). Nevertheless, progress has been made in the field of IIPs, showing their potential to enhance the translation efficiency of sa-RNAs.

Not only the mRNA but also the LNP has the capacity to stimulate the immune system, as the COVID-19 vaccines have showed us that the LNPs alone can be inflammatory. However, the mechanism leading to the adaptive immune response and the correlation with the inflammatory response, still has to be determined. Yet, it is possible to determine the mechanism underlying the induction of an efficient adaptive immune response and nanoparticles could be designed to stimulate particularly $CD8^+$ T cells or B cells. In order to move forward, it is very important to take a systematic approach, assessing every pathway that might be induced after vaccination, from known molecules such as MDA5 and RIG-I to less well-known molecules such as for instance non-RLR RNA helicases. Research should also not stop once a product is on the market, since often not every aspect of the mechanism behind the adjuvant activity has been fully elucidated, especially when taking into account the rapid approval of

COVID-19 mRNA vaccines. By starting to understand how today's mRNA vaccines stimulate the immune system, we can design a new generation of even better vaccines for the future.

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Advances in mRNA Delivery and Clinical Applications

Bo Hu, Abid Hussain, Qing Liu, Yuhua Weng, and Yuanyu Huang

Contents

Abstract Messenger RNA (mRNA) as a naturally occurring molecule is involved in the transmission of genetic information. It has shown great potential in field of vaccination, protein-replacement therapy, and treatment of genetic diseases. Compared with traditional therapeutic modalities, mRNA has various advantages such as safe expression pattern and rapid deployment ability and can be used for personalized treatment. However, during the last couple of decades, the mRNA vaccines were thought be difficult to develop due to many technical obstacles. Recently, due to the development in RNA modification, in vitro transcription (IVT), and, especially, delivery technology, the therapeutic mRNA got immense popularity. In this chapter, we described the latest advances in mRNA delivery systems as well as tried to figure

B. Hu · A. Hussain · Q. Liu · Y. Weng · Y. Huang (✉)
School of Life Science; Advanced Research Institute of Multidisciplinary Science; School of Medical Technology (Institute of Engineering Medicine); Key Laboratory of Molecular Medicine and Biotherapy; Key Laboratory of Medical Molecule Science and Pharmaceutics Engineering, Beijing Institute of Technology, Beijing 100081, China
e-mail: yyhuang@bit.edu.cn

Q. Liu
e-mail: 7520190063@bit.edu.cn

Y. Huang
School of Materials and the Environment, Beijing Institute of Technology, Zhuhai 519085, China

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_13

out the development trend of delivery platforms and also summarized the applications of mRNA therapeutics.

Keywords mRNA delivery · mRNA vaccine · mRNA therapeutics · Lipid nanoparticle · Polymer · Lipid–polymer hybrid

1 Introduction

Messenger RNA (mRNA) is a single-stranded RNA that is transcribed from DNA as a template and carries the genetic information to guide protein synthesis. The mRNA was first discovered by Brenner (1961). The mRNA-based therapy shares some important advantages including low risk of insertion mutations, transient production of the encoding protein, and reduced delivery barriers (Sahin et al. 2014). Having such advantages, the mRNA holds a great potential in vaccination, protein replace therapy, and genetic disease treatment. However, due to the complicated preparation and structure, it was hard to use mRNA as therapeutic unit (Fig. 1). In addition, with problems such as low stability, immunogenicity, and inability to enter cells, mRNA was not the leading candidate for drug development in last couple of decades. In recent years, advanced nucleoside chemical modification and new delivery strategies have largely overcome these shortcomings (Weng et al. 2020). Structurally, adding cap structure (5'-cap) and polyadenylic acid tail (3'-poly(A)) to the ends of open reading frame (ORF) of in vitro transcription (IVT) mRNA improves the resistance

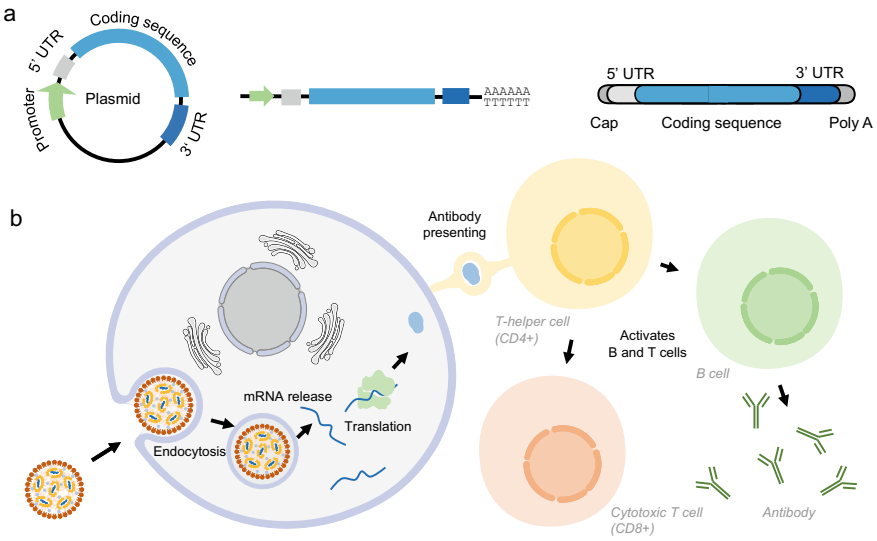

Fig. 1 mRNA working flow. **a** Synthesis of IVT mRNA from a DNA template. **b** Working mechanism of therapeutic mRNA, represented by mRNA vaccine

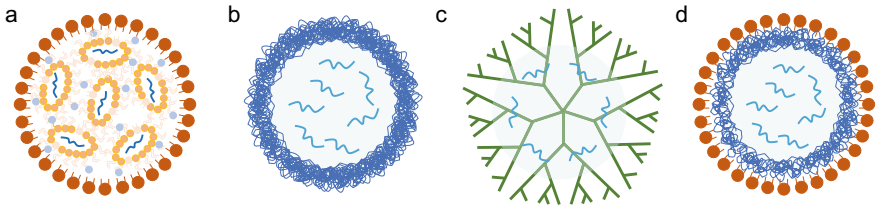

Fig. 2 Representative mRNA delivery systems. **a** Lipid nanoparticle, **b** polymer, **c** dendrimer, **d** lipopolyplex

of enzymatic degradation. Introducing 5' untranslated regions (UTR) and 3' UTR also contribute to mRNA stability. In addition, it was reported that UTRs increase in vitro transcriptional efficiency and regulates mRNA expression in certain cells (Jain et al. 2018). Incorporation of unnatural bases in the sequence not only further increases the stability of mRNA but also reduces the immunogenicity of mRNA (Kariko et al. 2005). Replacing the rare codon to synonymous frequently occurring codons in the sequence can result in order-of-magnitude change in translation efficiency (Presnyak et al. 2015; Thess et al. 2015).

So far, mRNA has demonstrated efficient transcription of functional proteins if they can enter into cells. Unfortunately, due to the large size, highly negative charge, and vulnerability to nuclease, mRNA cannot cross cell membrane by itself. Thus, it is necessary to use vehicle to deliver complete mRNA into target cells for its proper function. There are ample of attempts made to deliver mRNA by viral vectors; however, these studies did not go further due to their immunogenicity, low mRNA loading efficiency, difficulty to scale-up, and the risk of integrating encapsulated gene into genome. Meanwhile, numerous non-viral nucleic acid delivery systems have also been developed for gene therapy, such as lipid or lipidoid nanoparticles (LNPs) (Fig. 2), (Jayaraman et al. 2012; Sato et al. 2012; Semple et al. 2010; Whitehead et al. 2014) polymers (Fig. 2) (Rozema et al. 2007; Zhou et al. 2016a, b; Zorde Khvalevsky et al. 2013) or dendrimers (Fig. 2), (Ellert-Miklaszewska et al. 2019; Zhou et al. 2016a, b) exosomes, (Kamerkar et al. 2017) peptide, (Huang et al. 2016, 2015; Kim et al. 2010; Kumar et al. 2007) biomimetic nanoplexes, (Qiu et al. 2019) DNA carriers, (Li et al. 2019; Liu et al. 2019) and inorganic nanocarriers (Lin et al. 2013). Unfortunately, most of the aforementioned vehicles are not suitable for mRNA delivery. The unstable mRNA requires vectors to encapsulate it well to avoid the attack of nucleases, but the significant larger size of mRNA (orders of magnitude larger than siRNA or ASO) is making it difficult for the carriers to encapsulate mRNA. In addition, the mRNA loading efficiency and delivery efficiency also limit the use of some delivery platforms. Therefore, the most common mRNA delivery carriers are lipid nanoparticles (LNPs), polymers or dendrimers, and lipid–polymer hybrid nanoparticles. In addition, immune-active dendritic cells (DCs) are often used as mRNA carriers because therapeutic mRNA has a great potential as vaccine. Therefore, these delivery systems will be introduced in detail in following discussion.

2 Lipid Nanoparticles

Owing to the advantages such as robust delivery efficiency, high mRNA encapsulation, and low immunogenicity, LNPs are a class of most well-researched non-viral mRNA delivery platform. LNPs can be divided into cationic LNPs (cLNPs) and ionizable LNPs (iLNPs). The former keeps their cationic characters in pH independent manner. The latter shows no charge under neutral conditions while became positively charged when pH is lower than their pKa. Such features of iLNPs improve their safety profile by reducing non-specific binding with proteins in blood circulation while without delivery efficiency compromise. The components of LNPs basically include key lipid, phospholipid, cholesterol, and PEG-lipid. Phospholipid supports the lipid bilayer structure of LNP, introducing cholesterol will increase the stability of LNPs, and PEG can extend the circulation while decrease the toxicity of LNPs (Kauffman et al. 2016; Pardi et al. 2018). Changing the contents of each component will affect the binding with mRNA and delivery efficiency. It was reported that higher PEG density reduced in vivo immune-stimulation but also hindered efficacy of LNPs; (Kumar et al. 2014) the research suggested that this may be on account of the formation of steric barrier between LNPs and proteins including cytokine or chemokine and apolipoprotein E (ApoE). The ratio between 1,2-distearoylsn-glycero-3-phosphocholine (DSPC, a kind of phospholipid) and cholesterol decides the morphology of LNP, and the morphology of LNP further impacts nucleic acid encapsulation and delivery (Kulkarni et al. 2019). Besides, it has been discovered that the balance of cationic lipids and zwitterionic phospholipids is ideal for association with mRNA solvated with water molecules and salt ions (Leung et al. 2012).

Early works have focused on the development of cationic lipids, such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and 1,2-dioleoyloxy-3-trimethylammonium propane chloride (DOTAP), but recently, major researches prefer the use of ionizable lipids, because of their low toxicity and higher efficiency. Arbutus Biopharma and Alnylam Pharmaceuticals have developed a series of ionizable lipids represented by 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA) and (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA, MC3) (Fig. 3). Notably, ONPATTRO™ (patisiran), a siRNA drug used DLin-MC3-DMA as the key lipid of LNP, has been successfully marketed. Subsequently, many clinical tests confirmed that MC3-LNP could be used for mRNA delivery (Feldman et al. 2019; Pardi et al. 2017a). Besides, the C12-200-based (Love et al. 2010) and cKK-E12-based (Dong et al. 2014) LNPs (Fig. 3) were synthesized and employed for mRNA delivery (Dong et al. 2014; Kauffman et al. 2015).

To date, three representative mRNA pharmaceuticals use LNPs as delivery systems in their product pipelines. Moderna Inc. developed Lipid 5 (Fig. 3), SM-102 (Lipid H) (Fig. 3) as the key lipids of LNPs, and BioNTech and CureVac AG have partnered with Acuitas Therapeutics to develop multiple mRNA drugs using the ALC-0315 (Fig. 3) as key lipid of LNP platform. By modifying MC3, Moderna obtained a new lipid named Lipid 5, and they used ethanolamine as the

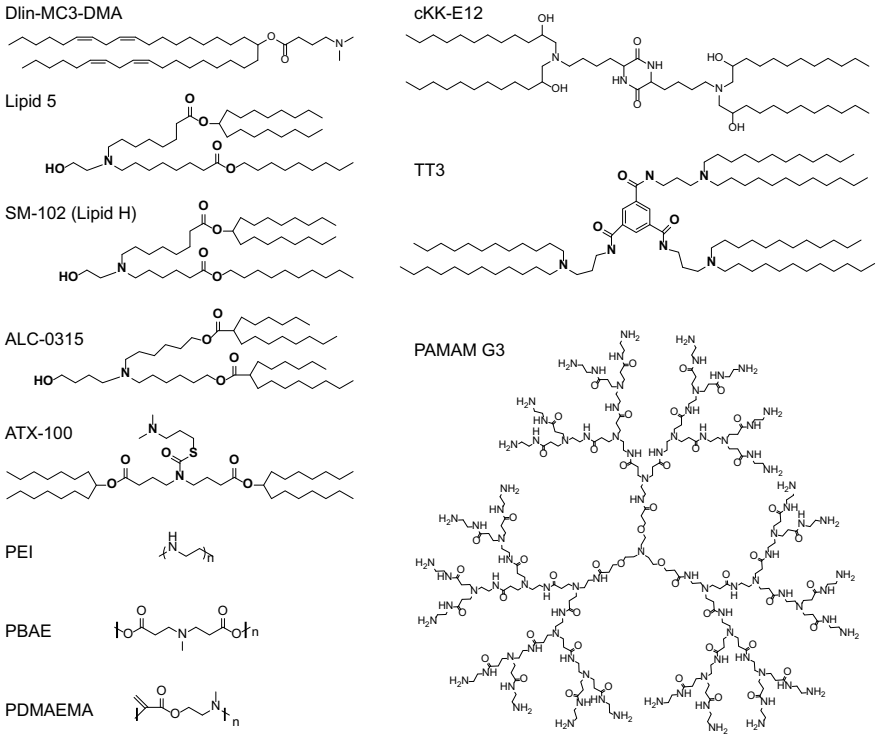

Fig. 3 Representative chemical structures of lipids, polymers, and dendrimer developed for mRNA delivery

hydrophilic head and introduced a biodegradable ester bond in each hydrophobic tail to accelerate its tissue clearance. During pre-clinical studies, Lipid 5 successfully delivered mRNA to non-human primate (NHP) and mediated the expression of luciferase and EPO in liver (Sabnis et al. 2018). Compared with MC3, Lipid 5 significantly improved the ability of tissue clearance, leading to significantly minor side effects and higher ability of dosing repeatedly with safety. Moreover, Moderna further refined Lipid 5 and thus obtained SM-102 for vaccine injection by changing the ester bond position and carbon chain length in the hydrophobic tail. Compared with the Lipid 5, the pKa of SM-102 is slightly higher from 6.56 to 6.75. It is believed that this change might be beneficial for intramuscular and intravenous administration (Buschmann et al. 2021). Based on this platform, Moderna launched project mRNA-1273, which is aiming to prevent COVID-19 by delivering mRNA coding for neutralizing antibody of SARS-CoV-2. Similarly, inspired by MC3, ALC-0315 (Fig. 3) also employed butanol ammonium as hydrophobic head and introduced four branched tails containing ester bonds to replace the original linoleic acid tail, and the branched structure makes the lipid form more obvious cone-shaped morphology when interacting with the membrane of endosomes or lysosomes. BioNTech and

CureVac have developed several projects represented as BNT162b2 and CVnCoV, respectively, and both these projects use the same delivery platform as explained above. Arcturus Therapeutics reported the successful replacement of human recombinant factor IX protein with mRNA in a mouse model of hemophilia B by using LNP delivery platform termed as lipid-enabled and unlocked nucleic acid-modified RNA (LUNAR) (Ramaswamy et al. 2017). LUNAR is composed of cholesterol, DSPC, PEG-lipid, and the key lipid ATX-100 (Fig. 3), which contains an ionizable amino head group and a biodegradable lipid backbone. Interestingly, after the injection of 0.3 mg/kg siFVIL@LUNAR to mice, a 97% reduction of expression of target protein was observed. Further evaluations proved that LUNAR is five times more potent than MC3 (Table 1).

Clinical drug developments have extremely high requirements on the safety and immunogenicity of final formulation; therefore, pharmaceutical companies are taking much care in the application of new platforms. On the contrary, many fundamental researches are imaginative. Inspired by the natural phospholipids in biological membranes, a library was designed containing 28 ionizable amine heads and 16 alkylated dioxaphospholane oxides (Liu et al. 2021). According to this library, 572 ionizable phospholipids for mRNA delivery were designed and screened. Another library containing eight polyamine as hydrophilic heads and three hydrocarbon chains with different lengths was also established. The resulting 24 novel ionizable lipids were synthesized and screened for luciferase mRNA delivery to Jurkat cells (Billingsley et al. 2020). The key lipid of top-performing formulation, C14-4, contains five ionizable tertiary amines and five C14 tails. Consequently, C14-4 LNP was selected for CAR mRNA delivery to primary human T cells. This work demonstrated that the ability of LNP to deliver mRNA to primary human T cells and showed the potential of LNPs to enhance mRNA-based CAR-T cell engineering methods. Similarly, fourteen novel lipid structures were designed and synthesized for siRNA delivery (Ramishetti et al. 2020). In the study, piperazine head groups with two ionizable amine groups or tertiary amine with single ionizable amine were selected as hydrophilic head and linoleic fatty acid or branched lipid chain as hydrophobic tails. In another separated study, four lipids from aforementioned study and one new structure based on same strategy for LNPs preparation were selected (Elia et al. 2021). All LNPs mediated at least 72 h luciferase expression via intramuscular and subcutaneous and intradermal injection. Furthermore, LNPs #2 and #14 based on lipid 2 and 14 elicited high anti-luciferase IgG expression, which paves the way for using these LNPs as mRNA vaccine delivery. There are also many new LNPs based on novel lipid structures which were reported for mRNA delivery, including iLP171, (Yang et al. 2020a) iLP181 (Li et al. 2021), 306Oi10 (Ball et al. 2018), and so forth. They will not be discussed in detail in this chapter.

In addition to developing novel lipid nanoparticle delivery systems, many researchers have focused on existing delivery system optimization by modification or adding new components to improve the properties of LNP delivery systems. The effects on mRNA vaccine delivery by changing the composition of LNP and the

Table 1 Representative active clinical mRNA therapeutics

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
Cancer vaccines	NA	Mesothelioma	MT1	Antwerp University Hospital	Dendritic cells	Intradermal	NCT02649829	I/II
	NA	Acute myelocytic leukemia	WT1	Antwerp University Hospital	Dendritic cells	Intradermal	NCT01686334	I/II
	NA	Glioblastoma	WT1	Antwerp University Hospital	Dendritic cells	NA	NCT02649582	I/II
	BNT111	Melanoma	NY-ESO-1, tyrosinase	BioNTech	Lipoplexes	Intravenous	NCT01684241 NCT02410733	I
	BNT112	Prostate cancer	NA	BioNTech	Lipoplexes	Intravenous Infusion	NCT04382898	I/II
	BNT113	HPV16, head and neck cancer	HPV16-derived E6 and E7	BioNTech	Lipoplexes	Intravenous Infusion	NCT04534205	II
	BNT122	Locally advanced or metastatic solid tumors	Neoantigen	BioNTech	Lipoplexes	Intravenous	NCT04813627 NCT04486378	II
	CV8102	Cutaneous melanoma, adenoid cystic carcinoma, squamous cell cancer of skin, head and neck	TLR7/8/RIG-1 agonist	CureVac	NA	Intratumor	NCT03291002	I

(continued)

Table 1 (continued)

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
	NA	Glioblastoma multiforme	pp65-LAMP	Duke University	Dendritic cells	Intradermal	NCT00639639	I
	NA	Glioblastoma	pp65-LAMP	Duke University	Dendritic cells	Intradermal	NCT02366728	II
	mRNA-5671	Colorectal cancer, pancreatic cancer	KRAS	Merck Sharp and Dohme Corp	LNP	Intramuscular	NCT03948763	I
	NA	Melanoma	Tyrosinase-related peptide 2	Memorial Sloan Kettering Cancer Center	Langerhans cells	Intradermal	NCT01456104	I
	NA	Myeloma	CT7, MAGE-A3, and WT1	Memorial Sloan Kettering Cancer Center	lanGerhans cells	Subcutaneous	NCT01995708	I
	mRNA-4157	Solid tumors	Neoantigens	Moderna and Merck Sharp and Dohme Corp	LNP	Intravenous infusion	NCT03313778 NCT03897881	II
	NA	Prostate cancer	hTERT and Survivin	Oslo University Hospital	Dendritic cells	NA	NCT01197625	I/II

(continued)

Table 1 (continued)

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
	NA	Melanoma	NA	University Hospital Erlangen	Dendritic cells	Intravenous	NCT01983748	III
	NA	Acute myelocytic leukemia, myelodysplastic syndromes	WT1	University of Campinas	Dendritic cells	NA	NCT03083054	I/II
	NA	Glioblastoma, malignant glioma	pp65-shLAMP	University of Florida	Dendritic cells	Subcutaneous	NCT02465268	II
Infectious disease vaccines	BNT162b2	COVID-19	SARS-CoV-2 spike protein	BioNTech, Pfizer, Fosun Pharma	LNP	Intramuscular	NCT04887948 NCT04816669 NCT04955626	Commercial
	CVnCoV	COVID-19	SARS-CoV-2 spike protein	CureVac	LNP	Intramuscular	NCT04860258 NCT04838847 NCT04652102	III
	CV7202	Rabies	Rabies virus glycoprotein	CureVac	LNP	Intramuscular	NCT03713086	I
	mRNA-1273	COVID-19	SARS-CoV-2 spike protein	Moderna	LNP	Intramuscular	NCT04860297 NCT04470427 NCT04649151	Commercial
	mRNA-1647	Cytomegalovirus	UL128, UL130, UL131, glycoproteins (gL, gH, gB)	Moderna	LNP	Intramuscular	NCT03382405 NCT04975893 NCT04232280	II

(continued)

Table 1 (continued)

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
	mRNA-1893	Zika	NA	Moderna	LNP	NA	NCT04064905 NCT04917861	II
	mRNA-1010	Influenza	NA	Moderna	LNP	Intramuscular	NCT04956575	I/II
	mRNA-1345	Respiratory syncytial virus	Prefusion F glycoprotein	Moderna	LNP	NA	NCT04528719	I
	mRNA-1653	Human metapneumovirus and parainfluenza virus 3	F protein	Moderna	LNP	Intramuscular	NCT04144348 NCT03392389	I
	mRNA-1644	HIV	NA	Moderna	LNP	Intramuscular	NCT05001373	I
Protein-replacement therapy	AZD8601	Myocardial ischemia	VEGF-A	AstraZeneca	LNP	Intracardiac	NCT03370887	II
	MEDI1191	Solid tumors	IL-12	MedImmune LLC	LNP	Intratumor	NCT03946800	I
	mRNA-2752	Solid tumors, lymphoma	OX40L, IL-23, IL-36γ	Moderna and AstraZeneca	LNP	Intratumor	NCT03739931 NCT02872025	I
	mRNA-2461	Solid tumor, lymphoma	OX40L	Moderna	LNP	Intratumor	NCT03323398	I
	BNT131	Solid tumors	IL-12sc, IL-15sushi, IFNα, and GM-CSF	Sanofi	NA	Intratumor	NCT03871348	I

(continued)

Table 1 (continued)

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
	MRT5005	Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator	Translate Bio	LNP	Inhalation	NCT03375047	I/II
Gene editing	Anti-mesothelin CAR-T cells	Solid tumor	PD-1 and TCR gene-knocked out	Chinese PLA General Hospital	CAR-T cells	Intravenous infusion	NCT03545815	I
	Universal dual specificity CD19 and CD20 or CD22 CAR-T cells	B cell leukemia and lymphoma	CD19 and CD20	Chinese PLA General Hospital	Dual specificity CAR-T cells	Intravenous	NCT03398967	I/II
	UCART019	B cell leukemia and lymphoma	CD19	Chinese PLA General Hospital	CAR-T cells	Intravenous	NCT03166878	I/II
	CTX001	β-thalassemia	BCL11A	CRISPR Vertex	Modified CD34 + hHSPCs	NA	NCT04208529 NCT03745287 NCT03655678	I/II
	SB-728mR-HSPC	HIV	CCR5	Sangamo	CD34 + hHSPCs	Intravenous infusion	NCT02500849	I

(continued)

Table 1 (continued)

Application	Therapeutic name	Indication(s)	mRNA coding	Sponsor	Delivery system	Administration route	NCT ID	Phase
	NA	Epstein-Barr virus-associated Malignancies	PD-1	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	Cytotoxic T lymphocytes	NA	NCT03044743	I/II

NA not applicable; *LNP* lipid nanoparticle; *CAR* chimeric antigen receptor

ratio of each component were explored. It was revealed that the optimized formulation led to about three times higher CD8 + T cell increase than original formulation. Further investigations showed lower levels of CKK-E12 resulted in higher T cell level and no clear correlations between DOPE, cholesterol, and the T cells. Some researchers have focused on cholesterol which is a major constituent within LNPs. Evidences showed that oxidized cholesterols altered LNP biodistribution. Based on this, nine cholesterol variants were synthesized and formulate them into CKK-E12 LNP (Paunovska et al. 2019). It was observed that the oxidized cholesterols helped LNPs deliver mRNA to liver environmental cells like hepatic endothelial cells and Kupffer cells at 0.05 mg kg^{-1}. Besides, three functional groups were conjugated with cholesterol, termed Chol-PEG400-selfpeptide (Chol-PEG400-SP), Chol-PEG400-Mannose (Chol-PEG400-Man), and Chol-PEG2000-W5R4K (Chol-PEG2000-WRK) (DeRosa et al. 2016). These functional cholesterols were then mixed into G0-C14 LNP. By utilizing concept of central composite design (CCD), the optimized formulation, O-LLN, was obtained with 6.29% Chol-PEG400-Man, 4.37% Chol-PEG400-SP, and 3.38% Chol-PEG2000-WRK, which can synergistically prolong systemic circulation, as well as increase liver targeting and hepatocyte uptake.

A strategy termed as selective organ targeting (SORT) was reported where lung or spleen targeted delivery was achieved by adding cationic lipid or anionic lipids into established LNP systems (Cheng et al. 2020). The clinical approved leukotriene-antagonist, MK-571, was discovered that it could enhance intracellular mRNA delivery (Patel et al. 2017). The MK-571 containing LNP (LNP-MK-571) resulted in 200% higher transfection efficiency in vitro and over twofold target protein expression in vivo. Unfortunately, this study did not explore the mechanism by which MK-571 mediates more efficient mRNA delivery. Besides, a formulation composed of DOTAP and cholesterol was formulated with immunopotentiator α-galactosylceramide (α-GC) (Verbeke et al. 2019). The α-GC was induced a pluripotent innate and adaptive tumor-specific immune response together with antigen-encoding mRNA and the glycolipid antigen. The new system resulted in seven times more tumor-infiltrating antigen-specific cytotoxic T cells and significantly lowered suppressive myeloid cells in the tumor microenvironment. LNPs can be visualized by adding MRI contrast agent; such system was formulated with TT3, DOPE, cholesterol, and DMG-PEG2000 for mRNA delivery and Gd-DTPA-BSA as MRI contrast agent (Luo et al. 2017). The key lipid was different to the classical structure of lipids which was composed of lipid-like compound N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide (TT) as the core and six lipid chains (Li et al. 2015). The introduced Gd-lipid endowed dual-functional LLN reduced longitudinal relaxation time (T1) and brighter images under magnetic fields, resulting in strong MRI signal was observed in vivo. Similarly, Gd as MRI contrast agent was as well as used in another LNP, named GARP. The LNP showed high cellular entry efficiency and rapid endosome and lysosome escape. In addition, GARP achieved imaging-guided and targeted siRNA delivery for cancer treatment (Guo et al. 2021). By leveraging the same lipid-like compound TT, TT3 was refined by combining TT3 with different types of biodegradable lipid chains (Zhang et al. 2020). The leading lipid FTT5 was

as well as formulated with DOPE, cholesterol, and DMG-PEG2000 at same molar ratio (20: 30: 40: 0.75). Large molecular weight mRNA (~4.5 kb and ~5.5 kb) was delivered in vivo. However, no comparisons of efficacy and safety between TT3 LLN and FTT5 LLN were performed.

From above discussion, it is easy to figure out that the trend in new lipid design is obviously the ionized hydrophilic heads with more than two biodegradable hydrophobic tails. This strategy may result in two main benefits: better safety profile and higher delivery efficiency. On one aspect, as mentioned above, the ionized amine head will help to reduce the adverse effects and immune response caused by LNPs. The introduced biodegradable tails also contribute to improving the safety of LNPs by accelerating tissue clearance. On the other aspect, increasing the number of tails is encouraging to improve the delivery efficiency of LNP, by which the interaction between LNPs and membrane of endosomal or lysosome is more likely to adopt a cone-shape morphology, leading to the loss of membrane integrity, and hence improved mRNA release.

3 Polymers

Since the original discovery by Folkman and Long (1964), polymers have occupied an important role in drug delivery, because they offered numerous opportunities to modulate the properties of drug delivery systems in addition to meet other criteria such as biodegradability, biocompatibility, and reproducibility (Qiu and Bae 2006). Polymers can be classified in various ways, but according to shape, the polymers can be divided as linear polymers, branched polymers, and grafted polymers and dendrimers. Moreover, the shape of polymers impacts their properties up to some extent; for example, linear polymers show high water solubility, and branched polymers mostly provide low critical micelle concentration (CMC) and controllable size, while grafted polymers and dendrimers have advantages in CMC, critical solution temperature (CST), and drug loading capacity.

Polymer in their simplest format usually has some key problems, which need to be addressed, such as polydispersity, safety, and biodegradability. In order to address these problems, the acceptable ways are lowering the molecular weight, changing the shape, and introducing PEG or biodegradable molecule. Taking PEI as an example, many groups successfully reduced the toxicity of PEI by modifying low-molecular-weight PEI with lipid tail. Compared with high-molecular-weight PEI, these polymers showed better safety performance (Dahlman et al. 2014; Lv et al. 2006; Zhao et al. 2016). A biodegradable polymer based on polycaprolactone (PCL) and PEI was developed, and it showed controlled degradation and was essentially non-toxic in cell studies. Notably, it also revealed much higher transfection efficiencies compared with PEI 25K (Arote et al. 2007). Moreover, PEI 10K with oleic acid was conjugated with carboxylate-terminated PEG (PEI_{10k}-Lin_{A15}-$PEG_{3.0}$). This modification created a pseudolipid which allows itself to form a micellar structure in aqueous solutions (Dunn et al. 2018). Compared with linear PEI, branched PEI was more stable under

salt-containing conditions (Wightman et al. 2001) and at a higher DNA concentration (Rudolph et al. 2005), which resulted in higher efficacy compared to linear PEI (Rudolph et al. 2005). In addition, other polymers were synthesized with PEI for nucleic acid delivery in vitro and in vivo (Guo et al. 2011; Huang et al. 2012). Poly(β-amino) ester (PBAE) (Fig. 3) is another well studied cationic polymer. Compared with PEI, biodegraded PBAE is more easier to synthesize and has lower bio-toxicity; (Xiong et al. 2018) however, instability in the serum is a problem for PBAE. It was found that the presence of serum could reduce the transfection efficiency of PBAE by 6 times (Zugates et al. 2007). PBAE can modify with alkyl side chains, and the resulting PBAE terpolymers facilitated non-covalent, hydrophobic interaction with PEG-lipid, leading to significantly increased stability, while avoiding the potential issues caused by PEG, such as condensation of damaged DNA and reduced uptake (Eltoukhy et al. 2013). Hyper-branched PBAE was synthesized for mRNA delivery, which resulted in efficient mRNA expression (101.2 ng/g luciferase protein in lung). In addition, PBAE with PEG-lipid showed potential for systemic delivery of mRNA to lungs (Kaczmarek et al. 2016). Notably, the further optimized formulation achieved a multiple order-of-magnitude increase in potency (Kaczmarek et al. 2018). Poly(dimethylaminoethyl methacrylate) (PDMAEMA) (Fig. 3) is another intensively investigated cationic polymer which is sensitive to temperature and pH. The polymer of PDMAEMA only observed weak mRNA binding, but PEGylation significantly improved mRNA binding and transfection efficiency (Uzgun et al. 2011). In addition, triblock copolymer based on PDMAEMA was synthesized, which resulted in efficient mRNA delivery. It was reported a class of materials, which involved cost effective synthesis, and named as charge-altering releasable transporters (CARTs). CARTs are serving initially as oligo (α-amino ester) cations that deliver mRNA and then boosting mRNA release through charge-neutralizing intramolecular rearrangement (McKinlay et al. 2017).

Dendrimers are featured by their hyper-branched structures, and they are having good application prospects because of the precise structure, molecular weight, and easily modifiable surfaces. In recent years, amphiphilic dendrimers are developed based on self-assembly strategy to form a nanosystems. The synthesis of amphiphilic dendrimers by using this strategy reduces the synthesis steps and the difficulty, which greatly improves the clinical application prospect of dendrimers (Lyu et al. 2020). Polyamidoamine (PAMAM) is the most well-known dendrimer, and the peptide-mimicking unit endows PAMAM dendrimer with water solubility and excellent biocompatibility. Derivatives of PAMAM are extensively used for nucleic acid delivery, especially for small interfering RNA (siRNA), but quite few studies have focused on mRNA delivery. PAMAM G1 dendrimer was modified with 2-tridecyloxirane, together with PEG-lipid, and the developed dendrimer nanoparticle showed advantages including rapid-response, fully synthetic, single-dose, and adjuvant-free and was used as a vaccine platform to deliver mRNA expressing antigens for Ebola, H1N1 influenza, Toxoplasma gondii, and Zika (Chahal et al. 2017; Cheng et al. 2018a).

4 Lipid–Polymer Hybrid Nanoparticles

In addition to aforementioned materials, many studies tried to hybrid polymers or dendrimers with lipids or other vehicles for mRNA delivery, and some of these hybrids have been tested in clinical trials, while others have shown promising translational potential. Stemirna Therapeutics have developed and patented the lipopolyplex (LPP) (Fig. 2) nano-delivery platform, which contains polymer-mixed mRNA as the core and lipid as the shell. The core–shell structure demonstrates better protection of mRNA, as well as gradually releases the mRNA as the polymers are degraded. Having said that, two of the LPP delivery programs have entered into clinical test (COVID-19 vaccine and personalized cancer vaccine) and at least seven candidates are under preclinical evaluation. Polymer–lipid hybrid nanoparticle was utilized to deliver therapeutics to transfect phosphatase and tensin homolog (PTEN) mRNA into tumors of mice (Islam et al. 2018; Lin et al. 2021), leading to significant tumor inhibition. The nanoparticle was employed cationic lipid-like compound G0-C14, co-formulated with poly(lactic-co-glycolic acid) (PLGA) and PEG-lipid in a polymer–lipid hybrid nanoparticle (Islam et al. 2018). In this system, the core of PLGA improved the stability of nanoparticle and G0-C14 was used for efficient mRNA complexation.

Enlightened by LNP, studies have tried to employ polymer as ionizable unit and assembled with helper lipids to formulate novel hybrid nanoparticles. Biodegradable amino-polyesters (APEs) via ring opening polymerization (ROP) of lactones with tertiary amino-alcohols were synthesized firstly, and then, the ionizable amino-polyesters were mixed with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol, and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (DMG-PEG) in molar ratios of 50: 25: 23.5: 1.5 to form APEs-LNP (Kowalski et al. 2018). Such nanoparticles showed significant ability to deliver mRNA to lung endothelium, liver hepatocytes, and splenic antigen-presenting cells with low in vivo toxicity. Besides, poly(glycoamidoamines) (TGAAs)-based TarN3C10 were developed and formulated with DSPC, cholesterol, and DMG-PEG in weight ratios of 5:2:2:1 into nanoparticle (Dong et al. 2016). By using dendrimer 5A2-SC8 with DOPE, cholesterol, and PEG-lipid as components, library of dendrimer-LNP formulations was established, and the leading formulation successfully delivered therapeutic FAH mRNA into mouse model of hepatorenal tyrosinemia type I and thus leads to obvious survival extension. Notably, the optimized formulation was efficacious at mRNA doses as low as 0.05 mg kg^{-1} in vivo. In addition, this study also reported that the optimal formation for mRNA delivery is different to that for siRNA delivery, lower ionizable lipid and higher zwitterionic phospholipid may render higher mRNA loading and release, which may be due to the fact that mRNA is larger and more flexible than siRNA, and reducing core lipid content prevents the lipid bilayer from binding too tightly to mRNA, resulting in the ability of mRNA release.

5 Polypeptides

Polypeptides for nucleic acid delivery are usually a kind of small peptides that are rich in positively charged amino acids. Mechanistically, cationic peptides condense negatively nucleic acids via electrostatic interactions. Protamine and mRNA can form tight complex by electrostatic interaction, and this complex can protect mRNA from being degraded. Furthermore, it was reported that such complexes can trigger immune response by activating several human blood cells in a toll-like receptor-dependent manner (Scheel et al. 2005). Thus, it can be developed not only as a delivery platform but also as an immune activator (Xu et al. 2020). Protamine was firstly reported for RNA delivery in 1961, (Amo 1961) and then, several projects have been entered in clinical testing (Alberer et al. 2017; Papachristofilou et al. 2019; Sebastian et al. 2019). CureVac developed a platform named RNActive®, (Kim et al. 2013) containing free and protamine-complexed mRNA, which can induce balanced adaptive immune responses as well as T cell-mediated immunity. Based on this technology, CureVac launched several projects, which are currently undergoing clinical trials. Cell-penetrating peptides (CPPs) are an arginine-rich protein, and it is hypothesized that positively charged CPPs promote clustering of the negatively charged glycosaminoglycans on the cell surface, which triggers macropinocytosis and lateral diffusion, or destruct the lipid bilayer directly (Verdurmen and Brock 2011; Ziegler and Seelig 2008). A CPP containing RALA motif for antigen-encoding mRNA delivery was reported, (Udhayakumar et al. 2017) in this study, and the RALA motif condensed mRNA into nanocomplexes and consequently achieved dendritic cell delivery. CPP Xentry can be fused with peptide which contains a truncated form of protamine (Bell et al. 2018). The fused peptide can transfect mRNA into human cells, and this is enhanced by the chloroquine and the toll-like receptor antagonist E6446. Polylysine (PLL) carries high positive charge because it is rich in amino groups on its side chain. It was widely used for plasmid DNA (pDNA) and siRNA delivery and has attempts for mRNA delivery. A complex containing mRNA, PLL, poly(*N*-isopropylacrylamide) (PNIPAM), and targeting ligand cRGD was reported. The mRNA and PLL were cross-linked though redox-responsive disulfide linkage, thereby improving the mRNA stability (Chen et al. 2017).

In addition, attempts have been made for polypeptides design with specific structures for improving the efficiency of mRNA delivery. The dendronized polypeptide (denpol) was used for mRNA delivery, (Oldenhuis et al. 2016) and the resulting system contained an L-lysine-di-cysteine polymer backbone and lysine dendrons. Delightfully, denpol efficiently delivered mRNA to various cell and showed the advantages of conformational flexibility, beneficial multivalent interactions of a dendrimer, and reduced responsiveness.

6 Other Carriers

Apart from aforementioned mRNA carriers, researchers have also been devoted to developing other platforms. Cells can be mRNA carriers due to their vesicular structures and specific biological functions. Dendritic cells (DCs) played a pivotal role in regulating both innate and adaptive immunity; (Constantino et al. 2017) specifically, they hold great ability to process proteins into peptides that presented on major histocompatibility complex (MHC) to trigger cellular immunity (Palucka and Banchereau 2012; Steinman and Banchereau 2007) and provide a complete antigen to B cells to trigger humoral immunity (Wykes et al. 1998). Such advantages making DCs as an ideal material for vaccine preparation. DCs usually can be engineered with mRNA in ex vivo way. To this end, DC precursor cell was isolated from the patient and then engineered with mRNA and activated. Finally, the functionalized DCs were re-administered to the patient. To date, several clinical tested projects employ DCs as mRNA carrier, resulting in better therapeutic outcomes. T cells also can be engineered. In one study, T cell was fused with DOTMA-lipoplex, and then, encapsulated mRNA vaccine encoding developmentally regulated tight junction protein claudin 6 (CLDN6) as a chimeric antigen receptor (CAR) target (Reinhard et al. 2020). The resulting system was able to deliver the CAR antigen into lymphoid compartments and stimulates adoptively transferred CAR-T cells. Gold nanoparticles functionalized with thiol-terminated DNA (AuNP-DNA conjugates) can deliver mRNA into cells by sequence complementary manner (Yeom et al. 2013). The delivered Bcl-2-associated X (BAX) mRNA efficiently expressed BAX protein in human cells and xenograft tumors in mice and inhibited tumor growth by inducing apoptosis. Another common delivery material, metal–organic framework (MOF), is also used for mRNA delivery. A dendritic cationic zirconium for MOF synthesis was developed and improved the colloidal stability of the mRNA assemblies and eventually leading to satisfactory mRNA transfection activities (Sun et al. 2018). Moreover, injecting mRNA by hollow microneedle is a kind of novel attempts. This method of administration only introduces therapeutic mRNA into body, leading to high safety profile. Additionally, the use of microneedles enables patient-friendly, painless, and efficient delivery of synthetic mRNA into the dermis (Golombek et al. 2018). The device prepared by microneedle with dissolving polyvinylpyrrolidone (PVP) can well-protect mRNA for at least two weeks under ambient conditions (Koh et al. 2018). Furthermore, it mediated targeted luciferase expression up to 72 h in vivo and induced cellular and humoral immune responses by injecting OVA mRNA. Humanized Gaussia luciferase (hGLuc) was successfully delivered by a commercialized microneedle device (MicronJet600 from NanoPass Technologies) to an ex vivo porcine skin model intradermally. (Golombek et al. 2018) The synthetic mRNA without delivery system was able to significantly express the target protein. In addition to aforementioned systems, materials such as extracellular vesicles, (Usman et al. 2018; Wang et al. 2018; Yang et al. 2020b; Yu et al. 2018) fluorinated peptoid crystals, (Song et al. 2018) chitosan-alginate gel scaffolds (Yan et al. 2019), and so forth have been designed for mRNA delivery.

7 Applications of Therapeutic mRNA

7.1 Vaccine

7.1.1 Cancer Vaccine

The most common application for therapeutic mRNA is vaccines, especially cancer vaccines. It was reported that intramuscular-injected carcinoembryonic antigen (CEA) mRNA can obtain protective anti-tumor immunity, leading to the exploration of mRNA inducing adaptive immune response in tumor (Conry et al. 1995). Since then, various mRNA codings for tumor-associated antigens (TAAs) have been developed for cancer therapy, such as MUC1, prostate cancer associated antigen (PSA), human telomerase reverse transcriptase (hTERT), Wilm's tumor-1 (WT1), glycoprotein 100 (gp100), tyrosinase, and survivin. In order to achieve enhanced adaptive immune response, many studies employ DCs as delivery system. In addition, CureVac has developed several projects currently in clinical trials using the protamine-based RNActive® platform. In the RNActive® system, protamine serves as a TLR 7/8 agonist to induce Th1 T cell responses, while modified mRNA serves as antigen generator. However, there is a clear trend to see that LNP or LPP is becoming absolutely dominant in the mRNA delivery system. Perhaps the robust delivery efficiency is an important factor in accelerating the decision to abolish immunogenic DCs and protamine.

It was reported only a few studies based on shared antigens have an overall clinical benefit of more than 25% (Chen et al. 2020; Wilgenhof et al. 2013). Basically, it is mainly attributed to such antigens which lack specificity and are not highly immunogenic (Esprit et al. 2020). Neoantigens are developed from somatic mutations in the cancer cell genome; they are specifically expressed on cancer cells; and therefore, this ensures on-target toxicity, without being off-tumor, and likely elicits high affinity T cells (Esprit et al. 2020). BioNTech pioneered in this field, and they firstly reported clinical data of neoantigen vaccine in 2017 (NCT02035956) (Sahin et al. 2017). BioNTech identified non-synonymous mutations by comparing whole genome/exome and RNA sequencing of tumor biopsies expressed by stage III and IV melanoma patients and healthy blood cells. They synthesized mRNA vaccine containing two IVT mRNA molecules, whereas each mRNA code five linker-connected 27-mer peptides. In the clinical test, patients after treatments were all developed immune response after maximum 20 dose of 0.5 or 1 μg per vaccination. Eight of fifteen patients had no detectable tumors, and no recurrence was detected in for the next 12–23 months. Moreover, the mRNA-4157 is a project launched by Moderna, in which the mRNA unit is encoding up to 34 neoantigens selected by a proprietary algorithm based on whole exome and RNA sequencing of tumor and blood samples. In published data of Keynote-603 (NCT03739931), (Moderna, 2020a, b) headneck squamous cell carcinoma (HNSCC) patients received mRNA-4157 and pembrolizumab showed no severe adverse effects and favorable outcomes compared with patients only received pembrolizumab treatment. Briefly, the overall

response rate (ORR) to mRNA-4157 and pembrolizumab was 50%, and disease control rate (DCR) was 90%, while the median progression-free survival (mPFS) was 9.8 months, which is favorable to the pembrolizumab that was 0%, 14.6%, and 2.0 months, respectively (Burtness et al. 2019; Cohen et al. 2019).

7.1.2 Infectious Disease Vaccine

Although traditional vaccines provide effective treatment and prevention to many diseases, there are still a few issues that hinder the development of vaccine, such as time and potential mutation of pathogen (Cao 2021). Such problems have been largely solved by mRNA vaccines because they have advantages of short development cycle, simple production process, easy to expand production, and so on. The outbreak of Zika and COVID-19 has demonstrated the advantages of mRNA vaccines to prevent the disease. Recently, since the World Health Organization (WHO) announced COVID-19 as a pandemic infectious disease that affecting the world, almost all pharmaceutical companies dealing mRNA have launched their vaccines against this disease. After that, the US FDA's Emergency Use Authorization (EUA) authorized two COVID-19 vaccines such as BNT162b2 and mRNA-1273 in December 2020 in the United States. It should be noticed that the whole cycle from initial development to drug approval took less than one year. BNT162b2 is an ALC-0315 LNP-based mRNA vaccine, which is co-developed by BioNTech and Pfizer. In a phase 3 study, the efficacy of 94% was observed at 28 days after the first administration and no serious adverse effects were observed (Pfizer and BioNTech 2020). The mRNA-1273 was developed by Moderna, which employed Lipid H LNP as delivery platform. Phase III clinical trial data demonstrated that vaccine efficacy was 93% and is durable up to six months (Moderna 2020b). Similarly, Curevac, Translate Bio, Stemirna, and Yuxi Walvax Biotechnology have also developed their COVID-19 vaccines and undergoing clinical trials.

Apart from COVID-19 vaccines, Moderna has the most extensive presence in the development of infectious disease vaccines such as vaccines against infectious cytomegalovirus (CMV), zika, influenza respiratory syncytial virus (RSV), human metapneumovirus and parainfluenza virus 3 (hMPV/PIV3), Epstein–Barr virus (EBV), HIV, nipah, and influenza H7N9 that were developed by using Lipid 5 or Lipid H LNP. Exception of BNT162b2 against COVID-19, the BioNTech used LNP as vehicle to deliver mRNA vaccine against influenza. Two projects against HIV and tuberculosis are under pre-clinical test. However, BioNTech did not disclose the selected delivery platform. In addition, five projects are developed by CureVac CV7202 for rabies(I), Lassa, yellow fever and Rota, malaria, and universal influenza which are currently in pre-clinical and clinical investigations.

7.2 Protein-Replacement Therapy

Protein-replacement therapy involves expressing antibodies or functional proteins (Weng et al. 2020). Although the idea has been proposed for years, but due to the advances of modification of nucleosides and improvement of purification, the development of mRNA therapeutics in protein-replacement therapy exceled. Unlike vaccines, mRNA for protein therapy needs to reduce their immune prototype as much as possible. In addition, chemical modification should be used to extend the half-life and improve the efficiency of transcription. Currently, Moderna and AstraZeneca are investigating an intramyocardially injected VEGF-A mRNA for heart regeneration after myocardial infarction (AZD8601). Pre-clinical and clinical data showed that the resulting VEGF-A promoted growth of blood vessels, therefore increased blood flow and partially restored cardiac function (Zangi et al. 2013). After this successful cooperation, they collaborated on the clinical study of MEDI11191. In this project, the engineered mRNA was integrated with two independently expressed IL-12B and IL-12A by a flexible linker, and the miR-122 binding site was added into 3' UTR to reduce the expression IL-12 protein in the liver (Hewitt et al. 2020). In the clinical test, patients received MEDI11191 showed IL-12 correlated IFN-γ change, which was consistent with proposed mechanism of action (Moderna 2021). Moderna carried out a program named mRNA-2572 containing three functional mRNA, which are OX40L, IL-23, and IL-36γ. OX40L is a transmembrane T cell co-stimulatory protein that can enhance T cell effector function, expansion, and survival (Croft et al. 2009). IL-23 and IL-36γ activate cells that bridge innate to adaptive immunity and promote DC maturation, respectively. To date, a phase I clinical trial is undergoing (NCT03739931) and few data on effectiveness has been published. However, the pre-clinical data showed that the extreme robust tumor shrinkage and significantly prolonged survival were achieved in mice. Besides to Moderna, Translate Bio is investigating an inhaled LNP formulation of cystic fibrosis transmembrane conductance regulator (CFTR) mRNA in a phase I/II clinical trial (NCT03375047).

7.3 Gene Editing

Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (CRISPR-Cas) are the powerful tools for gene editing that can be used in fields including genetic diseases treatment and animal model establishment. With those systems, sequences are deleted, replaced, or inserted at specific sites in the genome following the presence of DNA double-stranded break (DSB) by non-homologous end joining (NHEJ) or by homology-directed repair (HDR) (Doudna and Charpentier 2014). However, editing nucleases recognize their gene in a protein-DNA interaction-dependent manner (Xiong et al. 2018); thus, it required complicated protein engineering and restricts their broad applications (Pardi et al.

2017b; Stadler et al. 2017). Recently, high gene-editing efficiency has been achieved by using non-viral vectors to deliver pDNA expressing CRISPR protein (Cong et al. 2013; Ousterout et al. 2015; Wang et al. 2013). Compared with pDNA, mRNA-based CRISPR-Cas system showed higher editing efficiency, (Li et al. 2021) as well as the transient expression nature of mRNA also reduces the adverse effects and immune response caused by off-target cutting (Kowalski et al. 2019). Moreover, the addition of specific regulatory elements (e.g., k-turn motifs and microRNA binding sites) to the mRNA sequence further controls mRNA expression in certain cell types (Hewitt et al. 2020; Wroblewska et al. 2015).

Recent clinical efforts are mainly focused on ex vivo applications of gene-editing tools. CRISPR Therapeutics and its partner Vertex carried out program CTX001™ based on CRISPR-Cas9 system which is aiming to cure β-thalassemia (NCT03655678) and severe sickle cell disease (NCT03745287) by editing of BCL11A. Pre-clinical results showed that mice after treatment with CTX001™ increased their HbF levels in erythroid cells in vivo. Clinical data showed that the patients got benefit from treatment with CTX001™, which sustained increases in total Hb and HbF across genotypes. (Crispr Therapeutics 2020) CRISPR Therapeutics independently host the clinical evaluation of CTX110™, CTX120™, and CTX120™ (NCT04035434, NCT04244656, and NCT04438083) based on differentiated CRISPR-Edited allogeneic CAR-T design. Furthermore, Chinese PLA General Hospital is evaluating gene-edited dual specificity CAR-T cells or CAR-T cells for the treatment of B cell leukemia and lymphoma (NCT03398967 and NCT03166878). Editas Medicine and Intellia Therapeutics studies are in in early-stage clinical tests. Sangamo Therapeutics is making clinical efforts for mRNA-based ZFNs for gene editing in T cells (NCT02225665) and hematopoietic stem cells (HSCs) (NCT02500849) to treat HIV.

8 Discussion and Perspectives

With the development of chemical modification and in vitro transcription, the inherent disadvantage of mRNA is being gradually overcome. Meanwhile, the advantages of mRNA, such as relatively simple purification, wide range of applications, and safer expression patterns, have greatly attracted the attention of academia and the pharmaceutical industry. Thus far, clinical efforts have mainly focused on vaccination, since it showed advantages over conventional strategies such as the ability of rapid deployment. Similarly, the mRNA also has huge potential in the field of protein therapy. In addition, ZFNs and CRISPR-Cas translated by mRNA paving the way for genetic disease treatment. It seems that delivery technologies are the key factor determining the usage of the mRNA-based drugs. The requirements for mRNA delivery carrier are relatively stringent. At present, lipid–polymer hybrids are more accepted by drug companies. Such fact resulted in mRNA mainly expresses in the liver. How to achieve efficient extra-hepatic delivery of therapeutic mRNA is a key problem to be solved. There is no doubt that the focus on mRNA therapeutics is increased recently, and the

development of new nano-formulations using different materials will overcome this difficulty and greatly expand the application of mRNA drugs.

Acknowledgements This work was supported by the Beijing-Tianjin-Hebei Basic Research Cooperation Project (19JCZDJC64100), the Natural Science Foundation of Guangdong Province (2019A1515010776), the National Natural Science Foundation of China (31871003, 32001008, 32171394), the Beijing Nova Program from Beijing Municipal Science & Technology Commission (Z201100006820005), and the National Key R&D Program of China (2021YFE0106900). We thank Biological & Medical Engineering Core Facilities (Beijing Institute of Technology) for providing advanced equipment.

Competing Interests The authors declare no competing interests.

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Lipid Nanoparticles to Harness the Therapeutic Potential of mRNA for Cancer Treatment

Maria L. Guevara, Francesca Persano, and Stefano Persano

Contents

Abstract In recent years, mRNA has become an appealing platform for the development of therapeutic agents both for the prevention and treatment of cancer. Efficient delivery of mRNA into target cells is crucial for fully harnessing its therapeutic potential. However, mRNA possesses structural limitations, including its net negative charge and hydrophilicity, that impede its efficient cellular uptake. Likewise, mRNA is characterized by an intrinsic fragility, resulting in it being a highly instable molecule. Lipid nanoparticles (LNPs) have been successfully used for protecting and delivering mRNA encoding for various therapeutic proteins. This chapter is intended to give a comprehensive overview of the current approaches for mRNA synthesis and LNPs manufacturing. We provide an in-depth analysis of how mRNA technology is revolutionizing the area of cancer immunotherapy, critically reviewing the

M. L. Guevara
UOC Immunologia, IRCCS Ospedale Policlinico San Martino, Genova, Italy

Department of Experimental Medicine (DIMES), Center of Excellence for Biomedical Research, Università di Genova, Genoa, Italy

F. Persano
Department Matematica e Fisica 'Ennio De Giorgi', Università del Salento, Lecce, Italy

S. Persano (✉)
Istituto Italiano di Tecnologia (IIT), Genova, Italy
e-mail: stefano.persano@iit.it

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_14

major fields of application of nanoformulated-mRNA medications and addressing the advantages and drawbacks of each one. Finally, we offer a wide landscape of future possibilities and remaining issues of current mRNA-based therapies.

Keywords mRNA · Immunotherapy · Lipid nanoparticles · Vaccines · Cancer

1 Introduction

Messenger RNA (mRNA) is a subtype of RNA containing the genetic information necessary to produce a specific protein. The synthesis of mRNA occurs in the cellular nucleus through a process called transcription whereby genomic DNA serves as a template (Bentley 2014; Cramer 2019). Mature mRNA that has been subjected to splicing and post-transcriptional modifications is then exported to the cytosolic compartment where its translation begins upon binding with the ribosomal subunits (Cramer 2019).

Since its discovery in 1960, different attempts have been made to exploit mRNA as a therapeutic platform for the development of gene-based therapies (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020). mRNA-mediated transfection represents an appealing alternative to more conventional plasmid DNA (pDNA)-based strategies for inducing exogenous gene expression. This is because mRNA technology offers relevant advantages including superior transfection efficiency, especially in non-dividing and hard to transfect cells, without the risks of insertional mutagenesis (Leonhardt et al. 2014; Guevara 2019b). Indeed, unlike pDNA, mRNA does not need to enter the nucleus of the target cells to exert its function; it is sufficient that it reaches the cytosolic compartment for translation to occurs (Leonhardt et al. 2014; Andreev et al. 2016). Once mRNA has completed its function, it is rapidly degraded thus promoting only a transient expression of the protein of interest, which is convenient for safer and efficient therapeutic approaches (Huch and Nissan 2014).

However, mRNA's application as a therapeutic molecule was limited until the second half of the last decade due to the several limitations inherent to its high fragility, poor ability to enter cells because of its net negative charge, and high immunogenicity (Karikó et al. 2008; Guevara 2019b; Bidram et al. 2021). Although the intrinsic adjuvanticity of mRNA, ascribable to the interaction with innate immune receptors, has been exploited to enhance the efficacy of mRNA-based vaccines (Kranz et al. 2016), extensive efforts have been dedicated to reducing the immunogenicity and improving the stability of mRNA molecules by incorporating chemically modified nucleotides and regulatory elements (Karikó et al. 2008; Anderson et al. 2010; Nance and Meier 2021).

Despite modified nucleotides were demonstrated to minimize the susceptibility to degradation by ribonucleases and improve the translatability of "naked" mRNA, effective delivery remained the principal obstacle for ensuring adequate production of exogenous proteins upon systemic or local mRNA administration (Pardi et al.

2018). Recent advances in non-viral mRNA delivery technologies have broadened the application of the mRNA technology in preclinical and clinical settings (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020). A wide range of nanosized platforms has been investigated as mRNA delivery systems, such as polymeric, peptide-based, and lipid-based nanoparticles (McKinlay et al. 2017; Lou et al. 2019; Qiu et al. 2019; Kaczmarek et al. 2016; Sago et al. 2018; Veiga et al. 2018). Among these non-viral vehicles, lipid-based formulations represent the most advanced platform that has been successfully employed for in vivo mRNA delivery (Guevara et al. 2020; Pilkington et al. 2021; Schoenmaker et al. 2021).

The ongoing COVID-19 pandemic has further accelerated the development of mRNA technology, with two mRNA-based vaccines, BNT162b2 (BioNTech) and mRNA-1273 (Moderna), granted with the first historic authorization for clinical use by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), while another mRNA vaccine, CVnCoV (CureVac), is currently in phase 3 clinical trials (Risma et al. 2021; Uddin and Roni 2021). This success has drawn the interest of several pharmaceutical companies and research groups in acquiring the necessary capabilities to set up the manufacturing of nanoformulated mRNA-based therapies not only for cancer immunotherapy but also for other purposes (Martin and Lowery 2020; Dolgin 2021a, b). Currently, dozens of mRNA-based therapeutics are in preclinical and clinical phases of development, with promising outcomes in diverse types of cancers (Table 1).

In this chapter, we summarize the current methods utilized for mRNA preparation and synthesis, and the progress that has been made to increase the structural stability and translation efficiency of synthetic mRNA. We describe the preparation of lipid-based nanoparticles by standard nanoprecipitation technique for mRNA encapsulation, and the major formulation parameters that can affect its ability to induce transgene expression. Finally, we critically evaluate the different forms of RNA-based therapies that have been proposed.

2 Structure, Synthesis, and Purification of in Vitro Transcribed (IVT) mRNA

2.1 Structural Organization of IVT-mRNA

Synthetic mRNAs can be classified mainly in two types, the non-replicating (or non-amplifying) and the virus-derived self-amplifying RNA (saRNA) (or replicon).

The minimum structure of conventional non-replicating in vitro transcribed (IVT) mRNA consists of all those elements present in mature eukaryotic mRNA, including an open reading frame (ORF) region that encodes the desired protein, 5′- and 3′-untranslated regions (UTRs), and 5′ cap and 3′ poly(A) tail (Fig. 1) (Chaudhary et al. 2021).

Table 1 Clinical trials investigating lipid-based nanoparticles for mRNA-based cancer therapeutics

National clinical trial number	Disease	Status	Phases	Encoded proteins	Route	Sponsor
NCT03323398	Relapsed/refractory solid tumor malignancies or lymphoma	Recruiting	Phase ½	mRNA-2416 (mRNA encoding OX40L)	Intratumoral	Moderna
NCT03739931	Relapsed/refractory solid tumor or lymphoma	Recruiting	Phase 1	mRNA-2752 (mRNA encoding OX40L, IL-23, IL-36Y)	Intratumoral	Moderna, AstraZeneca
NCT02410733	Advanced melanoma	Active, not yet recruiting	Phase 1	NY-ESO-1, MAGE-C3, tyrosinase, gp100	Intravenous	BioNTech
NCT02316457	Triple negative breast cancer	Active, not yet recruiting	Phase 1	3 TAAs selected	Intravenous	BioNTech
NCT03289962	Melanoma, NSCLC, bladder cancer, CRC, breast cancer, etc.	Recruiting	Phase 1	Neo-Ag (mRNA)	Intravenous	BioNTech, Genentech
NCT04267237	NSCLC	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	Hoffmann-La Roche
NCT03815058	Advanced Melanoma	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	BioNTech, Genentech
NCT04486378	Stage II and III CRC (surgically resected)	Recruiting	Phase 2	Neo-Ag (mRNA)	Intravenous	BioNTech
NCT04161755	Pancreatic cancer (surgically resected)	Recruiting	Phase 1	Neo-Ag (mRNA)	Intravenous	Memorial Sloan Kettering Cancer Center, Genentech
NCT03313778	Resected/unresectable solid tumors	Recruiting	Phase 1	Neo-Ag (mRNA)	Intramuscular	Moderna, Merck

(continued)

Table 1 (continued)

National clinical trial number	Disease	Status	Phases	Encoded proteins	Route	Sponsor
NCT03897881	Complete resection of high-risk Melanoma	Recruiting	Phase 2	Neo-Ag (mRNA)	Intramuscular	Moderna, Merck
NCT03948763	CRC, NSCLC, pancreatic cancer	Recruiting	Phase 1	Mutated KRAS: G12D, G12V, G13D, G12C	Intramuscular	Moderna, Merck
NCT03468244	Advanced esophageal squamous carcinoma; gastric adenocarcinoma; pancreatic adenocarcinoma; colorectal adenocarcinoma	Recruiting	Phase 1	Neo-Ag (mRNA)	Subcutaneous	Changhai Hospital, Stemirna Therapeutics

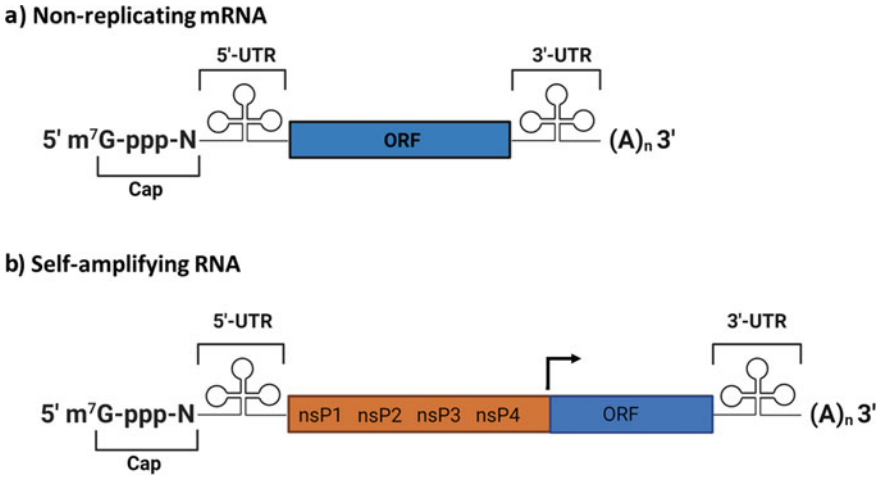

Fig. 1 Schematic illustration of the structural organization of non-replicating mRNA (**a**) and self-amplifying RNA platforms (**b**). Non-replicating mRNA is composed of a cap structure (m^7GpppN, where N can be any nucleotide), the 5′-UTR, an open ORF encoding a gene of interest, the 3′UTR, and a tail of 30–120 adenosine residues (poly(A) tail) (**a**). Self-amplifying RNA derives from an alphavirus genome and includes a 5′cap, nonstructural genes (nsP1–4), 26S subgenomic promoter (open arrow), the ORF encoding the desired protein, the 3′-UTR, and a poly(A) (**b**)

In contrast, saRNA derives from the genome backbone of an alphavirus, like the venezuelan equine encephalitis (VEE) virus, in which structural genes have been replaced by a transgene encoding the therapeutic protein of interest, whereas the genes responsible for RNA replication are maintained to preserve the auto-replicative capacity of the virus (Fig. 1) (Blakney et al. 2021). SaRNA is advantageous compared to conventional IVT-mRNA as it retains all the benefits of mRNA technology, such as rapid synthesis, transient activity, and suitability for customization, but the therapeutic effect can be achieved with a lower dose of RNA thanks to its self-replicative capacity (Vogel et al. 2018).

In the following sections, we will describe the role of the different regulatory elements in IVT-mRNA, how its sequence organization and composition can be optimized to maximize its therapeutic performances, and provide an overview of the preparation of synthetic mRNA by in vitro transcription.

2.1.1 *5′ and 3′ Untranslated Regions (UTRs)*

The UTR regions are non-coding elements located at the 5′ and 3′ ends of a mature mRNA (Leppek et al. 2018; Mignone et al. 2002). UTR sequences play a critical role in multiple processes conducting to mRNA translation into protein, including transport of mRNA from the nucleus to the cytosol, the assembly of the translation machinery, and mRNA decay (Leppek et al. 2018; Rabani et al. 2017; Mignone et al.

2002; Schuster and Hsieh 2019). Secondary structures (hairpins) in the UTRs are the major determinants of its regulatory function (Mignone et al. 2002). In UTRs of mRNA encoding proteins poorly expressed under normal conditions, hairpin structures are particularly abundant and form stable interactions with an average free energy of less than −50 kcal/mol (Mignone et al. 2002; Babendure et al. 2006). Secondary structures positioned in the proximity of the cap structure are more effective at inhibiting translation initiation; indeed, free energy of −30 kcal/mol is sufficient to impede the access of the 43S preinitiation complex, composed of the small ribosomal subunit (40S) bound by the initiation factors eIF1, eIF1A, eIF3, and the eIF2-Met-tRNAiMet-GTP ternary complex (eIF2-TC) (Mignone et al. 2002; Babendure et al. 2006; Pestova et al. 2007). On the other hand, secondary structures situated closer to the starting codon require a free energy higher than −50 kcal/mol to be able to inhibit translation (Mignone et al. 2002; Babendure et al. 2006). The preinitiation complex scans along the 5′-UTR until it encounters the AUG start codon; afterward, the larger 60S subunit joins the 40S to form an 80S initiation complex, and protein synthesis begins. This mechanism is known as cap-dependent translation and describes the initiation of mRNA translation in most organisms (Hinnebusch and Lorsch 2012). Some viral RNAs use a cap-independent mechanism for initiating translation, which involves an internal ribosomal entry site (IRES) able to attract ribosomal subunits independently of the cap structure (Martinez-Salas et al. 2018; Zhao et al. 2020).

The UTR sequences employed in therapeutic IVT-mRNA are retrieved from specific databases (i.e., http://utrdb.ba.itb.cnr.it/) or from coding sequences (CDS) available in sequence banks (e.g., https://www.ncbi.nlm.nih.gov/nucleotide/). Those from human α- and β-globin probably represent the most studied and characterized UTRs (Babendure et al. 2006).

Length is a critical parameter for the 5′-UTR, and an optimized sequence should not exceed 70 nt and could include the Kozak consensus sequence (GCC-(A/G)-CCAUGG) immediately upstream of the translation start codon (AUG) to enhance translation from the correct initiation codon (Asrani et al. 2018). Importantly, the start codon AUG within 5′ UTR is excluded to prevent alternative translation initiation and mutation of the amino acid sequence. In addition, secondary structure elements in the 5′ UTR region of the mRNA should be minimized to reduce the energy barrier for the scanning ribosome to reach the start codon.

The 3′-UTR has a great effect on mRNA's stability; indeed, its optimization results in increased mRNA half-life (Holtkamp et al. 2006; Wang et al. 1999). Accordingly, the duration of mRNA expression can be regulated by varying its composition. The introduction of AU-rich elements in the 3′-UTR causes mRNA destabilization, leading to rapid mRNA decay thus shortening protein expression, while mRNAs with enriched CG-content in the 3′-UTR sequence exhibit increased stability and translation efficiency. Likewise, the proper combination of 5′- and 3′-UTR sequences can enhance the translation efficiency (Ferizi et al. 2016).

2.1.2 The Function of the Cap Structure

The introduction of a 5′ end cap is a conserved post-transcriptional modification of eukaryotic mRNAs (Ramanathan et al. 2016). mRNA molecules are capped with a 7-methylguanosine (m^7G) connected by a 5′-to-5′ triphosphate bridge to the first nucleotide to form a cap 0 structure (m^7GpppN). Cap 0 regulates the translation of mRNA by preventing its degradation and facilitating the assembly of the translation machinery (Ramanathan et al. 2016). In mammals, the first transcribed nucleotide is methylated in the 2′ ribose position to form a cap 1 structure ($m^7GpppN_{2'Om}$) and, in approximately 50% of transcripts, also the second transcribed nucleotide is 2′ O-methylated in the 2′ ribose position to form cap 2 ($m^7GpppN_{2'Om}N_{2'Om}$) (Ramanathan et al. 2016).

While cap 1 structure is ubiquitously expressed in humans, the expression of cap 2 is restricted to specific tissue types, such as in striated muscles and at lower levels in brain, testes, lung, liver, and skin tissues. The function of cap structures remains largely unknown, but they are known to be involved in modulating nuclear export, splicing, turnover, translation efficiency, and decapping of mRNAs (Galloway and Cowling 2019). Cap 1 is important for self/non-self-discrimination, by preventing the recognition by interferon (IFN)-induced proteins with tetratricopeptide repeats (IFITs) or pattern recognition receptors (PRRs) (Galloway and Cowling 2019). IFIT proteins recognize non-methylated cap structures, like cap 0, and mRNA molecules with 5′-triphosphate or 5′-monophosphate ends. IFIT-1 sequesters non-methylated mRNA from the translational machinery by competing with EIF4E proteins for the binding to the cap structure (Galloway and Cowling 2019).

The importance of cap structures in preventing mRNA recognition by the innate immune system has been highlighted by the observation that cytoplasmic viruses often possess cap 1 structures and that the deletion of the viral methyltransferase responsible for the conversion of cap 0 into cap 1 resulted in viral attenuation (Bouvet et al. 2010). Retinoic acid-inducible gene 1(RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are responsible for the cytoplasmic recognition of double-stranded RNA (dsRNA) with 5′ppp and cap 0 ends, and their activation induce the expression of type I IFNs (IFN-I) (IFN-α and IFN-β) and other pro-inflammatory cytokines (Ramanathan et al. 2016; Galloway and Cowling 2019). Cap 1 modification abrogates both RIG-I and MDA5 recognition of dsRNA, preventing innate immune activation. Therefore, methylation of the first transcribed nucleotide is thought to be a molecular signature that discriminates self and non-self mRNA.

Several synthetic cap analogs have been developed for the capping of IVT-mRNA (Jemielity et al. 2010; Tang et al. 2019). The anti-reverse cap analog (ARCA) is widely used for the preparation of synthetic capped mRNA. ARCA possess a cap 0 structure with a 3′-O-methyl group on the sugar adjacent to the m^7G ($m^{7,3'\text{-}O}GpppG$), which prevents it from incorporating in the incorrect orientation (Tang et al. 2019; Warminski et al. 2017). The variety of cap structures has been recently expanded following the observations that up to 30% of caps in animals and viral mRNAs are also methylated at the first encoded nucleotide adjacent to the 7-mG cap to obtain N6-methyladenosine (m^6A) or N6,2′-O-dimethyladenosine (m^6Am). In addition,

multiple methylations also occur in the 5′ G cap (e.g., $m^{2,2,7}$GpppN) in viral RNAs and a subset of RNAP II-transcribed cellular RNAs (Warminski et al. 2017).

2.1.3 Role of the Poly(A) Tail

The poly(A) tail is a structure characteristic of mature mRNAs that plays a significant role in mRNA translation (Weill et al. 2012). It has been shown that a gradual increase in the poly(A) tail length of IVT-mRNA to 120 bases leads to increased translation, whereas shortening of the poly(A) sequence results in faster mRNA decay (patent WO 2017/059902 Al). A further increase in the poly(A) tail size beyond 120 residues does not enhance the translation efficiency.

The poly(A) tail is bound with high affinity by the poly(A) RNA binding proteins (PABPs), which interact with eIF4G and eIF4B to promote the circularization of the mRNA molecule and ribosomal recruitment to form a polyribosome complex (Weill et al. 2012). A sufficiently long poly(A) tail is necessary to ensure the circularization of mRNA via binding of PABPs to the poly(A) tail and the cap. The minimal length of poly(A) tail required for mRNA's stability has been determined to be 30 nt, which corresponds to the reported 25–30 nt footprint for a single PABP (Lima et al. 2017).

2.1.4 Modified Nucleotides

mRNA suffers from several limitations which impeded its use for a long time. In particular, limited stability and high immunogenicity were the most relevant issues limiting the therapeutic application of IVT-mRNA.

Chemically modified nucleotides are known to be present at low abundance in non-synthetic mRNAs (McCown et al. 2020). IVT-mRNAs incorporating modified nucleotides, commonly uridine, are termed modified mRNAs (modRNAs), while unmodified mRNAs (unmodRNA) do not contain chemically modified nucleotides. The most frequent naturally modified nucleotides are pseudouridine (Ψ), 5-methylcytidine (m^5C), N6-methyladenosine (m^6A), 5-methyluridine (m^5U), and 2-thiouridine (S^2U). The presence of modified nucleotides in the mRNA prevents its recognition as a foreign molecule by endosomal sensors, such as Toll-like receptors (TLRs) 3, TLR7, and TLR8 or cytoplasmic sensors, such as RIG-I and MDA5, responsible for the induction of type I IFNs, typically associated with antiviral responses (Karikó et al. 2005; Nelson et al. 2020). The activation of type I IFN signaling pathways causes the suppression of mRNA translation and its degradation, and it can even induce host cell death via apoptosis (Nelson et al. 2020; Palchetti et al. 2015).

It has been reported that the incorporation of modified bases in the mRNA sequence reduces innate immune activation, thus improving its translation and activity (Karikó et al. 2005, 2008). The replacement of uridine with Ψ is the predominant modification employed in the preparation of synthetic mRNA. Its incorporation into mRNA has shown to increase the resistance to RNase degradation and to limit

TLR activation, with a consequent improvement of its translatability and transfection efficiency both in vitro and in vivo (Anderson et al. 2011; Svitkin 2017; Roy 2021).

Nowadays, many types of modified bases have been developed, including m^5C, N1-methylpseudouridine ($m^1\Psi$), and Ψ, and the impact of these modified nucleotides on mRNA's activity can be sequence-dependent and cell-type-dependent. In this regard, particularly interesting has been a study in which transfection of THP-1 macrophages with m^5C/Ψ-modified mRNA encoding firefly luciferase (Fluc mRNA) resulted in a higher translation rate compared to unmodified Fluc mRNA, while the incorporation of m^5C/Ψ modified nucleotide into mRNA encoding enhanced green fluorescence protein (eGFP mRNA) caused a decrease in protein production (Li et al. 2016). The authors also showed that transfection with m^5C/Ψ-modified mRNA generated a significantly higher expression of Fluc in THP-1 cells than in hepatocellular carcinoma Hep 3B cells.

2.1.5 Codon Optimization of the ORF

Codon optimization relies on the degeneracy of the genetic code, according to which different codons code for the same single amino acid (Mauro and Chappell 2014). The optimization of the ORF sequence is intended to replace codons with low levels of charged tRNAs with codons recognized by abundant tRNAs, so that the exogenous mRNA can be translated with higher efficiency without causing modifications to the amino acid sequence (Mauro and Chappell 2014). Using luciferase and erythropoietin coding mRNAs as model, it has been demonstrated that codon usage optimization of the ORF improves the translation rate and consequently the activity of IVT-mRNA (Thess et al. 2015). The authors found that unmodRNA incorporating codons rich in guanosine and cytosine induced higher systemic levels of erythropoietin and stronger physiological effects compared to Ψ-modified mRNA. However, in some cases high translation rate of mRNA is not desired since some proteins require a slower translation to correctly fold into biologically active forms, and the inclusion in the ORF of codons with low frequency ensures the generation of protein products of higher quality (Brule and Grayhack 2017).

In conclusion, specific codon optimization strategies should be applied depending on the type of protein encoding by the ORF sequence to improve mRNA translation rate and concomitantly ensure optimal protein expression levels.

2.2 Synthesis of IVT-mRNA

IVT-mRNA compared to more traditional gene therapy platforms, such as viral vectors and pDNA, presents the advantage that its production requires simple procedures that can be easily engineered to the required scale. Besides, once the manufacturing process is established, in principle it can be applied for any RNA sequence with essentially no size limitations. mRNA is synthesized in a cell-free system by

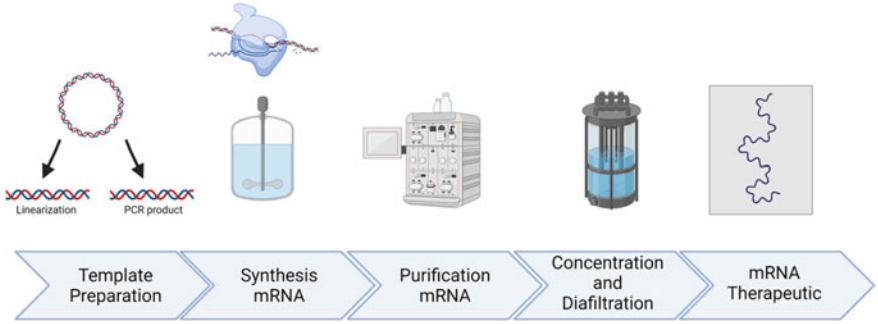

Fig. 2 Schematic illustration of the mRNA production process. The manufacturing of mRNA involves the preparation of a DNA template by PCR or pDNA linearization followed by a cell-free enzymatic in vitro transcription reaction. After synthesis, the mRNA is purified, concentrated, and diafiltered

in vitro transcription (Henderson et al. 2021), which does not foresee the use of hosts like bacteria, yeast, or mammalian cells, thus preventing the associated quality and safety concerns in the production (Fig. 2). Additionally, this host-free system allows to avoid complicated downstream purification procedures, consenting a rapid scale-up and cost-effective manufacturing. In vitro transcription utilizes a linearized pDNA or a PCR product containing a bacteriophage promoter (i.e., T7, T3 or SP6) as template for a bacteriophage DNA-dependent RNA polymerase, which recognizes the promoter within the DNA template and catalyzes the de novo synthesis of mRNA in the presence of ribonucleoside triphosphates (rNTPs) (Henderson et al. 2021). The mRNA sequence can be tailored to meet specific needs, concerning stability and translation efficiency, which can be modulated or enhanced by including further cis-acting elements such as 5′-cap structure and signal peptide (SP) or GSG linker, internal ribosome entry site (IRES) and 2A peptide sequences in multicistronic mRNAs (Mignone et al. 2002; Chng et al. 2015). Furthermore, the sequence of IVT-mRNAs can be optimized by changing the codon composition, and if needed, modified nucleotides can be inserted to improve its translatability and stability (Li et al. 2016; Thess et al. 2015). In vitro transcription has been commonly used for synthesizing both non-replicating mRNA and saRNA (Henderson et al. 2021; McKay et al. 2020).

The first step of in vitro RNA synthesis consists in the design of the DNA template containing a protein-encoding open reading frame (ORF) flanked by regulatory sequences (Henderson et al. 2021). Both pDNA and PCR products can be employed as a template for transcription. In a minimum composition, a pDNA must contain typical elements, such as a promoter sequence, an ORF sequence, 5′/3′-UTR sequences, a poly(A) tail, unique restriction endonuclease sites, a bacterial origin of replication (ori), and an antibiotic resistance gene (Avci-Adali et al. 2014). The ori and selectable marker in the vector backbone allow replication and selection of the plasmid in bacteria, potentially facilitating the establishment of a pDNA template bank.

In the case pDNA is utilized as template, purification, normally by chloroform/phenol extraction followed by ethanol precipitation (Dowhan 2012), and DNA linearization by digestion with a restriction enzyme producing blunt or 5′-overhang ends upstream the promoter sequence, are required steps (Henderson et al. 2021). PCR products for in vitro transcription are generated using a primer containing the desired bacteriophage promoter sequence (Henderson et al. 2021).

The RNA polymerase initiates transcription of the DNA template in the presence of natural rNTPs or with chemically modified rNTPs (5-methoxy-UTP, pseudo-UTP, etc.) to produce several copies of RNA molecules. The DNA template can be removed from the mRNA preparation by treatment with DNase (Henderson et al. 2021).

Functional RNA molecules require a cap structure at the 5′ end and a poly(A) tail at the 3′ end. Regarding the capping of the IVT-mRNA, it can be achieved by two different approaches, enzymatic capping, using vaccinia virus-derived enzymes, or a co-transcriptional method (Henderson et al. 2021; Muttach et al. 2017).

The enzymatic method utilizes a 2′-O-methyltransferase, which consents the generation of a cap 1 structure with a potential capping yield of 100%. However, the enzymatic method has exhibited some drawbacks that have recently limited its use, including a high variation in the capping efficiency, the requirement of an unstable temperature-labile cofactor (S-Adenosylmethionine), and high scale-up costs. For all these reasons, co-transcriptional methods are currently preferred for mRNA capping (Henderson et al. 2021).

In the co-transcriptional method, cap analogs are incorporated directly at the 5′-end of the IVT-mRNA by RNA polymerases, and erroneous internal incorporation of cap analogs during mRNA polymerization cannot occur since cap analogs lack a free 5′-triphosphate. The cap structure m^7GpppG represents the most largely employed cap analog (Muttach et al. 2017; Kocmik et al. 2018), but many other alternative cap analogs have also demonstrated good compatibility with commonly employed T7 bacteriophage RNA polymerase. A major limitation of this first generation co-transcriptional capping is that cap analogs compete with GTP as initiator nucleotide and, therefore, the method exhibits variable capping efficiency and reproducibility (Muttach et al. 2017; Kocmik et al. 2018). Additionally, the use of this first generation of cap analogs as nucleotide initiators can cause cap incorporation in reverse directions, due to the presence of a 3′-OH group on m^7G; thus, up to one half of the mRNA contains the cap in the wrong orientation and it is not translatable (Muttach et al. 2017; Kocmik et al. 2018).

This problem was solved by developing a second generation of cap structures named anti-reverse cap analogs (ARCA) with a methylated or deoxygenated 3′-OH group at the N7-methylguanosine ribose ($m^{7,3'\text{-O}}$GpppG or $m^{7,3'\text{-d}}$GpppG) (Kocmik et al. 2018). This prevents elongation at the "wrong" 3′-OH, avoiding the incorporation of ARCA analogs in the reverse orientation. Nevertheless, ARCA capping has the disadvantage that only cap 0 mRNAs can be prepared, and the cap structure is characterized by the presence of an unnatural 3′-O-methyl group that could be promptly recognized by innate immune receptors. The introduction of the ARCA cap analog is ensured by conducting the transcription reaction using an excess of ARCA over GTP (ARCA:GTP ratio of 4:1) (Kocmik et al. 2018; Liu et al. 2019). However, even if

working at optimized conditions, the capping efficiency rarely exceeds 80%, which means that at least 20% of IVT-mRNA possesses uncapped 5′-triphosphate ends, thus requiring additional purification steps. Due to the strict preference of bacteriophage RNA polymerases for G or A, depending on the promotor, artificial mRNAs starting with a U or C at the 5′-end cannot be prepared using in vitro transcription (Henderson et al. 2021).

Trilink BioTechnologies, a US biotech company, has recently developed CleanCap® mRNA, a third generation of co-transcriptional capping technology (Henderson et al. 2021). CleanCap® results in the incorporation of N6-methyladenosine methylated cap (m^6A or m^6Am) at the 5′-end. This method offers important technical advantages compared to previously proposed cap analogs, since it displays superior efficiency and reproducibility (94–99% of complete capping), yields a natural unmodified cap structure (cap 1) with reduced immunogenicity, possesses a greater cost-effectiveness than enzymatic capping, and is easily scalable for large-scale manufacturing (Henderson et al. 2021).

The addition of a poly(A) tail to the synthetic mRNA can be performed either post-transcriptionally using a poly(A) polymerase or co-transcriptionally by including a poly-A tail in the DNA template. While a poly(A) tail with a maximum length of 80 nt can be incorporated into the pDNA, since longer poly(A) sequences can give stability problems due to recombination events that may occur in the host bacteria, by using the poly(A) polymerase no inherent length limitation of poly(A) tail synthesis has been found (Chaudhary et al. 2021). However, the enzymatic incorporation of poly(A) does not consent a high reproducibility, and consequently, the poly(A) products display a larger and more variable size distribution compared to those obtained using a co-transcriptional technique. A solution to address this stability issue is to include a short linker sequence in the poly(A) tail (Trepotec et al. 2019). In addition, enzymatic polyadenylation is less affordable and inadequate for scaled-up manufacturing.

Thanks to the enormous progress in the field, 5′-capped and 3′-poly-adenylated mRNAs can be easily produced in “one-pot” reaction, achieving cap and poly(A) tail additions concomitantly during the in vitro transcription of synthetic mRNA (Henderson et al. 2021).

2.3 Purification of Synthetic mRNA

IVT-mRNA purification is required to ensure the removal of contaminants that may affect the therapeutic performance of the mRNA molecules. This includes the residual DNA template, unincorporated rNTPs and cap analogs, enzymes employed in the reaction, truncated mRNA products, and double-stranded mRNA. The method chosen for purification depends on the length and abundance of the IVT-mRNA, the type of impurities (nucleic acids and/or proteins), and the type of downstream application.

Standard purification methods include lithium chloride (LiCl) precipitation, alcohol-based precipitation (i.e., ethanol precipitation), and techniques based on

silica membranes (i.e., spin columns) (Henderson et al. 2021; Walker and Lorsch 2013). LiCl precipitation is employed to remove the majority of the unincorporated rNTPs and enzymes used for the synthesis of IVT-mRNA. Instead, ethanol precipitation can ensure the complete removal of free rNTPs, salts, and proteins (Walker and Lorsch 2013; Rio et al. 2010). Silica membranes selectively bind nucleic acids, allowing the elimination of salts, free rNTPs, and proteins (Baronti et al. 2018). Such membranes will retain the intact DNA template, and its removal requires pre-treatment with DNase after in vitro transcription.

None of these techniques are effective in the removal of truncated RNA and dsRNA impurities generated from abortive initiation of in vitro mRNA synthesis. Previous studies have identified dsRNA and truncated RNA as the contaminants that mostly affect IVT-mRNA's translation efficiency, as they trigger innate immunity through their recognition by RNA sensors. To date, the most common method employed to eliminate nucleic acid contaminants from IVT-mRNAs is reversed-phase high-performance liquid chromatography (HPLC) (Karikó et al. 2011). However, this procedure requires extremely toxic solvents and is not suitable for the large-scale production of mRNA. Moreover, reversed-phase HPLC is less efficient at purifying very large molecules of mRNA.

Alternative methods have been proposed for the purification of synthetic mRNA, including ion-exchange chromatography, oligo(dT) affinity chromatography, and other separation techniques that rely on differences in size, such as size exclusion chromatography (SEC) and cross-flow filtration (CFF) (Baiersdörfer et al. 2019).

Recently, a simple, fast, and cost-effective way to eliminate dsRNA contaminants from IVT-mRNA has been reported (Baiersdörfer et al. 2019). This method is based on the selective binding of dsRNA to cellulose in an ethanol-containing buffer. The authors showed that at least 90% of the dsRNA impurities can be removed with a good recovery rate (>65%), independently from the length and nucleoside composition of the IVT-mRNA. The different purification methods can also be combined to improve the purity of IVT-mRNA.

3 Lipid Nanoparticles for Therapeutic mRNA Delivery

The inability of naked mRNA to cross the outer membranes of cells thus to reach the cytoplasmic compartment, together with its lack of stability under physiological conditions, represent the major barriers impeding the complete exploitation of mRNA-based therapies (Guevara et al. 2020).

Various strategies have been proposed for enabling efficient delivery of mRNA into target cells, including chemical modification of mRNA (Zangi et al. 2013), ionic complexation with cationic polymers, physical methods (i.e., electroporation) (Van Tendeloo et al. 2001), and viral vectors (Segel et al. 2021), but so far, lipid nanoparticles (LNPs) have demonstrated the most encouraging results (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020; Kranz et al. 2016). The intense research

efforts dedicated toward the development of LNP as mRNA delivery systems recently culminated in the approval of two mRNA-based vaccines for clinical use (Dolgin 2021a, b), and the development of a growing number of clinical trials currently ongoing (Table 1).

LNPs have shown to efficiently protect mRNA from hydrolysis by RNases and at the same time allow endosomal escape so to achieve mRNA delivery into the cytosol of specific cells (Sago et al. 2018; Cheng et al. 2020). Once in the cytosol, it can be sequestered by the translation machinery for initiating protein synthesis.

Compared to the most popular viral vectors, LNPs are less immunogenic, they can carry larger genetic material payloads and are easier to manufacture. Therefore, even if usually LNPs exhibit lower transfection efficiencies than viral vectors, they are becoming the preferred tool for mRNA transfection.

LNPs are self-assembled nanostructures with a size of approximately 100 nm, consisting of different lipid components that can be grouped in three major types: ionizable or permanently cationic lipids, helper lipids, and stealth lipids (i.e., PEGylated lipids) (Fig. 3) (Guevara 2019b; Guevara et al. 2020). Ionizable cationic lipids are usually preferred over permanently charged cationic lipids, as they exhibit higher biocompatibility and efficiency. A great number of screening studies, testing a variety of ionizable lipids composed of different combinations of hydrophilic head groups and non-polar lipid tails, have allowed to increase the number of available lipids (Billingsley et al. 2020; Miao et al. 2020; Guimaraes et al. 2019; Carrasco et al. 2021). These studies showed that the performance of ionizable lipids is controlled by the chemical and structural characteristics of both the head group and lipid tail region. The main feature of ionizable lipids is their ability to respond to an acidic pH, which is usually defined by the pKa value. A single or a mixture of ionizable

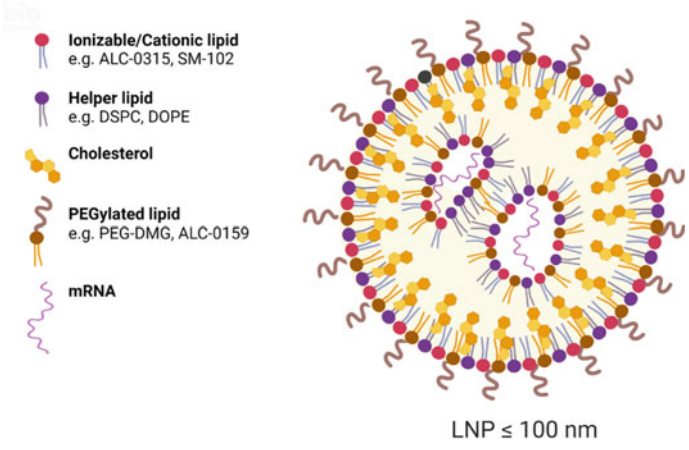

Fig. 3 Schematic of mRNA LNPs. LNPs are composed of four components, such as ionizable lipid (e.g., ALC-0315, SM-102), helper lipid (e.g., DSPC, DOPE), cholesterol, and PEGylated lipid (e.g., PEG-DMG, ALC-0159). The molecules of mRNA within the LNP are confined into aqueous regions

lipids determines the overall pKa of the LNPs that needs to be around 6.5, as established using the anionic fluorescent dye 2-(p-toluidino)-6-naphthalenesulfonic acid (TNS) binding assay (Guevara et al. 2020; Carrasco et al. 2021). At neutral pH, these lipids are in a zwitterionic form or do not possess any charged functional groups, and only upon internalization in the endosomal compartment of the cell, where the pH is near 5, the head group of the lipids is protonated and assumes a net cationic charge that promotes its interaction with the anionic endogenous endosomal phospholipids. Ionizable lipids with a conical shape are more desirable since this conformation is incompatible with a lipid bilayer organization, thus favoring the destabilization of the endosomal membrane and allowing the mRNA payload to be released into the cytosol (Carrasco et al. 2021). Regarding the lipid tail portion, a series of features like length of the hydrophobic tail, level of unsaturation, and presence of branches have been found to affect dramatically the transfection capability of LNPs (Carrasco et al. 2021).

Other components, like helper lipids, are included in the formulation to enhance the stability and delivery efficiency of LNPs, whereas PEGylated lipids are essential to reduce the opsonization of LNPs by serum proteins, which drive their undesired high accumulation in off-target organs (e.g., liver), and rapid clearance from bloodstream (Guevara et al. 2020; Patel et al. 2020). As recently demonstrated, the interaction of LNPs with serum proteins is dictated by mechanisms that are more complicated than it was initially thought and that do not rely only on the net charge of LNPs but involve other factors that still need to be determined and that deserve further attention (Miao et al. 2020).

The relative abundance of ionizable lipid, helper lipid, and stealth lipid critically determines the efficacy of LNPs and therefore needs to be opportunely optimized depending on the application and according to the administration route that is intended to be used (Guevara et al. 2020; Hassett et al. 2019; Ryals et al. 2020; Ndeupen et al. 2021). The number of possible options in the design of LNPs has been further increased due to the recent evidence that points out that different ionizable lipids can synergize in boosting mRNA transfection (Miao et al. 2020). Moreover, even if this has not been proven yet, it is highly plausible that the same synergism might be observed between helper lipids that are considered chemically and functionally equivalent.

In addition to the lipid composition, other features such as size and surface charge are known to have an enormous impact on the behavior of LNPs in vivo, and hence, it is pivotal to appropriately tune these parameters to achieve the desired therapeutic outcomes (Cheng et al. 2020; Ryals et al. 2020; Ndeupen et al. 2021; Nakamura et al. 2020; Hassett et al. 2021). The most common helper lipids that have been tested are 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol. DSPC is a phosphatidylcholine with saturated hydrophobic tails and a cylindrical shape that allows DSPC molecules to organize in a lamellar phase, which stabilizes the structure of LNPs (Guevara et al. 2020; Hou et al. 2021). DOPE is a phosphoethanolamine with a cis-unsaturated double bond in the two oleyl fatty acid chains. It exhibits a conical shape and

adopts an inverted hexagonal H(II) phase at acidic pH, which destabilizes endosomal membranes and facilitates the endosomal escape of LNPs (Guevara et al. 2020).

Nowadays, solvent injection, also known as nanoprecipitation (or antisolvent precipitation), is the most common technique used for the preparation of mRNA-loaded LNPs (Guevara et al. 2020; Hou 2021). In the last years, the implementation of microfluidic devices for LNP manufacturing has improved the reproducibility and scalability of the method. In these systems, the mixing of an organic solution, containing a mixture of lipids dissolved in an organic solvent (i.e., ethanol), with an aqueous solution, in which the mRNA molecules are dissolved, is realized in microchannels molded on a chip. The channels are designed in a way that the liquids are forced to flow in two separate microchannels and then come into contact at the crossing of channels, thus promoting the self-assembly of lipids into LNPs at the interfacial layer where the lipids are exposed to an environment with increased polarity (Fig. 4) (Guevara et al. 2020).

Microfluidic chips can be prepared with different mixing patterns, such as standard T- junction, microfluidic hydrodynamic flow focusing (HFF), microfluidic micromixer (MM), and staggered herringbone micromixer (SHM) (Riewe et al. 2020). The widely utilized NanoAssemblr™ platform employs a Y-shaped architecture incorporating SHM pattern. The channel configuration together with the flow rate are important parameters affecting dramatically the physicochemical characteristics of LNPs (Guevara et al. 2020; Riewe et al. 2020; Roces et al. 2020). LNPs with

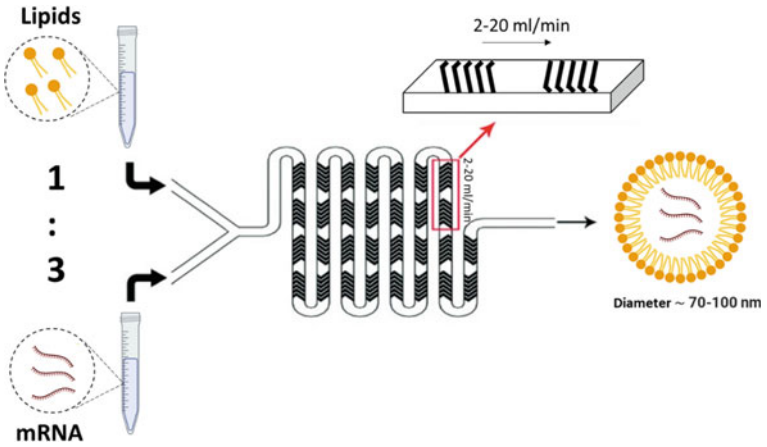

Fig. 4 Schematic of mRNA-loaded LNPs preparation by a microfluidic system with a SHM configuration. One volume of ethanol solution containing a mixture of lipids and three volumes of mRNA dissolved in an acidic aqueous solution are pumped separately into two distinct inlets of the SHM chip by a syringe pump at a flow rate of 2–20 ml/minute. The SHM design favors a rapid mixing of the ethanol and aqueous phases with consequent increases of the net polarity of the lipid solution. Above a certain threshold of polarity, the lipids precipitate as form of LNPs with a size usually ranging from 70 to 100 nm. The ionic interactions taking place between the negative charges of the phosphate groups (P) in mRNA molecules and the positive charges of the amino groups (N) in cationic/ionizable lipids are the force that drives the encapsulation of the mRNA into LNPs

a smaller size and narrow size distribution are typically produced with a higher flow rate (Roces et al. 2020).

4 mRNA-Based Cancer Immunotherapy: Antitumor Vaccines

Cancer immunotherapy represents the major area of research where mRNA has found application as therapeutic agent. It has been demonstrated that the mRNA technology can be utilized to create different immunotherapeutic products, such as vaccines, monoclonal antibodies, chimeric antigen receptor (CAR) cells, and immunomodulatory proteins (Persano et al. 2017; Stadler et al. 2017; Thran et al. 2017; Guevara 2019a; Kong et al. 2019; Rybakova et al. 2019; Wei et al. 2020; Lai et al. 2018).

Cancer vaccines are the most advanced application of mRNA, with both prophylactic and therapeutic potentials and relying on the capability of mRNA to simultaneously deliver genetic information and act as immunoadjuvant by interacting with innate immune receptors (Persano et al. 2017; Stadler et al. 2017; Kranz et al. 2016; Guimaraes et al. 2019). The immunomodulatory properties of mRNA can be particularly relevant for the development of antitumor vaccines that require overcoming of immune tolerance, a characteristic of many malignancies (Kranz 2016). On the other hand, upon recognition of the RNA by innate immune receptors, IFN-I triggers the expression of interferon-stimulated genes (ISGs), such as IFN-inducible double-stranded RNA-activated protein kinase (PKR), and 2′,5′-oligoadenylate synthetase (OAS), with consequent decreased translation and increased decay of mRNA (De Beuckelaer et al. 2016; Yang and Shah 2020). This could be extremely deleterious for other applications of mRNA, including chimeric antigen receptor (CAR) cell therapy and gene therapy, for which maximization of the expression might be essential for ensuring a therapeutic effect. In these cases, the use of modified nucleosides, such as pseudouridine, N1-methylpseudouridine, and 2-thiouridine, is highly desirable in order to increase the translation efficiency of synthetic mRNAs (Karikó et al. 2008; Svitkin et al. 2017).

Vaccines can be designed to target tumor-associated antigens (TAAs) (overexpressed antigens, tissue differentiation antigens, and tumor germline antigens) or tumor-specific antigens (TSAs) (oncoviral antigens and neoantigens), thus promoting an antitumor response that specifically attacks and destroys cancer cells and achieves a prolonged response and prevention of relapse due to the generation of an immunological memory (Kranz et al. 2016; Hollingsworth and Jansen 2019).

Recently, several studies have pointed out the importance of neoantigens as targets for immunotherapy (Sahin et al. 2017; Cafri et al. 2020; Blass and Ott 2021). The considerable progresses made in sequencing technologies and bioinformatic tools have permitted to reveal that neoantigen-specific $CD8^+$ and $CD4^+$ T cells are detectable in most tumors, independently if they have a viral etiology (Hollingsworth

and Jansen 2019; Sahin et al. 2017; Cafri et al. 2020; Blass and Ott 2021). Neoantigens are the consequence of somatic mutations that occur in malignant cells during cancer progression due to the high genomic instability typical of tumors. This class of TSAs is particularly appealing because, unlike TAAs, they are detectable only in cancer cells, therefore not subjected to central tolerance, and are characterized by a high immunogenicity, high affinity toward the MHC, and individual specificity (Hollingsworth and Jansen 2019; Blass and Ott 2021).

The first step in neoantigen identification is the comparison of whole exome sequencing (WES) or mRNA sequencing (mRNA-Seq) data from tumor and normal tissues obtained using high-throughput sequencing techniques (i.e., next-generation sequencing (NGS)) (Blass and Ott 2021; Esprit et al. 2020). Then, the data from the sequencing analysis are analyzed with bioinformatic tools to predict whether the identified mutations can generate tumor neoantigens (Esprit et al. 2020). Most of these bioinformatic softwares are based on HLA binding affinity and tend to ignore other important factors that have an impact on the antigen presentation process, such as the C-terminal cleavage by proteasome, efficiency of transporter associated with antigen processing (TAP)-mediated transport of peptides, expression abundance of neoantigens, tumor heterogeneity, heterogeneity and clonality of neoantigens, and loss of heterozygosity of HLA.

A vaccination platform capable of targeting multiple patient-specific antigens is highly desirable for developing personalized neoantigen vaccines with enhanced immunogenicity. In this regard, synthetic mRNA offers the possibility to easily incorporate multiple neoantigens in a single molecule that can be manufactured with a cost-effective and scalable approach.

Preliminary studies on mRNA-based neoantigen vaccines were conducted using mRNA-electroporated dendritic cells (DCs) (Wilgenhof et al. 2013). However, previous studies have reported that several factors may intervene in limiting the efficacy of DC vaccines including optimal maturation, subset of cells employed, antigen-loading efficiency, and the ability of DCs to migrate to vaccine-draining lymph nodes (Santos and Butterfield 2018). Therefore, currently most of the groups working in the area of mRNA vaccines are switching from DC platforms toward strategies that involve the delivery of neoantigen mRNAs into APCs directly in vivo, thus avoiding DC isolation and manipulation ex vivo.

The first proof of concept that in vivo delivery of tumor antigen-encoding mRNA into APCs is an achievable path for successful antitumor vaccination was reported only few years ago. For the first time, it was shown that DCs can be passively targeted in vivo upon systemic administration of mRNA-carrying lipoplexes displaying a negative net charge (Kranz et al. 2016). Vaccination with mRNA lipoplexes encoding tumor-specific or tumor-associated antigens stimulated strong type I IFN-dependent effector and memory T cell responses resulting in tumor rejection and protection from tumor rechallenge.

In situ vaccination is an alternative form of vaccine effective at eliciting antigen-specific T cell responses. This is achieved using a cytotoxic agent, alone or in combination with an immunoadjuvant, able to induce immunogenic cell death (ICD), which not only directly kills tumor cells, but also promotes the release of tumor antigens

and molecular signals that promote the activation of immune cells that are able to reach even distant cancer cells.

The use of mRNA-based therapeutics has also been proposed to induce ICD in tumor cells. For this scope, it has been utilized a mRNA encoding proapoptotic proteins, caspase or PUMA, and including in the 3′-UTR microRNA (miRNA) target sites to minimize the expression of the proteins in healthy hepatocytes, thus preventing side effects due to off-target expression (Jain et al. 2018).

4.1 mRNA-Loaded LNP-Mediated Monoclonal Antibody Delivery

Monoclonal antibodies are emerging as one of the most promising classes of cancer immunotherapy, so much so that several antibodies have received approval for clinical use for the treatment of several forms of cancers (Boyiadzis and Foon 2018; Mullard 2021). Antibodies have been designed to target specific proteins expressed on tumor cells and immune cells or released into the tumor microenvironment. The use of monoclonal antibodies for targeting immune checkpoints and inhibiting their functions (immune checkpoint inhibitors, ICIs) represents one of the most investigated areas of application.

Several types of ICIs have been or are currently under investigation, and many of them have received clinical approval for the treatment of different tumors (Gravbrot et al. 2019). Despite the encouraging outcomes from preclinical and clinical studies, and the fact that many patients have already benefited from the use of monoclonal antibodies, there are still concerns regarding this type of therapeutic agents that limit their wider application in the clinic (de Miguel and Calvo 2020; Palmieri and Carlino 2018; Chames et al. 2009; Hernandez et al. 2018). Major concerns are mainly related to the complex and expensive procedures required for their manufacturing and purification (Chames et al. 2009; Hernandez et al. 2018). Therapeutic antibodies are typically full-size immunoglobulins (Ig), mostly of the IgG type, which require a wide variety of post-translational modifications, including glycosylation, disulfide bond formation, and many other modifications that cannot be introduced synthetically (Jank et al. 2019; Yang and Li 2020; Lu et al. 2020). For this reason, their preparation is commonly realized in mammalian cell lines (Lu et al. 2020; Dangi et al. 2018). Then, a purification step is required to have an injectable antibody therapeutic free from any potential harmful contaminants. Given that these modifications can directly affect the functionality of monoclonal antibodies, it is of essential importance to implement analytical assays that can ensure the quality of the product. All these aspects contribute to the elevated costs of antibody-based treatments and make these therapies poorly affordable. To achieve the synthesis of functional antibodies in procaryotic expression systems, like *E. coli*, that can enable faster and cheaper production, different types of antibody fragments, such as single-chain variable fragments (scFv), heavy-chain-only VH (VHH) domains, and nanobodies, have

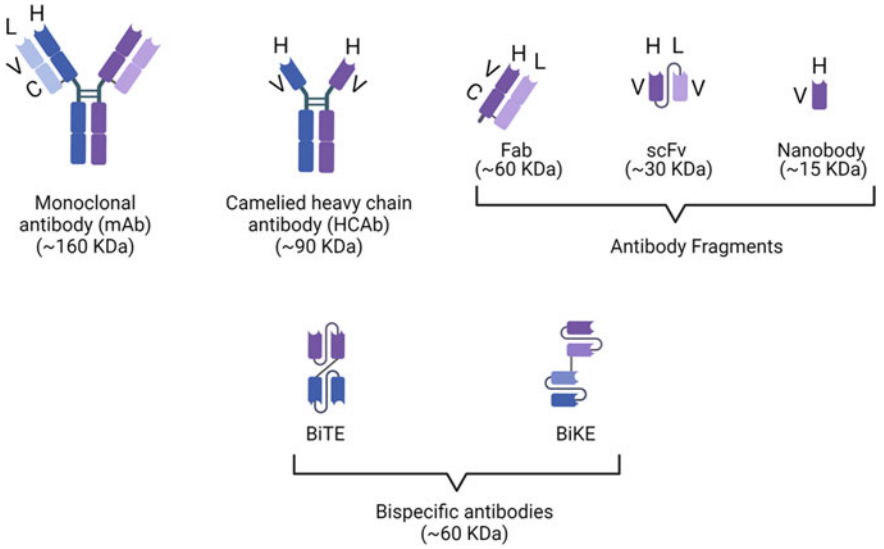

Fig. 5 Illustration of the different types of antibodies: monoclonal antibody, camelid heavy-chain antibody (HCAb), antigen-binding fragment (Fab), single-chain fragment variable (scFv), nanobody, bispecific T cell engagers (BiTE), and bispecific killer cells engagers (BiKE)

been developed (Fig. 5) (Jank et al. 2019). These are smaller than conventional antibodies and lack glycosylation. Another advantage of antibody fragments is that the small size can improve their penetration ability into tissues that are not reachable by conventional full-size antibodies, which is advantageous for many therapeutic applications. The antibody fragment technology has been also used to generate bispecific antibodies, and chimeric antigen receptors (CARs) usually consisting of scFv linked to intracellular signaling molecules, capable of triggering T cell effector activities (Hernandez et al. 2018; Jank e al. 2019; Yang and Li 2020). Yet, antibody fragments are cleared from circulation much more rapidly than conventional full-size antibodies, and since they do not have the Fc domain, fragments are unable to elicit Fc-mediated cytotoxicity (Jank et al. 2019; Yang and Li 2020).

The use of mRNA-loaded LNPs has recently emerged as a promising approach to overcome current limitations of monoclonal antibodies by consenting the production of a specific antibody directly in the body of the patient, circumventing the complicated and expensive purification steps and thereby avoiding the batch-to-batch variation that can be found when using antibodies (Van Hoecke and Roose 2019). Since IVT-mRNA contains all the instructions for appropriate folding and assembly, and for post-translational modifications, the antibodies generated from exogenous mRNA are perfectly functional. A potential limitation of this strategy is that only antibodies with natural modifications can be produced, and it cannot be employed for delivering antibodies conjugated to synthetic molecules, such as polyethylene glycol (PEG), which can extend the blood circulation half-life of antibody fragments. However, recent studies demonstrated that mRNA-mediated delivery can enhance the serum

half-life of both full-size and fragment antibodies which can improve the therapeutic efficacy of these treatments (Rybakova et al. 2019; Tiwari et al. 2018).

The first proof of the feasibility of mRNA-mediated delivery of therapeutic antibodies was reported in 2017 (Pardi et al. 2017). In that study, passive vaccination was achieved by systemic administration of LNPs carrying a modRNA encoding both light and heavy chains of an anti-HIV-1 neutralizing antibody. Similarly, the mRNA technology can be also exploited to obtain in vivo production of bispecific antibodies with two binding domains directed against a tumor antigen and CD3 marker, able to redirect and activate the antitumoral action of circulating T cells (Stadler et al. 2017). A single dose of 3–5 μg of formulated mRNA was sufficient to induce a rapid synthesis of bispecific antibodies and triggered complete eradication of advanced tumors. To obtain a comparable outcome with a recombinant bispecific antibody, it was necessary to give a dose three times higher than that of formulated mRNA. While most of the reported studies have achieved passive immunization through intravenous administration of mRNA-loaded nanoparticles, very recently it has been shown that intramuscular injection of formulated saRNA encoding an anti-Zika virus neutralizing human antibody (ZIKV-117) also induced high antibody titers and protected mice from Zika infection (Erasmus et al. 2018).

Taken together, these studies clearly show the potential of mRNA-loaded delivery systems for in vivo production of therapeutic antibodies, offering in this way a series of advantages compared recombinant antibodies, including reduced costs and prolonged serum half-life, thereby making antibody-based treatments more effective and accessible to a larger portion of patients.

4.2 mRNA-Loaded LNPs for CAR Immune Cell Engineering

CAR cell therapy is considered the most advanced modality of personalized immunotherapy in which immune cells, like T cells or NK cells, are isolated from a patient or a donor, genetically engineered ex vivo and ultimately infused into the patient (Sterner and Sterner 2021). The efficacy of adoptive T cell therapy has been demonstrated by numerous clinical trials showing remarkable outcomes in relapsed or refractory hematologic cancers. These clinical successes have led to the approval of CAR T cell products for children with acute lymphoblastic leukemia and adults with large B cell lymphoma by two of the major regulatory agencies, the FDA and the EMA (Sterner and Sterner 2021; Lin et al. 2021).

Despite these encouraging premises, CAR cell therapies suffer from considerable limitations concerning toxicity, primarily cytokine release syndrome (CRS) and neurologic adverse effects, and the high costs and complex procedures involved in the manufacturing of CAR cell-based treatments (Sterner and Sterner 2021; Lin et al. 2021). Interleukin 6 (IL-6) seems to be the major responsible for CRS since elevated levels of IL-6 have been observed in these patients and in murine models of the disease (Kishimoto 2021). While CRS may be alleviated through the administration

of tocilizumab, an anti-IL-6 receptor antibody, costs, and manufacturing challenges may be addressed with the advent of mRNA technology.

Viral transduction and electroporation are the most common techniques for CAR introduction into T cells (Van Hoecke and Roose 2019). However, both strategies present limitations. Viral vectors are associated with limited genetic cargo, safety concerns, and high costs, whereas electroporation can result in reduced viability, aberrant gene expression profile, and relative low transgene expression in the surviving transfected cells (Van Hoecke and Roose 2019). Therefore, alternative strategies for CAR delivery are highly desirable.

In recent years, ionizable lipid nanoparticles encapsulating mRNA encoding CAR have been extensively tested in preclinical studies for their ability to transfect immune effector cells either in vivo or ex vivo. A large screening study has allowed to identify seven distinct formulations capable of enhanced mRNA transfection of Jurkat T cells over lipofectamine (Billingsley et al. 2020). The best performing LNP formulation of these was tested with primary human T cells, displaying a CAR transfection efficiency equivalent to those observed with electroporation, but with a significantly inferior cytotoxicity. The potent killing activity of CAR T cells generated by transfection with mRNA LNPs was proven in a coculture assay with acute lymphoblastic leukemia cells.

Lately, in vivo targeting and transfection of lymphocytes have emerged as a fascinating viable route for simple and cost-effective generation of CAR T cells directly in the body of the patient. On this regard, an injectable LNP formulation functionalized on the surface with anti-CD3 antibody was developed for active targeting and mRNA transfection of circulating T cells, to induce transient expression of CAR or TCR recognizing disease-relevant targets (Smith et al. 2017).

5 Future Prospective and Conclusions

IVT-mRNA has the unprecedented potential to address major challenges of current immunotherapies and offers the basis for the development of innovative cancer therapies. The enormous progress in LNP formulations along with a better understanding of mRNA translation regulation has allowed the development of numerous mRNA-based treatments successfully tested in preclinical settings and currently under investigation in clinical trials.

Although considerable strides have been made in the design and manufacturing of LNP formulated mRNA-based therapies, to leverage the full potential of mRNA technology it is still needed to improve the transfection and targeting efficiency of LNPs and to increase the translatability of mRNA molecules by the engineering of the RNA sequence.

As discussed throughout this chapter, for its adequate activity, eukaryotic mRNA requires five structural elements, the cap structure, poly(A) tail, protein-coding sequence, and 5′ and 3′ UTRs. These elements are pivotal in regulating translation initiation, translation termination, stability, decapping, and post-transcriptional

modifications of mRNA. Thus, sequence optimization of IVT-mRNA can maximize the expression of the therapeutic protein in vivo.

In addition, alternative forms of RNA such as saRNA and most recently circular RNA (circRNA) have been proposed to enhance mRNA properties (Holdt et al. 2018; Wesselhoeft et al. 2019). circRNA is particularly appealing since, unlike linear RNA, it has no 5′ cap structure and poly(A) tail, and IRES sequences are harnessed to ensure maximum protein synthesis. Given that circRNA lacks free 5′ end cap and a 3′ poly(A) tail, this kind of RNA resists to exonuclease digestion and, therefore, has a longer half-life than conventional linear mRNA. Thus, the use of circRNA may further revolutionize the mRNA field in the coming years.

From the formulation standpoint, despite the encouraging results from passive targeting approaches by modulating physicochemical properties of LNPs, like charge and size, the ability of LNP to reach certain sites in the body or specific cell populations within organs with not relevant or absent off-target accumulation needs to be significantly improved. Indeed, it is clear from studies performed in small animal models that current LNP formulations generally suffer from low specificity, with the tendency to be sequestered by the reticuloendothelial system (RES) of the liver and spleen, or accumulate in the first draining organs (e.g., lungs) after intravenous administration. In addition, if compared to their viral counterpart, LNP-based non-viral vectors usually exhibit much lower transfection efficiencies.

The transfection efficiency of LNPs could be potentially improved, for example, by the rational design of ionizable lipids with optimized head groups and hydrophobic tails so to increase their ability to promote endosomal escape upon internalization by target cells. The incorporation of well-defined helper lipids into the formulation can also play a crucial role in enhancing the overall transfection efficiency of LNPs. Furthermore, hybrid delivery systems including, for instance, pH-responsive polymers (e.g., β-amino ester), or molecules that are known to enhance mRNA delivery by altering the endocytic pathway, can further enhance the endosomal escape of mRNA.

Selectivity of LNP formulated mRNAs can be improved by modulating the structure of the single lipids included in the formulation and the overall lipid composition of LNPs. For instance, modification of the alkyl length of ionizable lipids leads selective accumulation of mRNA-loaded LNPs in the liver or spleen (Fenton et al. 2018). In another study, the impact of cholesterol derivates on cell selectivity of LNPs was investigated. The results of this study demonstrated that the tropism of LNPs in liver endothelial cells, Kupffer cells, and hepatocytes strictly depends on cholesterol structures (Patel et al. 2020).

Finally, biodegradability and immunogenicity are important aspects that need to be considered throughout the design of novel lipid components. Indeed, biodegradability can promote fast elimination of the LNP components, thus minimizing any potential toxicity effect. mRNA-based medicines can either benefit from the intrinsic immunogenicity of lipids, especially in the case of anti-cancer vaccines, or this immunogenicity can be detrimental, by altering mRNA translation and/or causing undesirable adverse effects.

In summary, mRNA platforms are suitable for the treatment of a wide variety of pathologies since they allow the development of any protein-based therapy. However, despite the great therapeutic potential confirmed in a number of clinical trials with diverse applications, mRNA-loaded LNPs could still benefit from further studies aimed at improving the selectivity, transfection efficiency, and toxicity of LNP formulations and increase mRNA stability and translatability. All this together can ensure the development of next generation of mRNA-based therapies with superior therapeutic properties.

Acknowledgements The authors gratefully acknowledge partial financial support from the Horizon 2020 Marie Skłodowska-Curie Actions (H2020-MSCA) program (Grant No. 843838 to S.P.).

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RNA/Polymer-Based Supramolecular Approaches for mRNA Delivery

Eger Boonstra, Satoshi Uchida, and Horacio Cabral

Contents

Abstract Messenger RNA (mRNA) therapeutics garner growing attention, especially after the approval of two mRNA vaccine formulations for COVID-19. Meanwhile, as a therapeutic modality, mRNA still has issues of poor bioavailability, showing rapid enzymatic degradation in physiological environments and inducing uncontrollable inflammatory responses. Chemical modification of mRNA is a prevalent approach to these issues and effectively reduces mRNA immunogenicity. However, available modified nucleotide species are limited to protect mRNA from enzymatic attacks with preserved mRNA translational activity. Alternatively, in supramolecular approaches, mRNA can be formulated with other molecules to improve its bioavailability. This approach requires minimal modification of mRNA and thus preserves its translational activity. Nano-particulated mRNA formulations using lipids and polymers are widely studied. Among them, polymeric micelles effectively prevent enzymatic mRNA degradation in biological milieu after recent advances in polymer design, allowing safe and efficient mRNA delivery to various

E. Boonstra · H. Cabral (✉)
Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo 113-8656, Tokyo, Japan
e-mail: horacio@bmw.t.u-tokyo.ac.jp

S. Uchida
Medical Chemistry, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 1-5 Shimogamohangi-cho, Sakyo-ku, Kyoto 606-0823, Japan

Innovation Center of Nano Medicine (iCONM), Kawasaki Institute of Industrial Promotion, Kawasaki 210-0821, Kanagawa, Japan

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_15

organs. Supramolecular approaches also include mRNA formulation using complementary RNA oligonucleotides, which allows the installation of protective moieties to mRNA or crosslinking of several mRNA strands to improve mRNA stability against nucleases. This chapter reviews these strategies to effectively transport mRNA therapeutics to target cells in vivo.

Keywords RNA delivery · Polyplex · Polymeric micelle · RNA architectonics · Supramolecular assembly

1 Introduction

In the field of nucleic acid therapeutics, messenger RNA (mRNA) is a particularly attractive candidate by virtue of its superior safety compared to DNA and the ability to induce protein expression in hard to transfect, non-dividing cells. Advances in molecular techniques which allowed the in vitro generation of mRNA with any desired sequence opened up a new generation of molecular therapeutics (Sahin et al. 2014; Uchida et al. 2020). The ability to produce large amounts of therapeutic protein in situ is a very versatile tool in combating a wide variety of diseases. Consequently, great efforts have been devoted to developing therapeutic applications using mRNA. Recently, the remarkably rapid development and deployment of mRNA-based vaccines against COVID-19 illustrates the flexibility and therapeutic capabilities of this technology (Polack et al. 2020; Baden et al. 2021). However, to unlock the full potential of mRNA there are still hurdles to be overcome: The size and charged nature of the mRNA molecule prevent the passage across biological membranes, necessary for access to translation machinery. In addition, ribonucleases found ubiquitously in physiological environments cause rapid degradation of mRNAs when unprotected. The immunostimulatory effect of mRNAs through binding to pattern recognition receptors (PRRs) such as toll-like receptor 3 (TLR3) can also be considered a drawback in certain therapeutic settings. To address these concerns, scientists have devised a host of methods to modify or protect the mRNA, both covalently and non-covalently. Modifications to the mRNA itself can be made to improve ribonuclease resistance and reduce immunogenicity, through the introduction of alternative nucleosides such as pseudouridine, 5-methylcytidine, and N6-methyladenosine (Karikó et al. 2008; Kormann et al. 2011). Optimizations of different functional regions of the mRNA, such as the 5′ cap structure, 5′ and 3′ untranslated regions (UTRs), or the poly(A) tail, can be made to increase translation efficiency (Mockey et al. 2006; Wojtczak et al. 2018; Asrani et al. 2018). While these modifications to the chemistry of mRNA may represent an improvement over unmodified mRNA, they are usually not sufficient to mitigate the challenges of mRNA delivery. Therefore, many strategies exist where mRNAs are complexed with other molecules in order to improve physiological stability or transfection efficiency. Usually, these consist of charged macromolecules which interact with the mRNA in an electrostatic manner.

Lipids are a well-known class of nucleic acid carrier. The first generation of effective lipid-based nucleic acid delivery systems was represented by cationic lipids which could interact with the negative charges in the nucleic acid cargo (Felgner and Ringold 1989; Lu et al. 1994; Ma et al. 2005). Further improvements were made by introducing pH-responsive groups to the polar head groups of the lipids to enable enhanced stability in vivo and improve release characteristics (Bailey and Cullis 1994; Semple et al. 2001). The chemical structure of the lipids was also optimized to include unsaturated bonds in the hydrophobic tail and to fine-tune the pKa of the pH-sensitive hydrophilic head group (Heyes et al. 2005; Fenton et al. 2016). Finally, the biodegradability of the hydrophobic parts of the lipid molecules was improved by incorporating ester bonds, leading to the current generation of lipid nanoparticles (LNPs) (Fenton et al. 2017; Sabnis et al. 2018). While the newest LNPs are in advanced stages of development, with some LNP-based therapies approved for clinical use, challenges remain for the further application in terms of targetability with LNPs exhibiting strong liver tropism (Akinc et al. 2010; Francia et al. 2020). A comprehensive review of lipid-based nucleic acid delivery systems is outside of the scope of this chapter and has been published elsewhere (Samaridou et al. 2020).

Polymeric delivery systems represent the major alternative to lipids. These systems have gone through an extensive process of development and engineering over the past decades, characterized by increasing functionalization. Depending on the design of the carrier, a variety of nanostructures can be made, ranging from simple charge-based complexes or micelles to multi-layered nanoparticles or scaffold-based supramolecular structures (Kim et al. 2019; Uchida et al. 2020). This chapter will discuss the strategies for intracellular mRNA delivery using supramolecular assemblies of mRNA with polymeric carrier materials.

2 Polymeric Assemblies

2.1 Polyplexes

Polyplexes are formed by the interaction between negatively charged mRNA and cationic polymers. Polymers have several advantages for developing delivery systems for mRNA, such as relatively easy synthesis, high stability, and the possibility to introduce multiple functions into their chemical structure. Thus, polymers are able to protect mRNA from RNase (Choi et al. 2016), improve the cellular uptake, and mediate the endosomal escape of mRNA (Li et al. 2016). A myriad of polymers has been applied for developing mRNA delivery systems, including polyethyleneimine (PEI), poly(L-Lysine) (PLL), poly(β-amino ester)s, *N*-substituted polyaspartamides, charge-altering releasable transporters, and protamine.

PEI was one of the first polymers to be used for gene transfection (Boussif et al. 1995) and is still used widely for non-viral gene delivery, as it has high ability to complex with nucleic acids and strong endosomolysis (Lungwitz et al. 2005;

Boonstra et al. 2021). PEI-based polyplexes have been effectively used to deliver mRNA to a variety of cells, such as cancer cells and mesenchymal stem cells (Rejman et al. 2010; Johler et al. 2015). Moreover, PEI-based mRNA complexes have been applied in vivo, for example, by nebulization to transfect the lungs of mice (Johler et al. 2015). Because a major limitation of PEI is its high cytotoxicity, particularly at high molecular weight (Lv et al. 2006), various PEI derivatives have been synthesized to reduce toxicity and enhance the delivery efficiency (Taranejoo et al. 2015). Another major barrier for the use with mRNA is the strength of the interaction. In an in vitro translation assay Bettinger et al. (2001) found that using linear and branched versions of PEI to complex luciferase mRNA resulted in poor expression, owing to an overly stable polyplex.

PEI has also been blended with hydrophobic polymers to improve the encapsulation of nucleic acids in nano- and microparticles. For example, poly(lactic-co-glycolic acid) (PLGA), which is a hydrophobic polymer widely used for gene transfection because of its biodegradability through hydrolysis of ester linkages (Pai Kasturi et al. 2006; Silva et al. 2013; Zhang et al. 2013), has been mixed with PEI for improving the processability and loading efficiency of the formulations. PLGA is particularly useful for developing gene carriers due to its ability to render small size particles with structural integrity in a straightforward manner (Hølvold et al. 2013). Since the formation and loading of particles solely based on PLGA can be inefficient, with potential for damaging the cargo and carrier through hydrolysis, some research groups have used PEI to adsorb DNA onto the PLGA particle for improving the processability (Singh et al. 2003; He et al. 2005; Pai Kasturi et al. 2006). These modifications also enhanced the unpacking and subsequent protein production due to the efficient release of the nucleic acid, which can be a limitation of PLGA particles (Wang et al. 2004; Hølvold et al. 2013; Zhang et al. 2013).

As a DNA-transfection agent, PLL has been used since the 1970s (Laemmli 1975). Like PEI, PLL has a relatively large number of positive charges which presents the challenge of overcondensation and cytotoxicity. It has been suggested that decreasing the number of positive charges by using low MW PLL might improve mRNA delivery efficacy, as demonstrated by the finding that low molecular weight PLL yielded increased luciferase expression (Bettinger et al. 2001). Additionally, to address poor endosomal escape, researchers modified the PLL to contain weak basic groups such as histidine or imidazole (Ihm et al. 2005).

Derivatives of *N*-substituted polyaspartamide, such as poly{*N*-[*N′*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)) and poly(*N*-{*N′*-[*N″*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}aspartamide) (PAsp(TET)), have been shown to be safer than PEI based on their fast degradation capability (Itaka et al. 2010; Miyata et al. 2012). The protonation of the side chains of these PAsp derivatives at endosomal pH promotes the escape from endosomes and the cytosolic delivery of nucleic acids (Miyata et al. 2008; Kim et al. 2010). Moreover, it has been noted that the number of charged aminoethylene repeats in the side chain affects the binding with eIF4E and mRNA complexes, which is a significant advantage for initiating translation (Uchida et al. 2016a).

Poly(β-amino ester)s are also a safe option for nucleic acid delivery (Gao et al. 2016; Riera et al. 2019). Poly(β-amino ester)s modified with cationic or hydrophobic units can promote effective delivery into the cytosol after breaking endosomal membranes (Perche et al. 2011; Duncan and Gaspar 2011; Blanco et al. 2015; Capasso Palmiero et al. 2018). Thus, modified poly(β-amino ester)s have shown enhanced ability to deliver mRNA in vitro and in vivo (Capasso Palmiero et al. 2018; Fornaguera et al. 2018). For example, mRNA polyplexes of poly(β-amino ester)s having anti-CD3 antibodies complexed via poly(glutamic acid) effectively targeted macrophages in vivo to generate chimeric antigen receptor (CAR) macrophages upon delivery of mRNA (Krausgruber et al. 2011; Zhang et al. 2019).

Charge-altering releasable transporters are also safe polymers for mRNA delivery. These polymers present hydrophobic and cationic moieties, which can be rapidly degraded in endosomes to become small molecules promoting endosomal escape (McKinlay et al. 2018; Benner et al. 2019). For example, polymers containing 13 repeating hydrophobic groups and 11 repeating A moieties showed high mRNA translation in vitro and in vivo after intramuscular injection (McKinlay et al. 2018).

Protamine is an arginine-rich natural polymer that has been used for the delivery of nucleic acids, including mRNA (Brewer et al. 1999; Rettig et al. 2010). Particularly, protamine has been effectively applied for promoting the maturation of antigen-presenting cells (Scheel et al. 2004, 2005), which has been exploited for developing various vaccine formulations (Weide et al. 2009; Weber et al. 2011; Kowalczyk et al. 2016; Papachristofilou et al. 2019). These vaccines present enhanced Toll-like receptor 7 (TLR7) signaling and Th1 cytokines secretion, as well as high mRNA translation. Thus, protamine-based vaccines are being tested in humans against non-small cell lung cancer (Papachristofilou et al. 2019), prostate cancer (Kübler et al. 2015), and melanoma (Weide et al. 2009).

The polycations interacting with mRNA can be further engineered for generating compartmentalized architectures, such as polymeric micelles, which can load the mRNA in their core and effectively protect it from degradation, as well as improve its bioavailability in vivo. In the next section, we described the key features of such systems.

2.2 Polymeric Micelles

Polymeric micelles present high potential as drug carriers with the ability to control the distribution and function of the loaded agents inside the body (Cabral et al. 2018). Micelles can be designed for effectively overcoming biological barriers, such as extravasation in targeted tissues and intracellular access in specific cells. Due to these advantageous features, various polymeric micelles formulations are being evaluated in human clinical studies (Cabral and Kataoka 2014). Polymeric micelles can incorporate a wide range of nucleic acids, including mRNA (Lächelt and Wagner 2015; Cabral et al. 2018). Such polymeric micelles can be readily assembled by ionic and hydrogen bonding between block catiomers and negatively charged mRNA,

forming a mRNA-loaded core structure surrounded by a hydrophilic protective shell (Kataoka et al. 2001). In these polyion complex (PIC) micelles, the mRNA payload is present in a globular shape in the core (Uchida et al. 2013; Chen et al. 2017).

The mRNA-loaded micelles have the ability to deal with major challenges of mRNA delivery, including mRNA instability and immunogenicity, as well as translation efficiency. Thus, the polymers forming the micelles can be engineered for protecting the mRNA in their core from enzymes in biological environments and enhancing intracellular delivery. Moreover, it has been reported that polymeric micelles can reduce the immune responses of mRNA, most likely by avoiding the interaction of mRNA with TLRs or blocking TLR signals by endosomal disruption (Uchida et al. 2013). Thus, polymeric micelles mRNA delivery has been demonstrated in vivo after local or systemic administration. mRNA-loaded micelles based on the block catiomer poly(ethylene glycol)-P(Asp(DET)) (PEG-P(Asp(DET))) showed successful transfection of nasal epithelium, the central nervous system, and liver. The PEG-P(Asp(DET)) block copolymer was designed to protect mRNA from rapid degradation and improve the intracellular delivery of mRNA by polyion complexation and shielding with the PEG blocks. Moreover, the polyaspartamide block, i.e., P(Asp(DET)), presents 2 pKas (6.2 and 8.9), which allow for effective polyion complexation at pH 7.4 and enhance endosomal escape after protonation at endosomal pH (pH 5–6.5).

In physiological salt concentration (150 mM NaCl), the mRNA-loaded micelles are stable. However, polyelectrolytes in biological environments can disrupt them by interfering with the polyion complex between polymers and mRNA. Therefore, major efforts have been dedicated to stabilize the mRNA-loaded micelles. These approaches include crosslinking of the core by stimuli-responsive covalent bonds, or stabilizing the core of the micelles by increasing the hydrophobicity. For example, core crosslinking by reduction-responsive bonds, such as a disulfide group, can also enhance the stability: Polymeric micelles made with PEG-poly(l-lysine-1-amidine-3-mercaptopropyl) (PEG-P(Lys-AMP)), which has thiol groups in the polycation segment to cross-link the core of the micelles, are significantly more stable than the micelles prepared from PEG-P(Lys) block copolymers (Fig. 1a) (Dirisala et al. 2019).

Adding hydrophobic molecules to the core-forming segment can also promote the stability of mRNA-loaded micelles. Thus, micelles made from polymers with a cholesteryl group at the ω-end presented longer blood circulation compared to micelles prepared from polymers without the cholesteryl moiety (Fig. 1b) (Uchida et al. 2016b). The cholesteryl-stabilized micelles showed high tumor targeting, achieving high mRNA translation in pancreatic tumors. By delivering mRNAs encoding antiangiogenic sFlt-1, the cholesteryl-stabilized micelles achieved significant antitumor effects. The introduction of a hydrophobic polymeric layer between the mRNA-loaded core and the hydrophilic shell can also improve the stability. By using thermoresponsive poly(*N*-isopropylacrylamide)-poly(lysine-thiol) (PNIPAM-P(Lys(SH))) block copolymers, it is possible to assemble micelles in aqueous conditions after mixing them with mRNA at a temperature lower than the lower critical solution temperature (LCST). The stability of the micelles is significantly

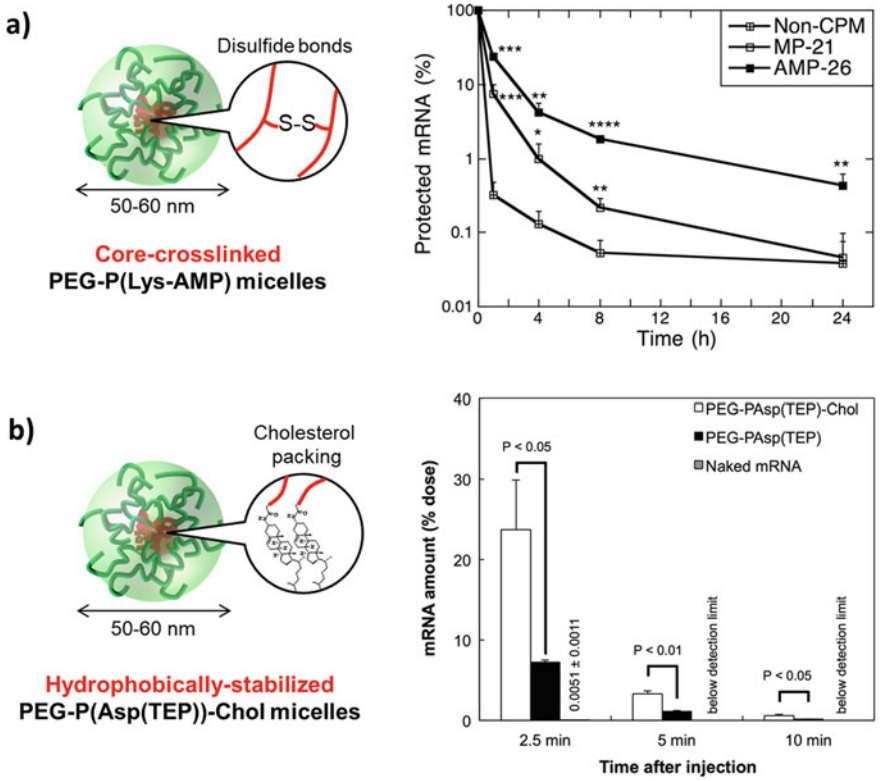

Fig. 1 Improving RNA longevity by incorporating stabilizing elements in polymer design. **a** Introducing disulfide linkers to the carrier polymer (PEG-P(Lys-AMP)) significantly extends intracellular half-life of encapsulated mRNA. **b** mRNA-loaded polymeric micelles stabilized with Chol moieties display a superior blood circulation profile compared to micelles without hydrophobic stabilization (adapted from Dirisala et al. (2019) and Uchida et al. (2016b))

improved compared to polymeric micelles prepared from PEG-P(Lys) block copolymers (Li et al. 2015). The surface of these micelles was further modified with cyclic RGD peptide for improving tumor accumulation and cellular uptake, which allowed effective mRNA in glioblastoma models (Chen et al. 2017).

The stability of mRNA-loaded micelles can be also enhanced by engineering the polymers to increase their affinity to mRNA. By controlling the rigidity of the backbone of the polycation segment, it is possible to promote the complexation of the polycation and mRNA. Simulations suggest that a polycation segment with higher flexibility can increase the entropic gain through water release when binding with RNA molecules (Yang et al. 2021). The effect of the polycation flexibility was recently exemplified by studying mRNA-loaded micelles made from PEG-poly(glycidyl butylamine) (PEG-PGBA) block copolymer with a relatively flexible polycation block, or PEG-P(Lys), which has a relatively more rigid polycation backbone (Miyazaki et al. 2020b). The micelles made with PEG-PGBA were more stable

Table 1 Isothermal calorimetry (ITC) analysis of the binding energies of mRNA-loaded polymeric micelles formed with flexible polymer (PEG-PGBA) and more rigid polymer (PEG-P(Lys))

Micelles	ΔH [kcal mol^{-1}]	ΔS [cal (mol K)$^{-1}$]	ΔG [kcal mol^{-1}]	K_A [M^{-1}]
mRNA/mPGBA	6.07 ± 0.06	57.7 ± 0.5	-10.5 ± 0.2	$(1.40 \pm 0.51) \times 10^8$
mRNA/mPLL	7.00 ± 0.19	52.8 ± 0.2	-8.27 ± 0.12	$(2.59 \pm 0.54) \times 10^6$

A 50-fold difference in binding constant indicates that backbone flexibility is an important factor in micelle stability (reproduced from Miyazaki et al. 2020b)

than the micelles from PEG-P(Lys), probably due to the 50-fold higher binding of PEG-PGBA to mRNA compared to PEG-P(Lys) (Table 1). The enhanced stability of the mRNA-loaded micelles made from PEG-PGBA promoted intracellular delivery, which increased translation in vitro and in vivo. Moreover, the micelles made from PEG-PGBA showed longer blood circulation than the micelles made from PEG-P(Lys) after intravenous injection, indicating the relevance of improving the stability of micelles for systemic administration.

Another promising approach for enhancing the stability of polyion complexes is engineering the valency between catiomers and aniomers (Carlson et al. 2013; Hori et al. 2018). This can be achieved by introducing guanidine groups, which strongly interact with phosphate groups via ion complexation and intermolecular hydrogen bonds (Calnan et al. 1991; Schug and Lindner 2005). Thus, mRNA-loaded micelles made from PEG-poly(glycidyl methyl guanidine) (PEG-PGMG) bearing guanidine moieties showed higher stability against polyanion exchange than the micelles prepared with the counterpart PEG-poly(glycidyl methyl amine) (PEG-PGMA) polymer, which has primary amines in the polycation segment (Miyazaki et al. 2020a). The micelles with guanidine groups also enhanced the protection of the loaded mRNA against nucleases. Moreover, in in vitro cellular experiments, the micelles with guanidine groups improved the intracellular delivery of mRNA and the translation efficiency compared to the micelles having polycations with amine moieties.

3 RNA Architectonics

Along with PIC formation with polycations, hybridization of mRNA with complementary oligo-/polynucleotide is another attractive strategy to introduce functional molecules to mRNA, which has been rarely pursued. RNA is preferred for hybridization to mRNA compared to DNA, as RNA/DNA heteroduplex is subjected to degradation by RNase H inside cells. Meanwhile, hybridization of long RNA may inhibit translational processes, cause RNA interference, and activate innate immunity against double-stranded RNA structure. To address this concern, functionalities of mRNA after hybridization with various lengths of RNA oligo-/polynucleotide were assessed after introduction to cultured cells (Yoshinaga et al. 2019b). While 23 nucleotides

(nt) or longer RNA impaired the efficiency of protein expression from mRNA and induced strong innate immune responses after hybridization to mRNA, 17 nt RNA showed minimal influence on translational activity and immunogenicity of mRNA. This result demonstrates the utility of 17 nt complementary RNA for introducing functional molecules to mRNA.

Using this strategy, PEG (12 kDa) was introduced to mRNA to prevent RNase attack (Yoshinaga et al. 2021a) (Fig. 2a). Hybridization of reporter mRNA with PEGylated 17 nt RNA increased the nuclease stability in serum by 15-fold and reporter protein expression efficiency by 20-fold in cultured cells. Importantly, translational efficiency of mRNA was preserved in rabbit reticulocyte lysate, which mimics the intracellular environment, even after hybridization of 20 PEG strands to 783 nt mRNA (Kurimoto et al. 2019). This study provided an example of mRNA stabilization strategy that does not rely on cationic materials.

Nuclease stability was also drastically improved just by bundling several mRNA strands without the use of additional materials other than RNA (Yoshinaga et al. 2019a). mRNA strands were crosslinked with each other using linker RNAs possessing two sequences (17 nt) complementary to mRNA at both 5′- and 3′-ends, sandwiching a 10 nt oligoadenine flexible sequence (Fig. 2b). The nanoassemblies of mRNA thus prepared exhibited an average size of below 100 nm and were composed of 7.7 mRNA strands on average. mRNA nanoassemblies showed around 100-fold

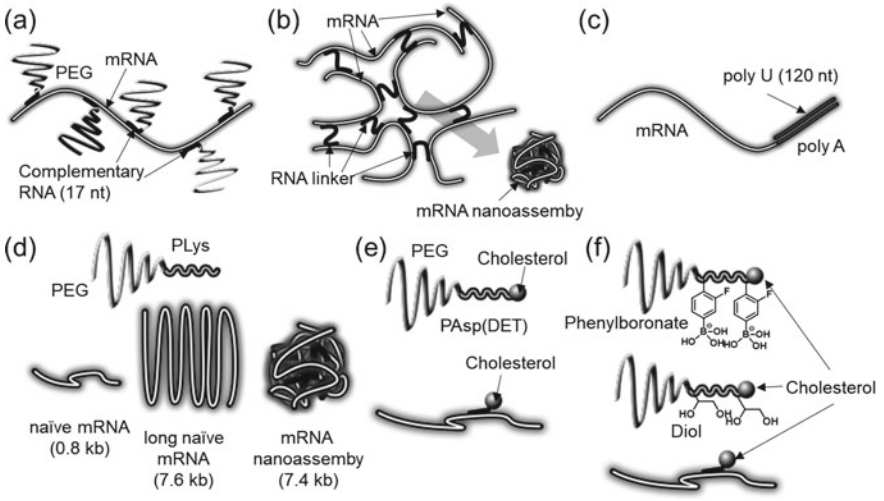

Fig. 2 RNA architectonics. **a** Introduction of PEG to mRNA using PEGylated 17 nt RNA complementary to mRNA. **b** mRNA nanoassemblies prepared by bundling several mRNA strands using RNA linkers. **c** Immunostimulatory mRNA hybridized with 120 nt poly U for vaccines. **d–f** Combination of RNA architectonics and polymeric micelle. **d** Encapsulation of mRNA nanoassemblies to polymeric micelles. **e** Introduction of cholesterol moieties to ω-terminus of block copolymer and mRNA. **f** Combination of cholesterol introduction **e** and phenylboronate ester crosslinking between block copolymers

enhancement in nuclease stability after incubation in serum compared to naïve (unhybridized) mRNA. Mechanistic analyses suggest that high-order structures, as well as secondary structures in nanoassemblies, contribute to enhanced nuclease stability of nanoassemblies. Importantly, translational activity was preserved in nanoassemblies. Indeed, mRNA nanoassemblies provided enhanced expression of reporter protein from mRNA after introduction to cultured cells and also after intraventricular injection in mice. Interestingly, these results suggest that nanoassemblies inhibit the recognition by RNase through steric hindrance, but still are accessible to initiation factors for mRNA translation. To get mechanistic insight into the translational process from nanoassemblies, the role of the 5′ cap was studied. Notably, nanoassemblies of mRNA with 5′ cap selectively disintegrate in the intracellular environment, while nanoassemblies of mRNA without 5′ cap fail to show intracellular dissociation. Considering that 5′ cap is essential for starting translation, and translational complexes have endogenous helicase activities; it is reasonable to hypothesize that translational complexes recognize accessible 5′ cap structures in nanoassemblies to trigger translation, which may unfold the nanoassemblies through endogenous helicase activity coupled with translational processes, thereby facilitating further translation. This explanation is consistent with recent biological findings that the secondary structure of endogenous mRNA is actively unfolded inside the cells (Qu et al. 2011; Rouskin et al. 2014).

Interestingly, the influence of RNA hybridization is dependent on the location of hybridization. Although 23 nt or longer RNA drastically reduces the translational efficiency of mRNA after hybridization to the coding region of mRNA (Yoshinaga et al. 2019b), translational activity was preserved after hybridization of 120 nt poly U to poly A (Uchida et al. 2018) (Fig. 2c). Meanwhile, the mRNA hybridized with poly U showed enhanced immunostimulation via recognition by innate immune receptors TLR3 and RIG-I. In mRNA vaccines, this formulation simultaneously expresses antigen and functions as an immunostimulatory adjuvant, improving vaccination effect in mice after injection in lymph nodes.

The functional mRNA described in this section can be administered without delivery carriers in delivery routes that require only modest resistance to RNases, including intraventricular and intranodal injection. Such a delivery approach without the use of cationic materials is favorable in terms of safety. Available delivery routes of such cation-free approaches will be expanded in the future with the progress in the technology. However, nuclease stability is still insufficient without the use of delivery carriers in various administration routes, such as intratracheal delivery to the lung and systemic delivery. In these routes, mRNA is exposed to a nuclease-rich environment for a long time before reaching target cells. The next section describes the combinatorial approaches of mRNA architectonics and polymeric micelles in these delivery routes.

4 Combination of RNA Architectonics and Polymeric Micelles

By functionalizing mRNA, RNA architectonics has a potential to synergize with polymer design in polymeric micelles, to improve mRNA bioavailability. The effect of mRNA bundling on micelle functionalities was studied by comparing micelles loading naïve mRNA (naïve/m) and those loading mRNA nanoassemblies (NA/m), after the preparation of micelles from PEG-polylysine (PLys) block copolymers (Koji et al. 2020) (Fig. 2d). Importantly, naïve mRNA and nanoassemblies differ in mRNA steric structure and also in size, with a total of 0.8 kb nucleotides in naïve mRNA and 7.4 kb in the nanoassembly (Yoshinaga et al. 2019a). To discriminate these two factors, micelles loading long naïve mRNA with a total of 7.6 kb nucleotides ($l_{7.6}$-naïve/m) was used as an additional control. All tested micelles showed an average size below 100 nm. Interestingly, naïve/m and NA/m exhibited spherical cores, while the core of $l_{7.6}$-naïve/m was distorted (Fig. 3a–c). The structuring of mRNA into nanoassemblies might facilitate the packaging process of long mRNA into the micelle core after addition of block copolymer. To evaluate enzymatic mRNA degradation after systemic injection to mice, mRNA concentration in the blood was measured with two methods: fluorescence measurement after injection of fluorescence-labeled mRNA, which quantifies the amount of mRNA including degraded mRNA, and quantitative real-time PCR (qRT-PCR), which leaves degraded mRNA undetected. NA/m exhibited more than a tenfold increase in the amount of mRNA detected by qRT-PCR, compared to naïve/m and $l_{7.6}$-naïve/m (Fig. 3d). Importantly, both fluorescence and qRT-PCR methods provided comparable values in the case of NA/m, indicating that the mRNA in NA/m is intact. In contrast, in naïve/m and $l_{7.6}$-naïve/m, the mRNA amount detected by qRT-PCR was less than 10% of that detected by the fluorescence method, indicating enzymatic degradation of mRNA in these micelles. Notably, increasing the mRNA length failed to improve nuclease stability in micelles, and thus the enhanced nuclease stability of NA/m may be attributed to the steric structure of the nanoassemblies rather than the increase in size. Further mechanistic analyses revealed that NA/m possesses a 2.2-fold higher density of PEG on the outer shell and a 1.5-fold higher density of mRNA in the core compared to naïve/m. This strategy of mRNA structuring is versatile to improve the nuclease stability of three different species of mRNA after encapsulation into micelles. Furthermore, mRNA nanoassemblies preserved translational activity to provide enhanced mRNA expression efficiency in cultured cells and in mouse brain after intrathecal injection.

Bioavailability of mRNA in micelles can also be improved by adding stabilizing functional moieties to mRNA and block copolymers. In this strategy, cholesterol (Chol) moieties were introduced to mRNA by hybridizing RNA oligonucleotides possessing a Chol moiety at 5′ or 3′ end (Chol-RNA), and to the ω-terminus of PEG-PAsp(DET) block copolymer, to obtain stacking between Chol moieties (Yoshinaga et al. 2021b) (Fig. 2e). Hybridization of only one Chol-RNA to a 783 nt mRNA

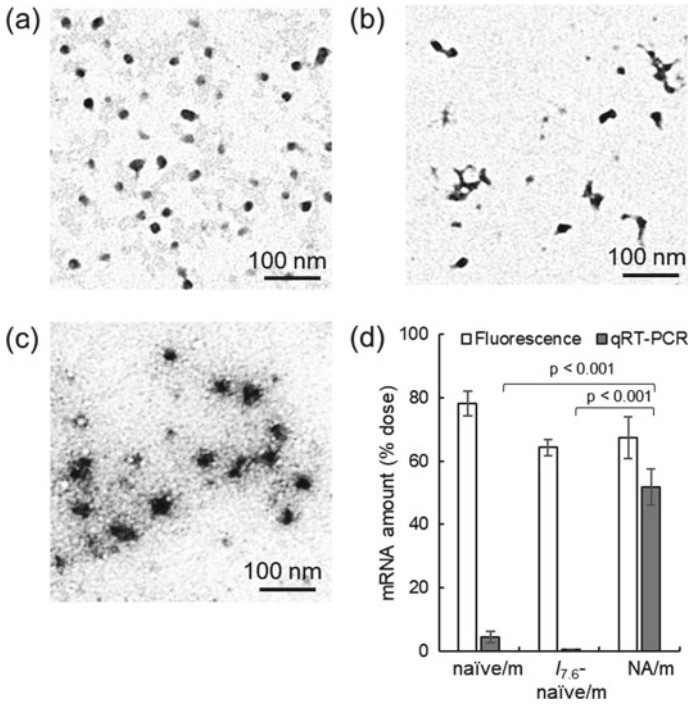

Fig. 3 Polyplex micelles encapsulating mRNA nanoassemblies. **a**–**c** Transmission electron microscopy images were obtained after staining with uranyl acetate, which selectively binds to mRNA to visualize the core of polyplex micelles. Images of micelles loading 0.8 kb naïve mRNA (naïve/m) (**a**), those loading 7.6 kb long naïve mRNA ($l_{7.6}$-naïve/m) (**b**), and those loading mRNA nanoassemblies (NA/m) (**c**). **d** Amount of mRNA in the blood 2.5 min after intravenous injection to mice, detected by two methods: fluorescence measurement after injection of fluorescence-labeled mRNA, which quantifies the amount of mRNA including degraded mRNA, and quantitative real-time PCR (qRT-PCR), which leaves degraded mRNA undetected (adapted from Koji et al. 2020)

resulted in enhanced condensation of mRNA after mixing with PEG-PAsp(DET)-Chol block copolymers, compared to the micelle without Chol introduction. Importantly, the condensing effect was not observed when Chol moieties were introduced to either one of mRNA or block copolymer, indicating the critical role of the interaction between the Chol moiety in the block copolymer and that in the mRNA for condensing mRNA. In accordance with this result, Chol moieties in the mRNA and the block copolymer synergistically improved micelle stability against nucleases in serum and against polyion exchange with dextran sulfate. Addition of non-hybridizing Chol-RNA or hybridization of RNA oligonucleotides without Chol moiety failed to improve micelle stability, highlighting that Chol introduction to mRNA is needed for micelle stabilization. Hybridization of Chol-RNA to reporter mRNA in the micelle improved reporter protein expression in cultured cells regardless of whether Chol was introduced to the 5′ or 3′ end of the RNA oligonucleotide complementary to the mRNA. Finally, the micelles were administered to mouse

lungs from the trachea. This administration route presents a harsh environment to micelles; anionic macromolecules and nucleases in respiratory mucus and alveolar fluid can destabilize the micelle structure and degrade the mRNA (Sanders et al. 2009). In this administration route, micelles loading mRNA hybridized with Chol-RNA showed enhanced reporter protein expression efficiency in the lung compared to those loading unhybridized mRNA.

Further stabilization of micelles is needed for systemic administration. For this purpose, the block copolymer structure was optimized before the employment of the Chol-based stabilization strategy. In this study, phenylboronate ester crosslinking was introduced at several different ratios to several species of polycation segments of block copolymers with different protonation degrees (Yoshinaga et al. 2019b) (Fig. 2f). For crosslinking, phenylboronic acid and polyol moieties were introduced to the cationic segment of the block copolymer. The crosslinking stabilizes micelles in extracellular environments and is selectively cleaved in ATP-rich intracellular environments via binding of the ribose ring in ATP to the polyol moiety. Entry of negatively charged ATP into the micelle core, as well as cleavage of the crosslinking, would facilitate mRNA release from micelles in the cytoplasm and protein translation from mRNA. The introduction ratio of crosslinking moieties and the protonation degree of the polycation may influence micelle stability and intracellular mRNA release. As overstabilization of the micelle structure may lead to impaired mRNA release inside cells, micelle optimization was performed to balance extracellular robustness and intracellular translational activity, resulting in the selection of one micelle formulation from 21 candidates, possessing different species of polycation segment or different introduction ratios of phenylboronic acid or polyol moieties. Then, Chol moieties were introduced to the mRNA and block copolymers in the optimal formulations. Ultimately, the Chol-introduced optimal micelles exhibited efficient mRNA introduction to cultured cells and prolonged blood circulation after systemic injection in mice. This study provides an example that RNA architectonics and polymer design are orthogonal approaches to bring synergy in improving mRNA bioavailability.

Worth noting, RNA architectonics can be combined with carriers other than polymeric micelles. Indeed, introduction of PEG onto mRNA allowed easy preparation of mRNA lipoplexes with average size controlled to be below 100 nm, just by mixing PEGylated mRNA and lipid components, which effectively prevented aggregation of the lipoplexes after systemic administration to mice (Kurimoto et al. 2019). While lipid-based mRNA delivery systems require laborious processes in preparation, this PEGylation method may provide promising solutions to this issue.

5 Conclusion

In the context of mRNA delivery, supramolecular strategies represent an ever-developing area of interest. By harnessing different functionalities of molecular components, a fine-tuned delivery system can be designed. The flexibility yielded

by these systems lends itself to application in a wide variety of therapeutic settings. Among these, polymeric micelles stand out for their excellent performance in protection against nuclease attack and show promising results in terms of safety and targetability compared to lipid-based systems. On the other hand, RNA architectonics allows for modification and functionalization of mRNA without the use of cationic materials, improving safety and biocompatibility. By combining RNA strands into nanoassemblies, resistance against enzymatic degradation was significantly reduced, while retaining translational activity. Using base-pair hybridization, it is possible to equip mRNA cargo molecules with functional groups without covalent modification or electrostatic complexation. Finally, combining the advantages of both polymeric micelles and RNA architectonics, a synergistic improvement in mRNA stabilization and deliverability was achieved. In the future, supramolecular delivery systems for mRNA should be further optimized for efficient in vivo trafficking and intracellular release. By customizing the components of the carrier, it can be outfitted with functionalities to meet the specific needs for each therapeutic approach.

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Delivery Vehicles for Self-amplifying RNA

Nuthan Vikas Bathula, Petya Popova, and Anna Blakney

Contents

Abstract Self-amplifying RNA (saRNA) is a next-generation nucleic acid technology that is structurally similar to mRNA, but capable of replicating upon delivery into the cytosol. This amplification results in high protein expression from a relatively low dose of saRNA (~100-fold lower than mRNA). The rapid, cost-effective, cell-free manufacturing, low dosage requirement, and acceptable safety profile have drawn spotlight on saRNA, which has recently entered clinical trials. However, similar to mRNA, saRNA formulations need highly protective and robust delivery vehicles to achieve a therapeutic effect. The delivery systems have an integral role on the therapeutic efficacy of the saRNA including, biodistribution, cellular uptake, protein expression, and immunogenicity. In the last three decades, a broad range of non-viral delivery systems for RNA have been investigated. Herein, we discuss the cutting-edge advancements in saRNA delivery platforms including the variety of delivery approaches that have been used for saRNA formulations to date, and the resulting

Nuthan Vikas Bathula and Petya Popova—These authors contributed equally to this work.

N. V. Bathula · P. Popova · A. Blakney (✉)
Michael Smith Laboratories, School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada
e-mail: anna.blakney@msl.ubc.ca

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_16

immunogenicity, biodistribution, cellular uptake, protein expression, and effect of route of administration.

Keywords saRNA · Lipid nanoparticles · Polymeric nanoparticles · Cellular uptake · Immune sensing

1 Introduction

Self-amplifying RNA (saRNA) originates from the genome of alphaviruses, such as Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Sindbis virus, and flaviviruses. The alphaviral genome has two open reading frames (ORFs) that encode proteins for the viral replicase, an RNA-dependent RNA polymerase that allows the replication of the gene of interest and structural proteins, which are substituted with a gene of interest (GOI). Upon entry into the cytoplasm, the saRNA strand is translated into the replicase, which then produces copies of complementary negative-strand RNA strand that is utilized for the production of more copies of the original positive-strand RNA. At the same time, the viral replicase binds the subgenomic promoter in the negative strand for replication of the subgenomic region, including the GOI (Fig. 1).

saRNA vaccine requires smaller doses in comparison with mRNA vaccines (McKay et al. 2020) and exhibits significantly longer protein expression, with duration of up to 60 days. (Ballesteros-Briones et al. 2020) However, a well-known limitation of saRNA vaccines is their relatively larger size compared to mRNA (~10,000 nt compared to ~1000 nt), and increased immunogenicity. This review will focus on different delivery vehicles for saRNA vaccines. We will discuss saRNA nanoparticle, endosome entry mechanisms into cells, active targeting, and the largely unexplored field of immune sensing of the delivery vehicle.

2 Delivery Vehicles

Despite the availability of a broad range of physical and chemical methods for in vitro transfection of nucleic acids (NA), the in vivo delivery of RNA to target tissue and cells is challenging and demands specialized delivery systems, including lipid nanoparticles, polymeric nanoparticles, cationic nanoemulsions, and electroporation (Fig. 2).

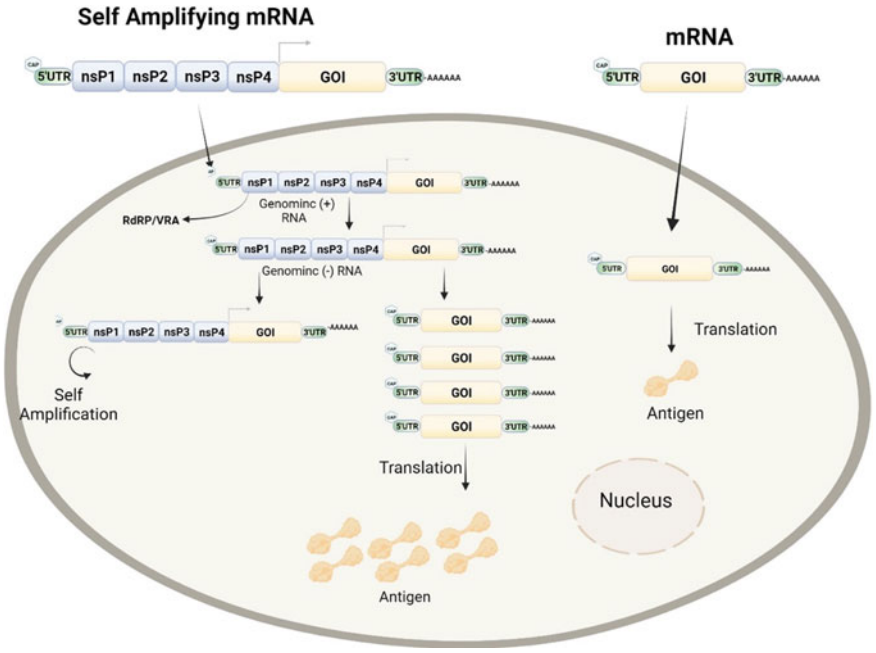

Fig. 1 Conventional mRNA (right) and self-amplifying RNA (left) both carry the gene of interest flanked by 5′ UTR and 3′ UTR along with a 5′ cap and 3′ poly(A) tail. Additionally, the saRNA carries genes encoding non-structural proteins and a subgenomic promoter that is derived from an alpha virus. Once delivered into the cytosol, the non-structural proteins make up the replicase machinery and are responsible to amplify the saRNA; thus, a single copy of delivered saRNA can be replicated into multiple copies and thereby translate higher quantities of antigen. On the other hand, a single copy of conventional mRNA delivered into the cytosol is limited by the initial delivered dose and lower translation of antigen

2.1 Lipid Nanoparticles

2.1.1 Development of Ionizable Lipid Nanoparticle Formulations

Lipid nanoparticle (LNP) formulations are the most advanced and clinically proven non-viral delivery vehicles for RNA. In the late 1990s, Cullis and colleagues developed novel ionizable cationic lipids—dialkylamino lipids and laid foundation for a new generation of revolutionary nucleic acids delivery system (Semple et al. 2001; Tam et al. 2016). The distinguishing feature of ionizable lipids is that they are positively charged at acidic pH and turn neutral under physiological pH (~7.3). The positive charge under acidic pH facilitates electrostatic interactions with anionic RNA giving excellent encapsulation efficiency (EE; >80%) (Lou et al. 2020) and triggers fusion with the endosomal membrane for efficient release of RNA cargo into the cytoplasm. Since the development of the first ionizable lipid- DODAP, they have been extensively explored and exploited to optimize the lipids as well as the

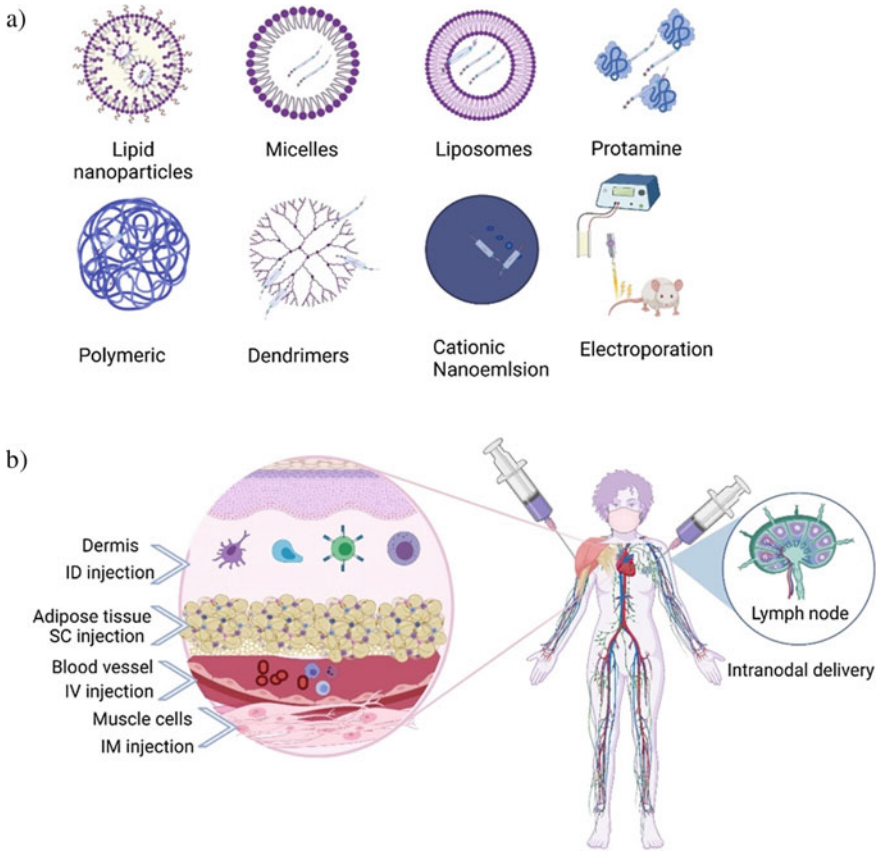

Fig. 2 **a** saRNA nanoparticle delivery formulations. **b** Routes of administration, where ID is intradermal, SC—subcutaneous, IV—intravenous, IM—intramuscular

formulations to achieve exceptional therapeutic efficacy with an appreciable clinical safety profile. Typically, an ionizable LNP (iLNP) formulation is composed of an ionizable lipid and several helper lipids, such as cholesterol, a phospholipid, and a PEGylated lipid (Cheng and Lee 2016). The LNP formulations that have been investigated for saRNA delivery to date are directly derived from those optimized for mRNA delivery (Blakney et al. 2019a, b).

2.1.2 Lipid Nanoparticles for saRNA Delivery

The therapeutic efficacy of an LNP formulation depends on several factors: the choice of ionizable lipid, helper lipids, solvents, their proportion and concentrations, manufacturing process, etc. Herein, cutting-edge innovations in LNP formulations to deliver self-amplifying RNA are discussed.

A model saRNA-LNP formulation was developed to explore the potential of iLNP to deliver saRNA as a vaccine candidate; DLinDMA (ionizable cationic lipid), DSPC (helper lipid), PEG-DMG 2000, cholesterol, formulated in 40:10:2:48 molar ratio gave uniform, monodisperse nanoparticles with mean diameter: 130–164 nm, and high EE (85–98%) of saRNA when produced by ethanol dilution process (Geall et al. 2012). Several saRNA vaccines against a broad range of diseased conditions were developed using the same formulation to investigate the therapeutic profile of the saRNA-LNP model (Lazzaro et al. 2015; Magini et al. 2016; Pepini et al. 2017). Respiratory syncytial virus (RSV) F-protein encoding saRNA, when encapsulated using the iLNP formulations, generated significant protection against RSV in a preclinical, murine RSV challenge model even at low dose compared to the benchmark viral replicon particle (VRP) vaccine (Geall et al. 2012). Similarly, when tested in non-human primates (macaques), the saRNA-LNP vaccine (encoding HIV envelop glycoprotein) demonstrated promising humoral and IFN-γ specific T cell response with acceptable toxicity compared to VRP and recombinant glycoprotein vaccines (Bogers et al. 2012). In addition to the safety and efficacy, the saRNA-LNP vaccine formulations facilitated an expedited development of a novel saRNA vaccine against the H7N9 influenza pandemic in only eight days, with excellent immunogenicity and protection (Hekele et al. 2013).

Furthermore, a panel of cationic lipids (cLNP) was tested against the benchmark iLNP for their efficacy in delivering saRNA as vaccine candidates (Lou et al. 2020). DOTAP and its analogues (DC-Chol; DDA; DMTAP; DSTAP; DOBAQ). The cLNP (DOTAP and DDA) and iLNP formulations generated similar IgG titers, post first and second immunization, whereas the iLNP generated significantly higher (TNF-α and IFN-γ producing) $CD4^+$ and $CD8^+$ T cells response against RVG compared to cLNP and commercial Rabipur.

Despite outstanding performance in in vitro and in vivo animal models, the LNP-delivered mRNA therapeutics had remarkably reduced efficacy in humans (Bahl et al. 2017). To address this, a human skin explant was used to optimize LNP formulations (C12-200 (ionizable lipid), DOTAP, DDA) by a DoE approach and it resulted in sevenfold higher saRNA expression compared to the unoptimized formulation (Blakney et al. 2019a, b). The parameters—type and concentration of lipid and the use of zwitterion (cephalin)—were found to influence the expression kinetics significantly. This variation illustrates the advantage and significance of analyzing and optimizing LNP formulations directly in human tissues.

While there are numerous mRNA clinical trials, currently, there have only been a few saRNA vaccine clinical trials to date, which have utilized an intramuscular (IM) route of administration and lipid nanoparticles as delivery vehicles. The first one, COVAC (NCT04934111) (McKay et al. 2020) by Imperial College London, studied doses of saRNA ranging from 0.1 to 10 μg which encodes the SARS-CoV-2 spike antigen, delivered with LNPs. In a follow-on study (NCT04702178), an optimized saRNA molecule sequence that can potentially combat undesired IFN responses was used with the goal of improving seroconversion. The final clinical trial (NCT04668339) is run by Arcturus Therapeutics, where a saRNA vaccine encoding

the spike glycoprotein of SARS-CoV-2 (STARR™) is delivered with LUNAR® (Ramaswamy et al. 2017) lipid nanoparticles (Low et al. 2021).

2.2 Polymeric Nanoparticles

2.2.1 Non-degradable Polymers

Polyethyleneimine Imine (PEI)

Compared to lipid NP, polymeric NP are not as clinically advanced. One of the most used cationic polymers for RNA delivery is the non-degradable molecule PEI. A pioneering study from 2016 (Demoulins et al. 2016) showed that saRNA, encoding influenza virus hemagglutinin (HA) and nucleocapsid (NP), forms complexes with linear PEI by inducing humoral and cellular responses against Influenza virus HA and NP proteins. (Ballesteros-Briones et al. 2020) A follow-up study was found that various Mw formulations of linear PEI and cell-penetrating peptides—Arg_9 and TAT 57—had different abilities to form complexes with saRNA and to translate proteins in vivo. Moreover, PEI and stimulator of interferon genes (STING)-agonist c-di AMP (cyclic-di-adenosine monophosphate) administration-induced potent immune responses in pigs (Démoulins et al. 2017). It was shown that high molecular weight PEI induces higher toxicity, while low molecular weight PEI does not mediate efficient RNA transfection. Interestingly, in a murine study comparing synthetic mRNA and saRNA encoding Influenza virus hemagglutinin, delivered with medium-length PEI, was shown that saRNA vaccination achieves equivalent levels of protection at 64 times lower doses compared to mRNA (Vogel et al. 2018). Using a similar formulation, VEEV saRNA was found to be more potent compared to SFV saRNA, encoding stabilized native-like HIV-1 Envelope glycoprotein (Env) trimers delivered to non-human primates (Aldon et al. 2021). For improving saRNA targeting with PEI platform mannosylated PEI polymers- (PEI-Ad-CD-Man_7) were introduced. The platform was based on cyclodextrin (CD) and adamantine (Ad) host–guest interaction in aqueous environment. The degree of mannosylation reduced transfection efficiency in vitro, but increased protein expression eightfold in human skin explants. It was indicated that mannosylated PEI particles could possibly aid receptor-mediated endocytosis of saRNA, therefore enhancing immune responses (Blakney et al. 2020a, b). The intrinsic properties of polymers are integral for RNA transfection and uptake. Intriguingly, it was found that polymer hydrophobicity affects saRNA translation in mice—lipophilic molecules mediate lower transfection efficiency, compared to hydrophilic copolymers in vivo (Gurnani et al. 2020).

Dendrimers

Another promising non-degradable delivery system for saRNA is dendrimers. Although applied in limited number of studies, dendrimers successfully delivered IM, saRNA molecules encoding antigens of Ebola, H1N1 Influenza, Toxoplasma gondii, and Zika virus generated protective immunity in mice (Chahal et al. 2016, 2017; Wang et al. 2021). The major drawback of dendrimer nanoparticles is cell-mediated toxicity due to their increased accumulation in cells. The inclusion of biodegradable cationic dendrimers with the amino acid ornithine could potentially solve this issue. For example, ornithine dendrimers could successfully deliver saRNA in skin cells. Interestingly, at N:P ratio of the dendrimer saRNA polyplexes mediated sevenfold higher protein expression compared to saRNA, delivered with optimized PEI nanoparticles.

2.2.2 Degradable Polymers

The potential of bioreducible polymers was unveiled for the delivery of saRNA in a study from 2020 that showed that increase of molecular weight of the linear, cationic polymer called "pABOL" -poly (CBA-co-4-amino-1-butanol) leads to a higher transfection efficiency both in vivo and in vitro, compared to delivery of PEI saRNA. Interestingly, paBOL saRNA vaccination resulted in high protein expression with both intradermal and intramuscular deliveries, while IM injection led to complete immune protection against Influenza virus in mice at doses of 1 ug. (Blakney et al. 2020a, b, 2021a, b).

Polymeric nanoparticles offer a promising delivery platform for saRNA, as they could potentially be optimized to target specific organs and tissues. Nevertheless, to completely utilize the platform, it is vital to explore their toxicological profile and mechanisms of immune sensing.

2.3 Cationic Nanoemulsions

Nanoemulsions (NE) are colloidal particles developed with oil phase dispersed into an aqueous phase and vice-versa. The fine droplets (oil-in-water or water-in-oil) are stabilized into confined thermodynamically stable particles by surfactants and co-surfactants (emulgents) (Jaiswal et al. 2015). Many recent studies using CNE formulations are based on MF59, Novartis' proprietary adjuvant, which is known for its safety and immunogenicity (O'Hagan et al. 2013), which occurs by triggering the tissue-resident monocytes to secrete chemokines and recruit APCs (O'Hagan et al. 2012).

2.3.1 Cationic Nanoemulsions for Self-amplifying RNA

Although CNE is well-studied and proven versatile tools for delivering siRNA, only a few studies explore the robust and clinically translatable CNE to deliver saRNA. A CNE delivery system, composed of DOTAP (cationic lipid) and MF59 (emulsion adjuvant), with sorbitan trioleate and polysorbate 80 as hydrophobic and hydrophilic surfactants, respectively, was developed to deliver saRNA encoding antigens for three infectious diseases (HIV, RSV, human cytomegalovirus (hCMV)) (Brito et al. 2014). Compared to the benchmark subunit vaccine (adjuvanted with MF59) and VRP, the CNE formulated saRNA vaccine candidate (against RSV and HIV) generated high IgG titers (geometric mean titer of 4.9×10^3) and viral neutralization even at low dose (0.015 μg). Similarly, when tested in non-human primates (rhesus macaques) against hCMV after two immunizations, strong antigen-specific cell-mediated protection was observed along with the humoral response. The inclusion of MF59 in the formulation increased the innate immune infiltration to the site of administration and led to an elevated immune response and protection (Brito et al. 2014).

In addition to eliciting potent humoral immunity, the novel formulation (as described by Brito et al.) generated a cross-reacting T cell response capable of protecting from heterologous influenza infection (Brazzoli et al. 2016). Besides viral infections, the CNE-saRNA vaccine was found to be safe and effective in providing protection against bacterial infections (Maruggi et al. 2017). APCs are vital to initiate tailored immune activation, and it has been shown saRNA vaccine immunogenicity can be enhanced by co-administering granulocyte–macrophage colony-stimulating factor (GM-CSF) (Manara et al. 2019). Co-administering saRNA-CNE (encoding GM-CSF) with saRNA-CNE (encoding nucleoprotein (NP) antigen of influenza A virus recruited a high number of APCs; however, no significant impact on the humoral response was observed, but an increased antigen (NP) specific T cells (especially $CD8^+$ T cells) immunity was generated. In addition, it also provided increased protection against a lethal heterologous PR8 influenza challenge than in mice administered with saRNA-CNE (NP) alone. Apart from robust transfection and immunogenicity, the saRNA vaccine delivery by the cationic nanoemulsions system holds appreciable biodistribution, tolerability, and safety profile (Stokes et al. 2020) and makes them an attractive and robust delivery system.

2.4 Electroporation

Electroporation is another method for saRNA delivery to cells that is dependent of brief electric pulses causing transient and reversible permeabilization of cellular membranes. Piggott et al. (2009) showed for the first time the potential of electroporation to enhance SFV formulation of saRNA vaccine delivery. In their study, saRNA encoding LacZ electroporated at optimized pulse conditions and was able to induce IgG1 response to LacZ in mice. Later, it was demonstrated (Huysmans et al. 2019a, b) that intradermal electroporation of the saRNA leads to longer antigen

expression 3–10 days compared to the one induced by electroporation of plasmid DNA or non-amplifying mRNAs or saRNA formulated with LNP and injected intradermally (Huysmans et al. 2019a, b). To evaluate efficacy in larger animal models, (Leyman et al. 2018) saRNA, pDNA modified, and non-modified mRNA were introduced in pigs with intradermal electroporation. Interestingly, saRNA mediated the highest and longest protein expression (12 days). (Ballesteros-Briones et al. 2020; Huysmans et al. 2019a, b) Electroporation allows saRNA delivery without the use of nanoparticles, which reduces toxicity and is broadly applied for transient protein expression of in different cell types. RNAse treatment before saRNA introduction was also found to increase expression efficiency (Huysmans et al. 2019a, b). Moreover, the higher targetability of electroporation could reduce the required saRNA dose even further.

3 Mechanistic Understanding of saRNA Nanoparticle Cellular Uptake and Entry

3.1 Cellular Uptake and Release Pathways

The end goal and final challenge for a delivery vehicle is to cross the cell membrane and release the cargo into cytosol; therapeutic efficacy of nucleic acids relies on efficient delivery into cytosol by crossing the cell membrane, escaping the endosomal pathway, and reaching the appropriate intracellular compartment. A range of cellular uptake mechanisms including phagocytosis, clathrin-mediated endocytosis, caveolae-dependent endocytosis are known to facilitate nanoparticle internalization by target cells; understanding the endocytic pathways and mechanisms aid in better designing cutting-edge drug-delivery vehicles with improved efficacy. Few studies have directly investigated the mechanisms of cellular uptake and release of saRNA nanoparticles.

3.1.1 Endocytosis and Endosomal Escape

Endocytosis is an active, multistep transport process across the cell membrane, wherein the cell membrane folds in to form endocytic vesicles and progressively undergo maturation from an early to late phase transition, during which the pH in endosomes gradually shifts from 6.0–6.5 (early endosome) to 5.0–5.5 (late endosome) and finally fuses with a lysosome (pH 4.0–4.7). However, in the intermediate stages, the endosomes that are equipped with receptor machinery identify and segregate the cargo and decide the destination (Mellman 1996). Endocytic pathways can be broadly classified into phagocytosis and pinocytosis (Fig. 3a).

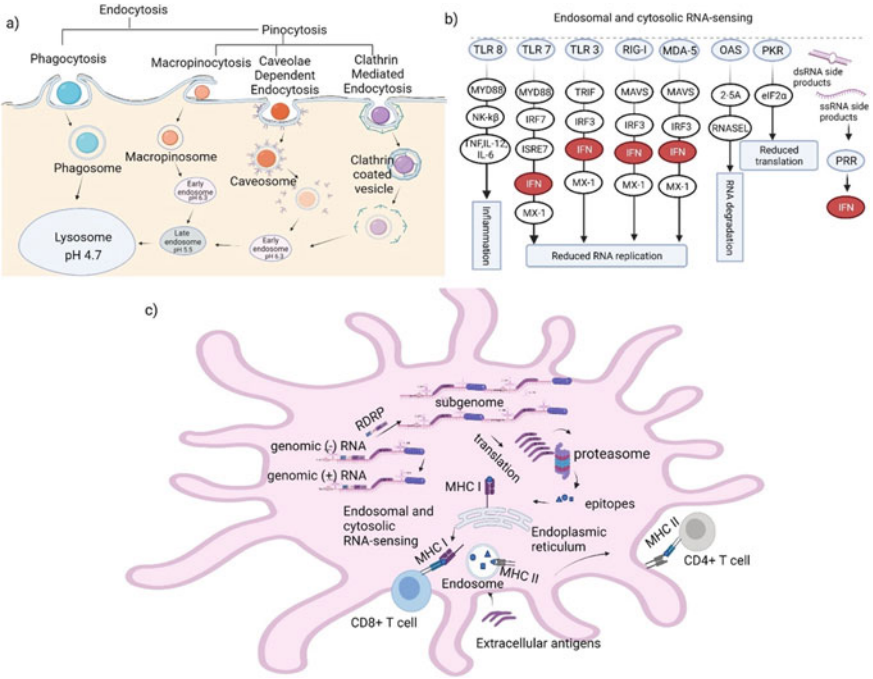

Fig. 3 **a** Cellular uptake pathways schematic of various cellular uptake mechanisms commonly followed to internalize nanoparticles. **b** saRNA endosomal and cytosolic saRNA-sensing, modified from (Sahin et al. 2014). In vitro transcribed saRNA-sensing by endosomal toll-like receptor 3 (TLR3), TLR7 and TLR8 (Jensen and Thomsen 2012) and cytoplasmic innate immune receptors—retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation-associated protein 5 (MDA5) 2′–5′-oligoadenylate synthase (OAS) (Nikonov et al. 2013) and protein kinase RNA-activated (PKR) (Blakney et al. 2021a, b). Downstream signaling leads to IFN activation tumor necrosis factor (TNF), interleukin-12 IL 12 and IL 6 activation, that results in inflammation and lower replication. Reduced translation is induced by downstream signaling from PKR through activation of eukaryotic translation initiation factor 2α (eIF2α). Overexpression of ribonuclease L (RNASEL) results in RNA degradation (Sahin et al. 2014). Legend: PRR—protein recognition receptors, IRF3 and IRF7, interferon regulatory factor; ISRE7, interferon-stimulated response element; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; MYD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor-κB; MX-1—MX Dynamin Like GTPase 1. **c** The image depicts adaptive immunity of saRNA vaccines. saRNA is self-amplified and antigen molecules are translated. Antigens, then are degraded by proteasome in cells, and the resulting fragments are presented by MHC-I to $CD8^+$ T cells. Extracellularly expressed antigen is taken up by endosomes and can undergo lysosomal degradation into fragments that are presented by MHC-II to CD4 + T cells (Kim et al. 2021). Legend: MHC-I and II—major histocompatibility complex I and II, CD4 T cells, CD 8T cells—cluster of differentiation 4T cell lymphocytes, cluster of differentiation 8T cell lymphocytes

The escape of RNA-loaded nanoparticles from the endocytic pathway prior to lysosomal fusion is critical and influences the efficacy of the therapeutic nanoparticle. However, it is not known how the route of internalization influences the endocytic escape of nanoparticle. A wide range of endosomal escape mechanisms have been investigated, with the ultimate aim of designing potential nanoparticle delivery systems with enhanced escape capability (Xu et al. 2021). The proton sponge effect (endosomal lysis by an increase in osmotic pressure) (Vermeulen et al. 2018) and fusion of nanoparticles (LNP and lipid-coated nanoparticles) with the endosomal membrane to release cargo into cytosol (Wittrup et al. 2015; Yang et al. 2017) are two well-explored endocytic escape mechanisms. The use of endosomolytic agents, such as peptides (melittin), toxins (exotoxin A), chemicals (chloroquine), loaded within nanoparticles to facilitate the endosomal escape was well-documented by (Ahmad et al. 2019). The following reviews discuss the most recent advancements in endosomal escape mechanisms (Cupic et al. 2019; Smith et al. 2018).

4 Routes of Administration

Vaccine routes of administration (Fig. 2b) are often determined by formulation design; they are vital for pharmacokinetics (clearance and distribution) and for the local/systemic and immune response. The majority of saRNA vaccine studies utilize the IM route of administration, including studies in non-human primates (Bogers et al. 2015) (Erasmus Jesse et al. 2020). There are few studies that utilize intradermal delivery and compare different routes of administration for saRNA vaccines. In one of them, both ID and IM deliveries of saRNA with pABOL lead to increased protein expression and cellular uptake, but only IM delivery resulted in protective immune responses in mice (Blakney et al. 2020a, b). Therefore, route of administration is of high importance for immunogenicity.

5 Innate Immune Sensing of saRNA Nanoparticles

5.1 saRNA Immune Sensing

Immune sensing of saRNA has been extensively studied and is well-described in various reviews (Pardi et al. 2018).

5.2 *Immune Sensing of Delivery Vehicles*

While RNA immune sensing is widely researched, saRNA delivery vehicles' interaction mechanisms with cells are largely unknown. It is presumed that stronger electrostatic interactions with cells result in a higher immune response, which explains the increased immunogenicity of cationic carriers, and their application as adjuvants. Interestingly, amino and hydroxyl groups increase the production of inflammatory cells in vivo to the highest extent. Increased endo- or phagocytosis is possibly due to the electrostatic interaction of cationic nanoparticles and the negatively charged cell membrane (Liu et al. 2017).

5.2.1 Immune Sensing of Lipid Nanoparticles

Cationic liposomes were shown to increase the expression of T helper type 1—TH1 cytokines—TNF-α, IFN-β, and IL-12, which might be beneficial for tumor targeting due to the tumor static effect (Elsabahy and Wooley 2013). Ionizable and cationic lipid nanoparticles were previously reported to induce the secretion of pro-inflammatory cytokines and reactive oxygen species, which depending on the dose of the delivery vehicles could become a safety concern. It is possible that toll-like receptors could activate downstream immune responses upon lipid nanoparticles internalization in cells (Hou et al. 2021). Interestingly, it was shown that DOTAP liposomes stimulated (cluster of differentiation) CD80 and CD86 expression in dendritic cells, but did not increase TNF-α, through NF-κB (Cui et al. 2005). Another study suggested the presence of multiple pathways for induction of toxicities and release of cytokines. In a study, where PI3K, mTOR, p38/AP1, and NF-κB were inhibited, the cytokines levels after siRNA-lipid nanoparticles introduction into cells were decreased (Elsabahy and Wooley 2013). In other studies, DinDMA lipid nanoparticle formulation specifically induced cytokine release in mice (IL-1α, IL-1β, IL-6, KC, IL-10, IFN-γ, and TNF-α). Lipid nanoparticle formulations for delivery of saRNA vaccines often include PEG which has been shown to elicit anti-PEG antibodies, in animals and humans, that can lead to severe allergic responses through anaphylatoxins release (Shimizu et al. 2020).

5.2.2 Immune Sensing of Polymeric Nanoparticles

The other frequently investigated delivery vehicles in RNA preclinical studies are polymeric NP, which can also lead to the induction of inflammatory cytokines (Mariani et al. 2019). There are several studies with linear PEI (Cubillos-Ruiz et al. 2009; Hu et al. 2013) which show its action as TLR5 agonist that can induce TLR5-inducible cytokine release in dose dependently in vivo. This could activate TLR5-mediated NF-kB signaling pathways like keratinocyte chemoattractant (KC) and IFN-inducible protein 10 (IP-10), similarly to bacterial Flagellin (Hu et al.

2013). However, more studies are still required to elucidate the mechanisms of PEI immunomodulation. (Elsabahy and Wooley 2013). In a recent study that compared bio reducible polymer pABOL and LNP for the IM delivery of saRNA, encoding SARS-Cov-2 spike protein and Influenzas HA, Blakney et al. showed that pABOL formulations induce in 100-fold higher protein expression, (Blakney et al. 2021a, b) Interestingly, LNP administered intramuscularly or intranasally, induced an increase in interleukin-6 expression, while pABOL NP did not. Both vaccine formulations resulted in the release of IFN-γ, IL-12, IL-5, and TNF-α 4 h after administration.

5.2.3 Immune Sensing of Mannosylated Nanoparticles

Various approaches aim at creating a delivery system with "pathogen-like" surface properties, including mannosylated lipid or mannosylated-chitosan nanoparticles. Mannose can target MR-mediated phagocytosis and lead to cytokine release. Some studies show that mannose on the surface of particles increases pro-inflammatory cytokines production IL-1β, IL-6 and TNF-α through activation of NF-κB (Chavez-Santoscoy et al. 2012; Elsabahy, Wooley 2013).

6 Conclusion

Even though there was extraordinary progress in mRNA and saRNA research in the last years, many aspects of saRNA vaccine delivery remain unknown—including the best viral sequence design and its effect on antigen production; saRNA structure optimization; and the driving forces of innate and adaptive immune sensing of delivery vehicles. Elucidating nanoparticles' interaction with the immune system is important for optimization of saRNA formulations. Some NP formulations, like pABOL, for example, may be better suited for protein replacement therapy, while other like LNPs with higher immunogenicity could be more suitable for vaccine delivery. Apart from NP design, it is important to modulate the elevated IFN response upon delivery of saRNA vaccines with incorporation of different IFN blockers, which would potentially benefit antigen expression. These topics are under-explored, and it will be beneficial to discover saRNA and NP interaction with the immune system and find methods for improvement of future saRNA vaccines or gene therapies.

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Nuclear Export of mRNAs with Disease Pathogenesis and Therapeutic Implications

Shalini Guha, Priyanka Barman, Aruniti Manawa, and Sukesh R. Bhaumik

Contents

Abstract In eukaryotes, RNA polymerase II genes are transcribed to mRNAs, processed in the nucleus, and then exported to the cytoplasm through the nuclear pore complex for translation to proteins. Thus, eukaryotic gene expression is regulated at multiple steps via coordinated actions of a large number of proteins in different cellular compartments. In this chapter, we describe how gene expression is controlled at the level of mRNA export from nucleus to the cytoplasm with implications for disease pathogenesis and therapeutic development.

Keywords NXF1 · TREX · CRM1 · mRNA export · Cancer · Therapeutics

Abbreviations

4E-SE	eIF4E sensitivity element
AD	Alzheimer's disease

S. Guha · P. Barman · A. Manawa · S. R. Bhaumik (✉)
Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale IL-62901, USA
e-mail: sbhaumik@siumed.edu

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_17

AML-1	Acute myeloid leukemia
AREX	Alternative mRNA export
ARE	AU-rich elements
ALS	Amyotrophic lateral sclerosis
ASOs	Antisense oligonucleotides
CBC	Cap-binding complex
CETN2/3	Centrin2/3
CIP29	29 kDa cytokine-induced protein
CRM1	Chromosome region maintenance 1
DBP5	DEAD-box protein 5
DSS1	Deletion of Suv3 suppressor 1
DUB	De-ubiquitination
ENY2	Enhancer of yellow 2
FACT	Facilitates chromatin transcription
FDA	Food and drug administration
FTD	Frontotemporal dementia
GANP	Germinal –center-associated nuclear protein
GLE1	GLFG (glycine–leucine-phenylalanine–glycine) lethal
HD	Huntington's disease
HECT	Homologous to E6AP carboxyl terminus
HuR	Human antigen R
IP6	Inositol hexaphosphate
iPSC	Induced pluripotent stem cells
LAAHD	Lethal arthrogryposis with anterior horn cell disease
LEF-1	Lymphoid enhancer-binding factor 1
LCCS1	Lethal congenital contracture syndrome 1
LRR	Leucine-rich repeat
LRPPRC	Leucine-rich pentatricopeptide repeat-containing
MAP1	Microtubule-associated protein 1
MBLN1	Muscleblind-like 1
MDS	Myelodysplastic syndromes
MDM2	Mouse double minute 2 homolog
mRNP	Messenger ribonucleoprotein
NEDD8	Neural precursor cell expressed developmentally down-regulated 8
NES	Nuclear export signal
NPC	Nuclear pore complex
NTF2L	Nuclear transport factor 2-like
Nups	Nucleoporins
NXF1	Nuclear RNA export factor 1
NXT1	Nuclear transport factor 2-like export factor 1
PCID2	PCI domain containing 2
Ran	Ras-related nuclear protein
RanBP3	Ran-binding protein 3
Ran-GAP	Ran GTPase activating protein
RBD	RNA-binding domain

RING	Really interesting new gene
RRM	RNA recognition motif
SAGA	Spt-Ada-Gcn5-acetyltransferase
SCF	Skp1-cullin1-F-box
SCA7	Spinocerebellar ataxia type 7
SGF73	SAGA-associated factor 73 kDa
SINE	Selective inhibitors of nuclear export
TDP43	TAR DNA-binding protein 43
THOC1	THO complex 1
TREX	Transcription export
UAP56	U2AF65-associated protein 56
UBA	Ubiquitin-associated
UBM	UAP56-binding motif
UIF	UAP56 interacting factor
UPS	Ubiquitin proteasome system
USP2	Ubiquitin-specific protease 2
UTR	Untranslated region

1 Introduction

Expression of the eukaryotic protein-coding genes is a complex process that begins with transcription to mRNAs in the nucleus, followed by co-transcriptional mRNA processing (i.e., mRNA 5′-end capping, 3′-end polyadenylation, and splicing) and nuclear export, and finally translation to proteins in the cytoplasm (Durairaj et al. 2009, 2017; Guha and Bhaumik 2021). Thus, eukaryotic gene expression is tightly controlled at multiple steps in different cellular compartments by a variety of proteins/factors. Misregulation of any of these steps would alter gene expression, which is often associated with diseases. Such cellular compartmentalized gene expression process requires proper transport of mRNAs from nucleus to the cytoplasm, and thus, nuclear mRNA export plays a crucial role in the regulation of eukaryotic gene expression. Nuclear export of mRNAs occurs through the nuclear pore complex (NPC) at the nuclear membrane. NPC is a large complex comprising of ~ 30 different proteins, known as nucleoporins (Nups), with an eightfold symmetry (Kabachinski and Schwartz 2015). It has a central cylindrical transporter channel, cytoplasmic fibrils, and a nuclear basket. Such NPC is permeable to small molecules, but bigger biomolecules including mRNA cannot freely move through it. Thus, export of mRNA from the nucleus to cytoplasm through NPC occurs via the coordinated actions of a number of proteins/factors. Mutations/malfunctions of these factors would alter mRNA export to the cytoplasm and hence gene expression, leading to cellular pathologies or disease states. In this chapter, we concisely describe nuclear export of mRNAs with disease pathogenesis and therapeutic implications.

2 Nuclear Export of mRNA

Export of mRNA from nucleus to the cytoplasm is an evolutionarily conserved process from yeast to humans, which occurs through NPC via the interaction of export receptor with mRNA and NPC, and involvement of other proteins and interactions (Fig. 1). Bulk mRNAs in humans are exported by the export receptor, NXF1 (also known as TAP; Mex67 in yeast), while the export receptor, CRM1 (*C*hromosome *r*egion *m*aintenance 1; also called Xpo1; Crm1 and Xpo1 in yeast), transports a subset of mRNAs from nucleus to the cytoplasm (Fig. 1), as described.

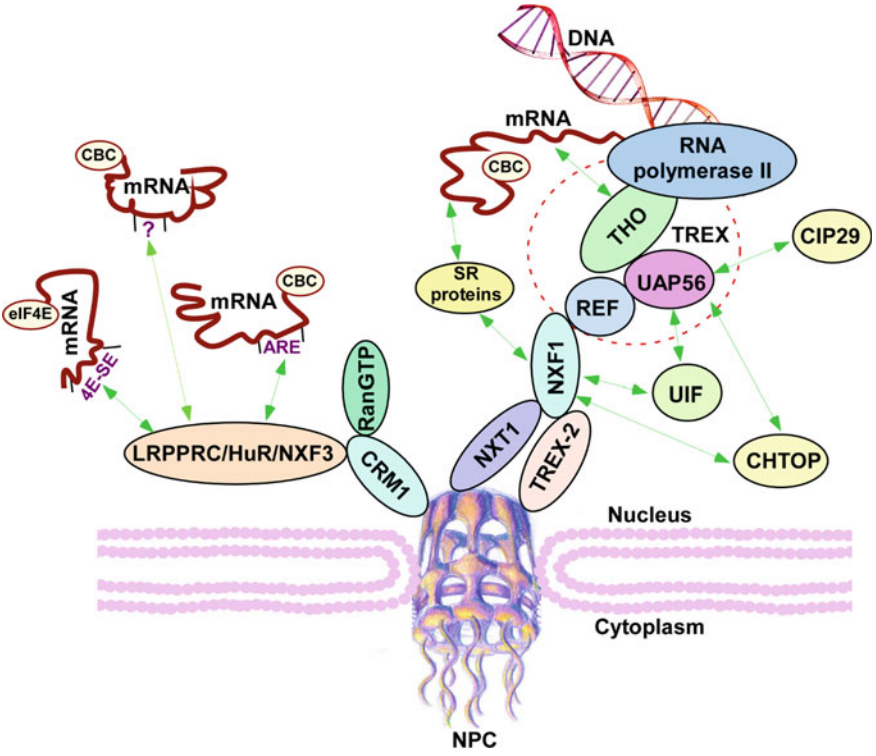

Fig. 1 Schematic diagrams showing distinct mechanisms of nuclear export of mRNA. Export of mRNA from nucleus to the cytoplasm occurs through NPC via the interaction of export receptor with mRNA, NPC, and other factors. Bulk mRNAs are exported by the export receptor, NXF1, while the export receptor, CRM1 transports a subset of mRNAs from nucleus to the cytoplasm. Double-headed arrow represents interaction. CBC, cap-binding complex; TREX, transcription export; TREX-2, transcription export 2; UIF, UAP56-interacting factor; ARE, AU-rich element; and 4E-SE, eIF4E-sensitivity element

2.1 NXF1-Mediated mRNA Export

NXF1 was first discovered as an export factor for the Mazon Pfizer viral RNA and was later characterized as a cellular mRNA export factor (Gruter et al. 1998; Katahira et al. 2015). It has multiple domains such as *R*NA-*b*inding *d*omain (RBD), *R*NA *r*ecognition *m*otif (RRM), *l*eucine-*r*ich *r*epeat (LRR), *ub*iquitin-*a*ssociated (UBA), and NTF2-*l*ike (NTF2L) (Valkov et al 2012; Katahira et al. 2015). The RRM of NXF1 has a low affinity to RNA and thus needs adaptor proteins for efficient interaction with mRNA for transport (Viphakone et al. 2012; Aibara et al. 2015). An important adaptor of NXF1 is ALY/REF (Yra1 in yeast) that is an integral component of TREX [*Tr*anscription *ex*port; an evolutionarily conserved complex of THO (that consists of HPR1/THOC1, THO2/THOC2, TEX1/THOC3, THOC5, THOC6, and THOC7; and Hpr1, Thp2, Tho2, Mft1, and Tex1 in yeast), UAP56/DDX39B (Sub2 in yeast) and ALY/REF] involved in transcription and mRNA export (Heath et al. 2016; Guha and Bhaumik 2021). Thus, such requirement of ALY/REF by NXF1 couples transcription to mRNA export. In addition to interaction with adaptor protein, NXF1 also interacts with another protein, NXT1/p15 (Mtr2 in yeast), via its NTF2L domain for interaction with phenylalanine–glycine (FG) repeat-containing Nups at the nuclear basket of NPC for transport of mRNA through NPC with the help of nucleocytoplasmic shuttling protein, RAE1/GLE2 (Rae1/Gle2 in yeast), at the periphery of the NPC channel (Bachi et al. 2000; Braun et al. 2002; Blevins et al. 2003; Durairaj et al. 2017; Guha and Bhaumik 2021). Further, the LRR domain of NXF1 contributes to its interaction with mRNA (Liker et al. 2000; Aibara et al. 2015). In addition, the UBA domain of NXF1 enhances the interaction of NXF1 with NPC (Fribourg et al. 2001). Therefore, multi-domain-containing NXF1 plays important roles in transporting mRNA through NPC via its interaction with several factors. Following mRNA transport to the cytoplasm, NXF1 is dissociated from mRNA with the help of a DEAD-box helicase, DBP5/DDX19B (Dbp5 in yeast), inositol hexaphosphate (IP6), and Gle1 in yeast (GLE1) (Lund and Guthrie 2005; Alcazar-Roman et al. 2006; Weirich et al. 2006; Montpetit et al. 2011; Durairaj et al. 2017; Guha and Bhaumik 2021). The signaling molecule, IP6, forms the complex with GLE1, which promotes the binding of DBP5 with cargo mRNA to trigger ATP hydrolysis and mRNA release (Alcazar-Roman et al. 2006; Weirich et al. 2006; Montpetit et al. 2011; Durairaj et al. 2017; Guha and Bhaumik 2021). Thus, mRNA export can be influenced by the signaling pathway at the level of mRNA release in the cytoplasm.

As mentioned above, NXF1 interacts with the TREX component, ALY/REF, which was initially discovered as a transcriptional coactivator of T-cell receptor α-gene, because of its ability to bind *l*ymphoid *e*nhancer-binding *f*actor 1 (LEF-1) and *a*cute *m*yeloid *l*eukemia 1 (AML-1) forming an enhancer-stimulating complex (Bruhn et al. 1997). ALY/REF was later characterized as a molecular chaperone able to promote the dimerization of basic leucine-zipper transcription factors in order to induce their DNA-binding activities (Virbasius et al. 1999; Mertz et al. 2007; Osinalde et al. 2013). The N- and C-terminal transient helices of ALY/REF, called UAP56-binding motif (UBM), are required for its interactions with UAP56 (Hautbergue et al.

2009). ALY/REF also has a central RRM that binds to mRNA (Golovanov et al. 2006) and provides ALY/REF the ability to bridge interactions between mRNA and NXF1. The RRM of ALY/REF has two unstructured arginine-rich regions on its two sides that act as the RNA-binding sites (Golovanov et al. 2006). These sites also interact with NXF1 (Hautbergue et al. 2008). Arginines in these sites are also modified by methylation, which reduces the ability/propensity of ALY/REF binding to mRNA. Such decreased propensity of interaction is required for the timely dissociation of mRNA from ALY/REF to be handed over to NXF1 for export (Hautbergue et al. 2008; Hung et al. 2010). Thus, ALY/REF plays important role in transporting mRNA from the site of its synthesis to the nuclear pore for export to the cytoplasm (Bjork and Weislander 2015). ALY/REF is responsible for exporting both spliced and intronless mRNAs (Rodrigues et al. 2001). Although ALY/REF is a major player in mRNA export, it has functional redundancy with the UAP56-interacting factor (UIF) that interacts with the histone chaperone, *Fa*cilitates *c*hromatin *t*ranscription, that regulates transcription (FACT), for its recruitment onto the mRNA (Hautbergue et al. 2008). UIF interacts with NXF1 and UAP56, similar to ALY/REF (Hautbergue et al. 2008). Like UIF and ALY/REF, CHTOP appears to function as mRNA export adaptor as it interacts with NXF1 and competes with ALY/REF for binding to UAP56 (Chang et al. 2013). Further, CIP29 interacts with UAP56 of TREX in an ATP-dependent manner (Dufu et al. 2010). CIP29 also interacts with its paralog, DDX39A/URH49, to form AREX (*A*lternative m*R*NA *ex*port; that contains THO, URH49, CIP29, and ALY/REF) for mRNA export (Dufu et al. 2010; Yamazaki et al. 2010). Thus, NXF1 interacts with TREX, TREX-interacting proteins (CHTOP and UIF) and AREX for mRNA export. In addition, NXF1 also interacts with serine–arginine (SR) proteins (e.g., 9G8 and SRp20 that bind to specific RNA sequence) for mRNA export (Huang et al. 2003; Hargous et al. 2006).

Like ALY/REF, the UAP56 component of TREX also plays an important role in mRNA export via its ATP-dependent helicase activity (a DEAD-box helicase). UAP56 was initially discovered as an essential pre-mRNA splicing factor (Fleckner et al. 1997). It is present on the pre-mRNAs during splicing, and its ATPase activity is stimulated by RNA. In its ATP-bound form, UAP56 interacts with ALY/REF to enhance ALY/REF's association with mRNA to be exported. It is, thus, a part of the *m*essenger *r*ibo*n*ucleo*p*rotein (mRNP) during mRNP translocation to the nuclear pore, but is displaced from mRNP prior to the nucleocytoplasmic translocation (Kiesler et al 2002; Durairaj 2017; Guha and Bhaumik 2021). Such displacement is caused by the binding of NXF1 to ALY/REF (Hautbergue et al. 2008).

In addition to its role in mRNA export via its ALY/REF and UAP56 components, TREX is also involved in transcription elongation (Dominguez-Sanchez et al. 2011; Durairaj 2017; Guha and Bhaumik 2021). TREX complex is assembled during transcription elongation to facilitate transcription (Li et al. 2005; Dominguez-Sanchez et al. 2011; Durairaj 2017; Guha and Bhaumik 2021). Further, mRNA processing and capping have also been implicated in TREX assembly and hence mRNA export (Hammell et al. 2002; Hocine et al. 2010). For example, mRNA cap-binding complex (CBC) and splicing factors are involved in the formation/recruitment of TREX on the mRNA (Heath et al. 2016; Sen et al. 2019; Guha and Bhaumik 2021). Further,

mRNA 3′-end formation machinery has also been implicated in facilitating mRNA export via assembling TREX (Heath et al. 2016; Guha and Bhaumik 2021). Therefore, TREX is a master regulator at the crossroad of transcription, mRNA processing, and mRNA export.

Like TREX, another transcription export complex, namely *tr*anscription *ex*port 2 (TREX-2), is also involved in mediating mRNA export via its interaction with mRNA export receptor, NXF1, not for promoting interaction of NXF1 with mRNA, but for NXF1 docking to the NPC for mRNA export (Jani et al. 2012; Durairaj 2017; Guha and Bhaumik 2021). TREX-2 is a well-conserved complex from yeast to humans. In mammals, it is composed of GANP (*G*erminal center-*a*ssociated *n*uclear *p*rotein; Sac3 in yeast), PCID2 (*PCI d*omain containing 2; Thp1 in yeast), DSS1 (*D*eletion of *S*uv3 *s*uppressor 1; Sem1 in yeast), ENY2 (*En*hancer of *y*ellow 2; Sus1 in yeast), and CETN2/3 (*Cen*tri*n*2/3; Cdc31 in yeast). GANP interacts with NXF1 (Wickramasinghe et al. 2010), forms the scaffolding platform to hold other subunits together in the formation of TREX-2 (Wickramasinghe et al. 2010; Jani et al. 2012), and associates with the nuclear basket of the NPC with the help of NUP153 and TPR proteins (Rajanala and Nandicoori 2012; Umlauf et al. 2013). The ENY2 component of TREX-2 is also an integral component of the transcription coactivator, *S*pt-*A*da-*G*cn5-*A*cetyltransferase (SAGA), and thus, functions at the interface of transcription and mRNA export, hence providing the functional link between transcription and mRNA export (Jani et al. 2012; Durairaj et al. 2014a; Guha and Bhaumik 2021). Both GANP and ENY2 are indispensable for the stability and localization of the TREX-2 at the NPC. However, other TREX-2 components such as PCID2 and CETN3 appear to be dispensable for the TREX-2 stability and localization (Umlauf et al. 2013).

2.2 CRM1-Mediated mRNA Export

Although mRNA export receptor, NXF1, is responsible for bulk mRNA export, the transport of a subset of mRNAs from nucleus to the cytoplasm occurs via the export receptor, CRM1 (Guha and Bhaumik 2021). CRM1 interacts directly with NPC, but requires adaptor proteins for interacting with mRNA. Such adaptor proteins have RBD as well as leucine-rich *n*uclear *e*xport *s*ignal (NES) for interaction with mRNA and CRM1, respectively, in the presence of *Ra*s-related *n*uclear protein Ran-GTP. This interaction network is required for transporting mRNA through the NPC. There are several adaptors involved in CRM1-mediated mRNA export. One such adaptor is *hu*man antigen *R* (HuR), that is, responsible for exporting mRNAs by binding to their *A*U-*r*ich *e*lements (AREs) at the 3′UTR (*U*n*t*ranslated *r*egion) with the help of nucleocytoplasmic shuttling proteins, pp32 and APRIL (Guha and Bhaumik 2021). Another adaptor protein, *l*eucine-*r*ich *p*entatrico*p*eptide *r*epeat-*c*ontaining (LRPPRC), binds to the 4E-eIF4*E*-*s*ensitivity *e*lement (SE element) at the 3′UTR of mRNA with eIF4E bound to 5′-end cap for nuclear export (Guha and Bhaumik 2021). NXF3 also functions as a CRM1 adaptor to export mRNA. However, the specific mRNA recognition sequence of NXF3 is unknown. Thus, CRM1-mediated

mRNA export does not occur in association with TREX or TREX-2. Further, the interaction of CRM1 with its adaptor proteins for mRNA export does not happen co-transcriptionally, unlike NXF1-mediated mRNA export. CRM1 requires Ran-GTP for high-affinity binding to its cargoes. It forms a trimeric transport complex with its export cargo and Ran-GTP inside the nucleus. Such transport complex formation is the rate-limiting step in CRM1-mediated transport. *Ran-b*inding *p*rotein 3 (RanBP3) promotes this complex formation (Nemergut et al. 2002) for exporting them out of the nucleus. After exiting the nucleus, the transport complex is met by *Ran-G*TPase *a*ctivating *p*rotein (Ran-GAP) in the cytoplasm. Ran-GAP interacts with CRM1 and hydrolyzes Ran-GTP to Ran-GDP in the presence of Ran-BP2 (nucleoporin present in the cytoplasmic fibrils containing Ran-binding domains) or sometime Ran-BP1 (soluble protein), which results in the dissociation of CRM1-mRNA cargo complex (Bischoff et al. 1995; Kehlenbach et al. 1999). Such cargo release helps recycle the used mRNA export factors back to the nucleus.

2.3 Ubiquitin–Proteasome System (UPS) Regulation of mRNA Export

We have described above how mRNA is exported out of the nucleus by distinct pathways. Bulk mRNAs are exported in a transcription-dependent manner via NXF1. Such pathway of mRNA export is further controlled by the UPS. The ubiquitination was implied in regulating mRNA export by showing that Tom1, a *h*omologous to *E*6AP *c*arboxyl-*t*erminus (HECT) domain-containing E3 ubiquitin ligase, is involved in the export of mRNA (Duncan et al. 2000). Later studies in yeast (Neumann et al. 2003; Rodriguez et al. 2003; Gwizdek et al. 2005) implicated Rsp5, another HECT domain-containing E3 ubiquitin ligase, in regulating mRNA export. In these studies, the THO subunit, Hpr1, was shown to be poly-ubiquitinated by Rsp5 and consequently degraded by the 26S proteasome. Subsequent studies involving depletion of *HPR1* or inactivation of Rsp5 resulting in the stabilization of Hpr1 correlated with the defects in the mRNA export (Gwizdek et al. 2005 and 2006). Further, studies (Shukla et al. 2009) in yeast demonstrated for the first time that Mdm30, an F-box protein [a component of *S*kp1-*c*ullin 1-*F*-box (SCF) ubiquitin ligase complex involved in substrate recognition] regulates mRNA export. Subsequent studies in yeast demonstrated that the Sub2 component of TREX in yeast is ubiquitylated with the help of Mdm30 and proteasomally degraded to help promote the process of mRNA export (Durairaj et al. 2014b). Similar proteasomal degradation was also observed in human cells (Xiong et al. 2012), increased level of Sub2 homologue (i.e., UAP56), and decreased mRNA export in *Xenopus* oocytes (Luo et al. 2001). Like TREX, NPC has also been targeted by UPS in regulation of mRNA export. For example, Nup96 has been found to be ubiquitylated and degraded by the 26S proteasome in controlling mRNA export in mice (Chakraborty et al. 2008). All these

studies support the regulation of mRNA export by ubiquitylation and proteasomal degradation.

Further, ubiquitylation has been shown to promote mRNA export independently of proteasomal degradation. For example, yeast homologue (i.e., Yra1) of ALY/REF undergoes ubiquitylation that does not signal 26S proteasomal degradation, but favors its dissociation from appropriately packaged mRNPs prior to export (Iglesias et al. 2010). Likewise, ubiquitylation of histone H2B (that does not undergo 26S proteasomal degradation) facilitates mRNA export (Vitaliano-Prunier et al. 2012). The *de-ubi*quitination (DUB) module of SAGA participates in maintaining histone H2B ubiquitylation. Sgf73/Ataxin-7 is an integral component of SAGA, and anchors DUB to the rest of SAGA (Shukla et al. 2006; Lee et al 2009). Thus, depletion of Sgf73/Ataxin-7 disrupts DUB integrity/function, thereby resulting in the elevated level of histone H2B ubiquitylation that is associated with reduced transcription and hence mRNA export (Shukla and Bhaumik 2007; Kohler et al. 2008; Vitaliano-Prunier et al. 2012; Sen and Bhaumik 2013). Indeed, loss of Sgf73/Ataxin-7 is found to reduce mRNA export (Kohler et al. 2008), elevate histone H2B ubiquitylation (Lee et al 2009), and impair SAGA's overall structural integrity (Shukla et al. 2006; Kohler et al. 2008; Lee et al 2009) and transcription (Helmlinger et al. 2006; Shukla et al. 2006; Shukla and Bhaumik 2007; Sen and Bhaumik 2013). Intriguingly, Sgf73/ataxin-7 has been shown to promote TREX-2 assembly to enhance mRNA export (Kohler et al. 2008). Further, it has been recently reported that Sgf73 is involved in transporting stress-inducible transcripts through NPC in a timely manner under environmental stress (Kim et al. 2019). In yeast, Sgf73/ataxin-7 interacts with Yra1 in the presence of environmental stress and enhances nuclear export of the stress-induced mRNAs (Kim et al. 2019). Moreover, Sgf73 also facilitates mRNA export under normal conditions (Kim et al. 2019). Thus, Sgf73 plays an important role in mRNA export, in addition to its well-established functions in regulating SAGA's integrity, histone H2B ubiquitylation, pre-initiation complex formation, and transcription. Together, all these studies reveal that mRNA export is controlled by ubiquitylation in a proteasome-dependent and -independent manners. However, most of these studies were carried out in yeast, and it remains unknown whether similar regulatory mechanisms exist in humans. Based on the evolutionary conservations of mRNA export machinery/factors from yeast to humans, the above UPS regulatory mechanisms of mRNA export are likely to be present in humans, which remains to be elucidated.

Overall, mRNA is processed co-transcriptionally via 5′-end capping, splicing, and 3′-end polyadenylation and then exported out of the nucleus to the cytoplasm through NPC. Such co-transcriptional mRNA export is mediated via the export factor, NXF1. Bulk mRNAs are exported to the cytoplasm through this pathway. However, a small subset of mRNAs are exported via export receptor, CRM1. Further, UPS regulates mRNA export in a proteasome-dependent and -independent manners. In addition, mRNA 5′-end capping, splicing, and 3′-end polyadenylation also regulate mRNA export. Thus, mRNA export is a tightly controlled process in regulation of eukaryotic gene expression. Impaired mRNA export and/or inappropriate mRNP formation lead to the retention of mRNAs in the nucleus, which can be dealt with

nuclear exosome that is made of $3' \rightarrow 5'$ exonuclease and accessory factors (Hilleren et al. 2001; Libri et al. 2002; Vasudevan and Peltz 2003; Fan et al. 2018).

3 Nuclear Export of mRNA in Disease Pathogenesis

As described above, mRNA export is a highly regulated process, and impaired/aberrant mRNA export alters gene expression, leading to cellular pathologies/diseases. For example, downregulation/hijacking of endogenous mRNA export machinery by viruses leads to successful viral infection (Guha and Bhaumik 2021). Further, mutations/malfunctions of mRNA export factors are found to be associated with cancer, neurodegenerative and cardiovascular disorders, and eye and other diseases (e.g., Nousiainen et al. 2008; Capelson and Hetzer 2009; Di Gregorio et al. 2013; Kumar et al. 2015; Gasset-Rosa et al. 2017; Berson et al. 2019; Borden 2021). Moreover, since splicing is an important processing event for mRNA to become export-competent, mutations in the pre-mRNAs impairing recruitment of the splicing factors can result in incomplete mRNA splicing, and hence mRNA export defect, leading to the accumulation/retention of transcripts in the nucleus. For example, mutation in the collagen pre-mRNA sequence causes it to be improperly spliced, which results in its retention in the nucleus. This leads to the decreased cellular level of the collagen protein, developing a disorder, namely osteogenesis imperfecta type I with increased fragility of bones (Johnson et al. 2000). Another disorder caused by pre-mRNA mutation is the myotonic dystrophy type I which results in severe muscle wasting disorder due to the aberrant nuclear retention of some mRNAs (e.g., MBNL1 [*M*uscle*bl*i*n*d-*l*ike 1]-regulated mRNAs) (Mateos-Aierdi et al. 2015). Likewise, mutations/malfunctions of the splicing factors impairing mRNA export are also associated with diseases (e.g., Scott and Rebel 2013; Saez et al. 2017). Thus, impaired/aberrant mRNA nuclear export, splicing defect, and mutations/malfunctions of mRNA export factors are associated with a variety of diseases. Some examples for the association of mRNA export factors with cancer and neurodegenerative disorders are described below.

3.1 Links to Cancers

Alteration of mRNA export is associated with a number of cancers. For example, the mRNA export receptor, CRM1, is over-expressed in gliomas, cervical, lung, gastric, prostate, colorectal, and pancreatic cancers (Shen et al. 2009; Turner et al. 2012). An increase in CRM1 level promotes the export of a subset of mRNAs involved in cell proliferation and survival, thereby aiding in cancer progression (Van der Watt et al. 2009). However, the mechanism of such over-expression in these cancers is not well understood. There have been genetic studies which hint at the roles of MYC and p53 in CRM1 regulation (Golomb et al. 2012). Further, along with mRNA export, CRM1

also functions in the transport of proteins including both tumor suppressors (e.g., BRCA1/2, p27, and p53) and oncoproteins (e.g., SNAIL, cyclins, TERT/telomerase, SURVIVIN, DNA topoisomerases, c-ABL, and YAP1) (Taagepera et al. 1998; Fu et al. 2013; Gravina et al. 2014; Kim et al. 2016). Therefore, up-regulation of CRM1 can cause cancers and other diseases through altered mRNA and protein transport across NPC. Further, mutations in CRM1 are also linked to tumorigenesis via the gain-of-function, which converts it into an oncogenic driver, specifically in B-cell malignancies (Camus et al. 2017). Recurrent mutations in CRM1 such as E571, R749, and D624 were identified mostly in B cell malignancies from the whole exome and genome sequencing analysis (Chang et al. 2016 and 2018; Taylor et al. 2019). $CRM1^{E571K}$ mutation has been found to be associated with MYC and BCL2 for promoting lymphomagenesis (Taylor et al. 2019). CRM1 mutations are also observed in 0.5–2.9% of solid as well as hematopoietic tumors (Consortium 2017). Therefore, CRM1 is involved in cancer via its mutations as well as up-regulation.

Like CRM1, its adaptor, LRPPRC, is also modulated in cancers. LRPPRC is up-regulated in a number of cancer tissues and cell lines such as prostate cancer, gastric cancer, lung adenocarcinoma, esophageal squamous cell carcinoma, colon cancer, and mammary and endometrial adenocarcinoma and lymphoma (Jiang et al. 2014 and 2015; Zhang et al. 2017). The LRPPRC level positively influences the tumor grade, metastasis, and the prostate-specific antigen level in the serum of prostate cancer patients (Jiang et al. 2014; Zhang et al. 2017). Studies (Jiang et al. 2014) suggested the contribution of LRPPRC in autophagy inhibition for facilitating tumorigenesis in prostate cancer. This is because high LRPPRC has been observed to be coupled with low *m*icrotubule-*a*ssociated *p*rotein 1 family member (MAP1S) level (Jiang et al. 2015). MAP1S is responsible for connecting mitochondria with microtubules in order to aid in autophagy and increased the suppression of tumorigenesis by influencing autophagosomal biogenesis and degradation (Jiang et al. 2015). Its level is negatively associated with patient survival in gastric cancer (Zhang et al. 2017). Another factor, eIF4E, associated with CRM1-mediated mRNA export is also found to be increased in different types of cancers and patient cells including M4 and M5 AML and lymphomas (Topisirovic et al. 2003; Hariri et al. 2013).

Similar to up-regulation of CRM1 and associated factors such as LRPPRC and eIF4E, NXF1-associated TREX and TREX-2 components are also found to be up-regulated in different types of cancers. For example, the level of the THOC1 component of TREX is elevated in lung, colon, breast, and ovarian cancers (Yang et al. 2008; Dominguez-Sanchez et al. 2011). Such elevation of THOC1 level is found to be directly correlated with the tumor size and metastatic state in breast cancer (Guo et al. 2005). THOC1 is also found to be reduced in skin and testes cancer specimens (Yang et al. 2008; Dominguez-Sanchez et al. 2011). Like THOC1, expression of the THOC5 component of TREX is also up-regulated in primary hepatocellular carcinoma (Saran et al. 2016). Further, phosphorylation of THOC5 is found to be increased in patient stem cells having chronic myeloid leukemia (Griaud et al. 2013). Similar to THOC components, the level of the ALY/REF component of TREX is increased in oral squamous cell carcinoma (Saito et al. 2013). Expression of ALY/REF is also increased in glioblastoma cells (Nagy et al. 2021; Wang et al. 2021) and drives

carcinogenesis by binding to the *MYC* mRNA (Nagy et al. 2021; Wang et al. 2021). ALY/REF also stabilizes a neuroblastoma protein, MYCN, via USP3 to drive cancer (Nagy et al. 2021). Like TREX, the components of TREX-2 are also upregulated in cancer. For example, the level of the GANP component of TREX-2 is increased in mantle cell, diffuse large B cell, and Hodgkin's lymphomas (Fujimura et al. 2005). Other subunits of TREX-2 such as DSS1 and PCID2 are also altered in cancers such as breast cancer and colorectal cancer, and their over-expression has been correlated with increased cancer cell survival (Rezano et al. 2013; Zhang et al. 2021). Thus, TREX and TREX-2 components are associated with various cancers. Further, Sgf73/ataxin-7 that facilitates mRNA export via TREX-2 regulates DUB activity and histone H2B ubiquitination, alteration of which is associated with cancers (Kapoor 2013; Melo-Cardenas et al. 2018; Zhou et al. 2021). Indeed, Sgf73/ataxin-7 is altered in various cancers such as lung, kidney, bladder, uterine, ovarian, and cervical cancers (cBioPortal; https://www.cbioportal.org/). Like Sgf73/ataxin-7, TREX, and TREX-2 components, NXF1 is also altered in cancer including *c*hronic *m*yeloid *l*eukemia (CML) (Puente et al. 2015). Further, nuclear basket or nucleocytoplasmic shuttling protein, Rae1, is also found to be elevated in breast cancer (Chin et al. 2006).

In addition to mRNA export factors, the components of NPC are also altered in cancer. For example, Nup88 involved in CRM1-mediated export is elevated in numerous malignancies such as breast (Agudo et al. 2004), head, and neck cancers (Singh et al. 2020). Likewise, Nup62 is also up-regulated in prostate and ovarian cancers (Kinoshita et al. 2012; Karacosta et al. 2016). Like upregulation, downregulation of NPC component is also associated with cancer. For example, decrease in Nup96 level reduces export of specific mRNAs causing an accelerated cell cycle progression and cancer development (Chakraborty et al. 2008). Thus, alteration of NPC components via up- or down-regulation is found in different cancers (Culjkovic-Kraljacic and Borden 2013). NPC components can also be altered/modulated via chromosomal translocations causing formation of Nup fusion proteins (Yu et al. 2000; Graux et al. 2004; Capelson and Hetzer 2009; Xu and Powers 2009; Mendes and Fahrenkrog 2019). Such chromosomal translocations have been reported to be aiding in multiple human malignancies (Yu et al. 2000; Graux et al. 2004; Capelson and Hetzer 2009; Xu and Powers 2009; Mendes and Fahrenkrog 2019). For example, Nup214 translocations have been observed for some rare acute myeloid and acute non-lymphoblastic leukemias (Graux et al. 2004; Saito et al. 2008). The C-terminal region of Nup214 frequently fuse with chromatin remodeling proteins such as SET and DEK (Mendes and Fahrenkrog 2019). These fusion proteins, namely SET-Nup214 and DEK-Nup214, disrupt nuclear export (Mendes and Fahrenkrog 2019;). Like Nup214, Nup98 also undergoes chromosomal translocation (Capelson and Hetzer 2009; Xu and Powers 2009). Most of the 14 Nup98 translocations have been linked to human malignancies such as AML, CML, and *m*yelo*d*ysplastic *s*yndromes (MDS) (Capelson and Hetzer 2009; Xu and Powers 2009). Thus, mRNA export factors and/or NPC are altered/modulated in a variety of cancers and can be used as biomarkers for cancer pathogenesis.

3.2 Links to Neurodegenerative Diseases

Cells have an increased reliance on mRNA export machinery, TREX, during rapidly proliferating and differentiating conditions, like in cancers, tissue differentiation, and embryogenesis. This is because with increased proliferation, cells transcribe and process mRNAs more, leading to a high demand of mRNA export and protein translation. When such reliance or dependence of cells on TREX is somehow disrupted, various neuronal disorders emerge. However, such impairment of TREX dependence is not always direct. Thus, along with mutations in TREX subunits causing neuronal disorders, there can be mutations in other genes which encode for proteins developing TREX dependence in a diseased state. Some examples of TREX mutations affecting the cells directly are missense mutations in the *THOC2* gene which leads to the development of syndromic intellectual disability (Kumar et al. 2015). Further, a de novo translocation event occurring on the X chromosome near the *THOC2* gene has been linked to cerebellar hypoplasia, ataxia, and retardation in children (Di Gregorio et al. 2013). Mutations in *THOC6* are linked to intellectual disability (Amos et al. 2017; Mattioli et al. 2019). Such *THOC6* mutations cause the protein to be mislocalized in the cytoplasm (Beaulieu et al. 2013). Another component of TREX having links to neurodegenerative diseases is ALY/REF (Berson et al. 2019). An unbiased screen in *Drosophila* for RBD genetically interacting with *T*AR *D*NA-binding *p*rotein 43 (TDP-43) revealed that Ref1 (fly orthologue of ALY/REF) is a possible contributor of *a*myotrophic *l*ateral *s*clerosis (ALS) and *f*ronto*t*emporal *d*ementia (FTD) (Berson et al. 2019). This study revealed that Ref1, when down-regulated, reduced TDP-43-induced toxicity. Furthermore, depletion of Ref1 caused mitigation of the toxicity due to the expression of the *C9orf72* GGGGCC repeat expansion (Berson et al. 2019), which has been found in ALS (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Thus, mutations/malfunctions of TREX components are associated with neurodegenerative disorders.

Further, as described above, malfunction of Sgf73/ataxin-7 (that promotes mRNA export via TREX-2 under stressed and normal conditions) leads to *s*pino*c*erebellar *a*taxia type 7 (SCA7) that is a rare autosomal dominant neurodegenerative disorder characterized by progressive cerebellar ataxia, dysarthria, dysphagia, cone-rod dystrophy, and retinal degeneration leading to blindness (La Spada 1998). This disease is caused by the expansion of a CAG trinucleotide repeat within the *SCA7* or *ATXN7* (gene for ataxin-7), which encodes a poly-glutamine tract within ataxin-7 and promotes toxic accumulation of ataxin-7 within neuronal nuclei. Further, the loss of ataxin-7's function in maintaining the DUB activity alters development of neuronal tube and visual system (Mohan et al. 2014). In Zebrafish, *atxn7* inactivation causes ocular coloboma, a structural malformation responsible for visual impairment (Carrillo-Rosas et al. 2018). Altered expression of *atxn7* also affects optical nerves (Carrillo-Rosas et al. 2018). At the final stages of retinal development, ataxin-7 deficiency shows alteration in the expression of crx, a transcriptional activator required for terminal differentiation of immature photoreceptors to mature

ones (Carrillo-Rosas et al. 2018). These findings together dictate the essential functions of ataxin-7 in vertebrate eye morphogenesis and photoreceptor differentiation, indicating that the loss of ataxin-7 function may contribute to the development of human coloboma. Further, increased expression of Sgf73/ataxin-7 is also associated with attention-deficit/hyperactivity disorder (Helmlinger et al. 2006; Carrillo-Rosas et al. 2018; Dela Pena et al. 2019).

Apart from TREX and Sgf73/ataxin-7, alterations of nuclear pore or nuclear envelop via mislocalization of Nups contribute significantly to neurodegenerative diseases such as AD (*A*lzheimer's *d*isease that comprises 60–80% of all dementia cases and is caused by extensive build-up of 42-amino-acid long amyloid-beta plaques and hyperphosphorylated species of microtubule-associated protein tau containing neurofibrillary tangles), HD (*H*untington's *d*isease that exhibits the symptoms consisting of dementia, personality changes, and involuntary movement), ALS and FTD (Boehringer and Bowser 2018; Fahrenkrog and Harel 2018). For example, Nup62 formed a smooth circle in the nuclear envelope in control tissue samples, while it formed an uneven nuclear envelope in AD patient tissue samples (Boehringer and Bowser 2018). Mouse models of HD also showed nuclear envelope defects (Gasset-Rosa et al. 2017). Such mouse models had CAG trinucleotide repeats within one/both huntingtin (*Htt*) alleles, which resulted in poly-glutamine aggregates with intranuclear inclusions of nucleoporins (e.g., Nup62 and Nup88) (Gasset-Rosa et al. 2017). Consistently, nuclear envelope abnormality was also seen in *i*nduced *p*luripotent *s*tem *c*ells (iPSC) derived neural progenitor cells and in the motor cortex in HD patient tissue samples (Gasset-Rosa et al. 2017). Similarly, in cell culture models of HD, NPC components were found in the poly-glutamine aggregates (Sakuma et al. 2017). Further, Nup155 that interacts with mRNA export factor, GLE1, is found to be mutated in cardiac disorder (e.g., atrial fibrillation) via GLE1-mediated mRNA export defect in mice (Zhang et al. 2008). Likewise, GLE1 is seen to be mutated in two motor neuron diseases, namely *l*ethal *c*ongenital *c*ontracture *s*yndrome *I* (LCCS1) and *l*ethal *a*rthrogryposis with *a*nterior *h*orn cell *d*isease (LAAHD) (Nousiainen et al. 2008). These two diseases result in phenotypes that include fatal reductions in the anterior horn motor neuron development, wasting of the ventral spinal cord and skeletal muscle atrophy (Nousiainen et al. 2008). The mutation in *GLE1* is caused by a single nucleotide substitution in the *GLE1* intron 3 that results in mis-splicing event and adds 3 amino acids in the GLE1 protein sequence, thereby changing the structure of GLE1, and hence mRNA export (Nousiainen et al. 2008). Thus, mRNA export machinery is associated with neurodegenerative diseases, when misregulated.

4 Therapeutic Strategy Targeting mRNA Export Machinery/Factors

As described above, the dysregulation of mRNA export or export factors/regulators is associated with cancer, neurodegenerative disorders, and other diseases. Thus,

mRNA export factors/machinery can be targeted for therapeutic development. For example, TREX and TREX-2 components are altered in various cancers and/or neurodegenerative disorders, as described above. Thus, these components can be targeted for therapeutic development for treatment. Further, as described above, CRM1 is over-expressed or mutated in a number of cancers. Additionally, factors involved in CRM1-mediated mRNA export are also altered in cancers. Thus, CRM1 can be targeted for therapeutic development.

CRM1 inhibitors can be developed as therapeutic agents for treatment. Leptomycin B is found to inhibit CRM1, but it has severe toxicity, and thus cannot be used clinically (Sun et al. 2013). However, leptomycin analogs, such as ratjadones, anguinomycins, and KOS2464, have shown less toxicity with significant therapeutic efficacy in a number of solid and hematopoietic tumors (Koster et al. 2003; Mutka et al. 2009; Sun et al. 2013). Further, synthetic selective inhibitors of nuclear export (SINE) compounds such as KPT-185, KPT-276, KPT-335, KPT-330 (Selinexor), KPT-8602 (Eltanexor), SL-801 (Felezonexor or CBS9106) have been developed in the recent years (Sun et al. 2016; Camus et al. 2017), and these compounds bind to the Cys528 at the cargo-binding groove of CRM1 to impair its function (Sun et al. 2013 and 2016). Several SINE compounds such as KPT-330, KPT-8602, and SL-801 have proceeded to clinical trials, and KPT-330 has been *f*ood and *d*rug *a*dministration (FDA)-approved for treating resistant and relapsed multiple myeloma (Vogl et al. 2018; Richter et al. 2020). KPT-330 has further proceeded to multiple clinical trials alone or in combination with other drugs for treatment of a wide range of cancers including solid tumors, multiple myeloma, liposarcoma, non-Hodgkin lymphoma, and AML (Abdul Razak et al. 2016; Kuruvilla et al. 2017; Wang et al. 2018). Thus, FDA-approved KPT-330 for treating multiple myeloma may also emerge as a new drug for other cancers.

Like CRM1, eIF4E that is involved in CRM1-mediated mRNA export is also elevated in cancers, and thus, can be therapeutically targeted for treatment. Indeed, ribavirin (an antiviral drug), an mRNA cap competitor that interferes with eIF4E function, was used for the treatment of refractory and relapsed myeloid leukaemic patients in a multi-centre phase II clinical trial (Assouline et al. 2009). This was the first clinical study which targeted eIF4E in a human malignancy showing clinical activity along with molecular responses in leukemic blasts (Assouline et al. 2009). Such treatment caused a reduction in eIF4E-mediated mRNA export and thus reduced mRNA export which was manifested clinically as cancer remission (Assouline et al. 2009). In fact, 5 out of the 11 evaluable patients in the study manifested clinical improvement following the ribavirin monotherapy such that 1 showed complete remission, 2 showed partial remissions, and 2 showed blast responses (Assouline et al. 2009). Out of the other 6, 4 had stable diseases and 2 had progressive diseases (Assouline et al. 2009). In spite of objective clinical responses, cells can become resistant to ribavirin, either by the impairment of drug entry or by some covalent modification of the drug. Under such resistance, eIF4E can cause a re-entry into the nucleus which correlates with elevated mRNA export, which can manifest patient relapsing.

As mentioned above, UPS is an important regulator of the mRNA export, and thus, can be targeted for therapeutic development. Multiple proteasome inhibitors are on the verge of FDA-approval with some already approved (Frankland-Searby and Bhaumik 2012; Park et al. 2018). The first of its kind is Bortezomib which docks onto the chymotrypsin active proteolytic site in the 20S proteasomal subunit of the 26S proteasome, and inhibits proteolytic activity. It has been approved by FDA for the treatment of multiple myeloma as it acts specifically on the myeloma cells by inducing apoptosis after excessive protein accumulation (Hideshima et al. 2011). The myeloma cells produce high amounts of immunoglobulin and show increased activation of apoptotic caspases, cell cycle arrest, DNA fragmentation, and cell death following bortezomib treatment (Hideshima et al. 2011). Relapsed or refractory myeloma can be treated by another proteasome inhibitor, carfilzomib (Jakubowiak et al. 2012). It shows near-complete clinical response in most of the untreated multiple myeloma patients in combination with lenalidomide and dexamethasone (Jakubowiak et al. 2012). Marizomib (also called NPI-0052) is emerging as a potent proteasome inhibitor affecting the chymotrypsin, trypsin, and caspase activities of the 26S proteasome (Chauhan et al. 2005). Although proteasome inhibitors can be used as therapeutic agents, inhibition of the proteasome can have secondary effects, as it is involved in many cellular processes (Frankland-Searby and Bhaumik 2012). Therefore, efforts are being put to develop agents by targeting the specific enzymes associated with UPS such as E2 ubiquitin conjugase, E3 ubiquitin ligase, and de-ubiquitinase. Further, proteins involved in regulating E3 ubiquitin ligase activity are also being specifically targeted for therapeutic development. For example, NEDD8 needs to be covalently bonded to the cullin component of cullin-RING (*r*eally *i*nteresting *n*ew *g*ene) E3 ligases. Thus, a compound, named as MLN492, has been developed to act as a potent inhibitor of NEDD8, leading to inhibition of E3 ligase activity (Liu et al. 2018a, b). Cullin-RING E3 ligase activity also needs Cdc34 working as an E2 conjugase for degradation of a number of cellular proteins such as the tumor suppressor p27 (Liu et al. 2018a, b). A drug, CC0651, has been developed to target Cdc34, which helps suppress p27 ubiquitination. Furthermore, compounds like serdemetan, nutlin-3, and NSC-207895 have been developed to target E3 ubiquitin ligases such as MDM2 and MDMX (for tumor suppressor protein p53), and they exhibit in vitro anticancer activities (Weathington and Mallampalli 2014). Examples of some inhibitors of DUB activity include p5091 and p220077 (Altum et al. 2011; Chauhan et al. 2012). These inhibitors target USP7 specifically and show in vitro response by enhancing ubiquitination and degradation of MDM2, thereby causing p53 levels to increase and cell apoptosis to occur when treated on cancer cells and myeloma cell lines (Altum et al. 2011; Chauhan et al. 2012). Similarly, many small molecules specific to USP2, USP14, USP5, and F-box proteins are being developed to target the UPS for cancer therapy (Aleo et al. 2006; Kapuria et al. 2010). Future studies identifying additional E3 ubiquitin ligases and de-ubiquitinases involved in mRNA export, and their alterations in diseases would reveal new biomarkers with specific targeted therapeutic potentials.

Further, as described above, Sgf73/ataxin-7 is involved in cancer and neurodegenerative disorders, and thus, can be targeted for therapeutic development. Proteolytic

cleavage is a well-known mechanism of neurodegenerative diseases including polyglutamine diseases (Sambataro and Pennuto 2017). The protease, caspase-7, has been suggested for proteolytic cleavage of ataxin-7 in SCA7 (Guyenet et al. 2015; Young et al. 2007). Thus, the inhibition of ataxin-7 proteolytic cleavage could be an effective therapeutic strategy, using genetic or pharmacological intervention. Further, non-allele-specific silencing of *ATXN7* using RNAi has been shown to improve the disease phenotypes in a SCA7 mouse model (Ramachandran et al. 2014). Consistently, Niu et al (2018) developed a strategy to treat visual impairment in SCA7 via inhibition of mutated ataxin-7 in retina, using *a*nti*s*ense *o*ligonucleotides (ASOs). Intravitreal injection of ASOs specifically targeting the mutated ataxin-7 reduced protein expression in the retina and ameliorated pathology and vision loss in a SCA7 mouse model. Further, yoga can also be a potential therapeutic technique for SCA7 (Mooventhan and Nivethitha 2017). Yoga has shown to be beneficial in reducing cramps in patients who have a type of progressive ataxia (Mooventhan and Nivethitha 2017). Due to the lack of mobility for 1 year, the patient developed ischial pressure ulcers (Mooventhan and Nivethitha 2017). Within 3 weeks, the patient was able to mitigate the pain and heal the ischial pressure ulcers by undergoing Iyengar yoga therapy (Mooventhan and Nivethitha 2017). In addition, other drugs can be developed by targeting Sgf73/ataxin-7. Therefore, a number of therapeutic agents can be developed targeting mRNA export factors/regulators for disease treatments.

5 Concluding Remarks

The mRNA export is an important, necessary, and ubiquitous cellular process for eukaryotic gene expression. Nuclear export of mRNAs is controlled at multiple steps such as mRNP assembly/packaging in the nucleus, interaction with nuclear basket, transit through central channel of NPC, release of mRNA in the cytoplasm, and recycle of export factors to the nucleus. These steps can be further influenced by extracellular factors/stimuli, intracellular signals or stress, thus implicating convergence of signaling pathways in the regulation of mRNA export. We describe above how mRNA export is regulated by various factors/processes. Altered mRNA export or mutations/malfunctions of mRNA export factors/regulators are associated with various cancers, neurodegenerative disorders, and other diseases. Thus, mRNA export machinery/regulators can be therapeutically targeted for treatment. Multiple therapeutic approaches and agents are discussed above for treatment. However, many of the above factors also function in other cellular processes in addition to regulating mRNA export. Thus, therapies targeted to these factors may have considerable secondary or adverse side effects. Thus, future studies on mRNA export in various diseases or patient samples in comparison with normal/healthy cells with mechanistic insights would provide additional biomarkers and specific targets for therapeutic development with relatively less side effects to maintain normal cellular functions.

Acknowledgements The work in the Bhaumik laboratory was supported by National Institutes of Health grants (1R15GM088798-01, 1R15GM088798-02, and 1R15GM088798-03), a National Scientist Development Grant (0635008N) from American Heart Association, Grants-in-aid from the American Heart Association (10GRNT4300059 and 15GRNT25700298), a Research Scholar Grant (06-52) from American Cancer Society, a Mallinckrodt Foundation Award, and multiple internal grants from Southern Illinois University. Shalini Guha is supported by the doctoral fellowship of Southern Illinois University, Carbondale. We apologize to the authors whose work could not be cited owing to space limitations.

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Preparation of Messenger RNA-Loaded Nanomedicine Applied on Tissue Engineering and Regenerative Medicine

Hsi-Kai Tsou, Cheng-Chung Chang, Tomoji Maeda, and Chin-Yu Lin

Contents

H.-K. Tsou · C.-C. Chang · C.-Y. Lin (✉)
Institute of New Drug Development, College of Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan
e-mail: geant@mail.cmu.edu.tw

H.-K. Tsou
Functional Neurosurgery Division, Neurological Institute, Taichung Veterans General Hospital, Taichung, Taiwan

T. Maeda · C.-Y. Lin
Tsuzuki Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung, Taiwan

T. Maeda
Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, Saitama, Japan

H.-K. Tsou
Department of Rehabilitation, Jen-Teh Junior College of Medicine, Miaoli County, Taiwan

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_18

Abstract Tissue engineering holds great promise for regenerative medicine; the multipotent cell lineage, biocompatible tissue-compliant biomaterials, and critical differentiation cues compose the critical factors for successful tissue engineering applied to regenerative medicine. The critical differentiation cues could be provided by recombinant proteins, alternative novel therapeutics, and gene therapy, including viral and non-viral-based methodology. One of the emerged gene therapeutics is messenger RNA (mRNA) administration, which holds critical advantages, such as tentative drug expression, no DNA backbone remaining in the cytosol, and easy to be manufactured as cocktail therapeutics and scaled up in a good manufacture process (GMP) factory. However, the successful mRNA medicine applied to tissue engineering remains challenging, including sophisticated mRNA medicine carriers, endosomal escape capability, targeted delivery, fine-tuned gene expression duration, and subsequent immune responses. This chapter will discuss the up-to-date technologies addressed on mRNA manufacture, sophisticated carrier design, mRNA medicine endosomal escape, nuclease resistance, and sustained expression. Furthermore, combined with tissue engineering, we will also introduce the mRNA medicine selection for therapeutic purposes, applied on the diseased animal model, mRNA activated matrix construction, and subsequent evaluation criteria will also be addressed.

Keywords Messenger RNA · Gene therapy · Regenerative medicine · Polyplex nanomicelle · mRNA-based therapeutics

1 Introduction

RNA therapy has recently gained more attention in new medicine development, especially for an intractable disease eager to find a new therapeutic medication. RNA medicine has many different chemical formulas, structures, and therapeutic mechanisms, such as siRNA, microRNA, and messenger RNA. The most critical function of mRNA is to provide and synthesize the target protein of interest through translation machinery in mammalian cells. Gene therapy using mRNA is a promising alternative with several advantages over that of plasmid DNA (pDNA) and has been extensively investigated in preclinical and clinical studies (Boczkowski et al. 1996; Chan et al. 2019; Lin et al. 2016). The preparation of therapeutic mRNA, loaded in

a drug carrier, endosomal escape scenario, and application in regenerative medicine will be comprehensively depicted.

2 Preparation of Therapeutic mRNA

The most popular and convenient method utilized to synthesize the mRNA is in vitro transcription (IVT), which is entirely proceeded by chemically and enzymatically reaction and is affordable for a general laboratory. The other method is chemically synthesized like a polymerization reaction. Here, we will describe the comprehensive synthesis process of mRNA through IVT in vitro.

2.1 DNA Template

The first step for mRNA synthesis by IVT is to prepare a pure and linearized DNA template for RNA polymerase binding to proceed with the RNA polymerization. The linearized DNA template is often collected from plasmid DNA after particular restriction enzyme digestion, which should be removed or entirely inactivated through heating, EDTA incubation, or proteinase K digestion and column purification. Then, the circular plasmid DNA should be entirely digested by a restriction enzyme, followed by separation through electrophoresis purification. The purity of the DNA template is a human manipulation controllable factor that plays a determinant role in the quality of the subsequently synthesized mRNA. In our experience, after we examined the quality of synthesized mRNA, most of the inferior mRNA quality was attributed to the quality short DNA template. In our experimental data, the gel electrophoresis and DNA bioanalysis show a unique and highly concentrated DNA template prepared for the production of runx1 mRNA (Fig. 1a and b). Besides, the double-strand plasmid DNA contamination in the final mRNA medicine product would also essentially interfere with the outcome of therapeutic efficacy, specifically in the immune activation (Nelson et al. 2020).

2.2 In Vitro Transcription (IVT)

Although the entirely chemical polymerization can be utilized to synthesize the mRNA transcript in an in vitro scenario, the chemically synthesized mRNA length is limited, and the process is complex, usually limited to hundreds of base-pair in a reaction (Nagata et al. 2010) and needs further enzymatic ligation to prepare longer mRNA transcripts (Pradere et al. 2017). Besides, the whole process is expensive and does not yet have sufficient demonstration to examine the feasibility of scaling up the reaction. On the other hand, in vitro transcription is the primary process

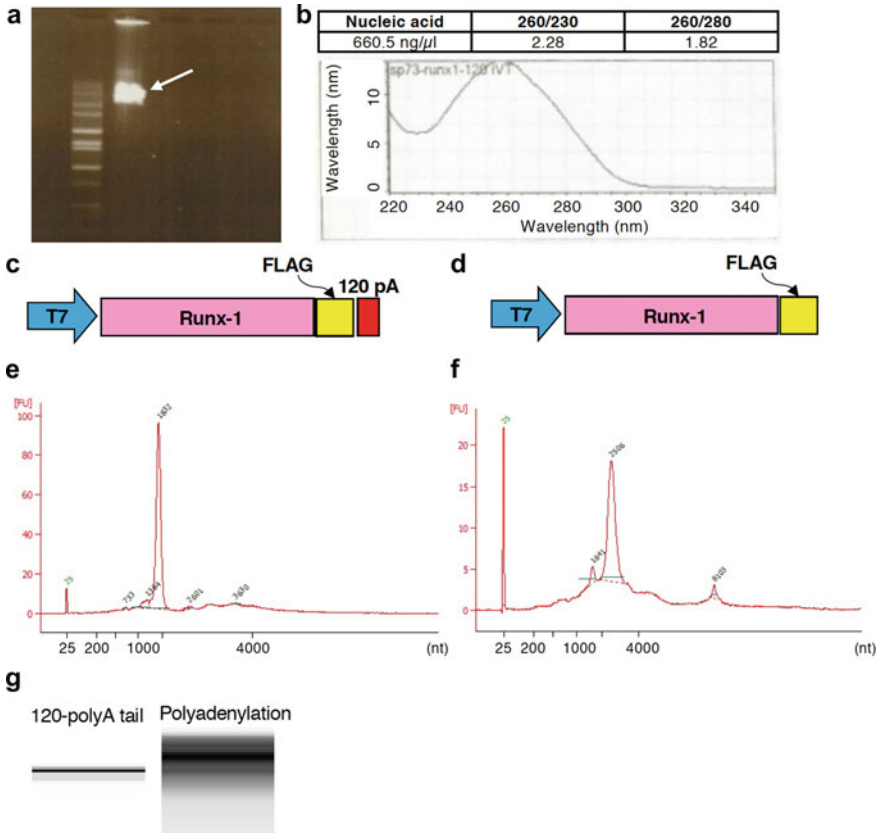

Fig. 1 Preparation of IVT vector template. **a** Plasmid DNA linearized by restriction enzyme, collected by agarose gel electrophoresis. Arrow indicates the linear plasmid with high concentration. **b** Examine the DNA concentration and quality. The O.D. 260/280 ratio higher than 1.8 represents good quality. **c** DNA template vector map with restricted polyA tail. The Runx-1 mRNA fused with the FLAG signal will be encoded under T7 promoter control. **d** DNA template vector map without restricted polyA tail. The Runx-1 mRNA fused with the FLAG signal will proceed with post-transcriptional polyadenylation. **e** Bioanalysis of the Runx-1 mRNA with restricted polyA tail. **f** Bioanalysis of the Runx-1 mRNA with post-transcriptional polyadenylation. **g** Agarose gel electrophoresis of the Runx-1 mRNA with restricted polyA tail and polA tailing through polyadenylation

to manufacture mRNA medicine, particularly facing large-scaled pandemics, such as the current COVID-19 outbreak, which requires billions of doses of vaccines within months. Thanks to the cell-free characteristic of the IVT process, allowing the promptly, large-scaly, and cost-effectively manufacture of the mRNA vaccine combating the COVID-19 pandemic. A 5 L bioreactor can produce almost a million mRNA vaccine doses in a single reaction (Kis et al. 2020). Besides, the mRNA transcript used in the mRNA medicine formulation could be adjusted rapidly to fulfil the required critical proteins combating the different diseases or pathogens

(Chaudhary et al. 2021). The mRNA medicine without DNA backbone in the final formula, attributing the mRNA medicine to be easily prepared as a cocktail to address multi-typed diseases at one shot. Due to the cell-free manufacturing process, the purchasing and maintenance cost of GMP-grade compliant manufacturing apparatus of mRNA medicine is cheaper than that of the plasmid-, virus-related and cell-containing manufacturing apparatus. The IVT process utilizes the recombinant RNA polymerase derived from T7, T3, or SP6 bacteriophages and prepares the synthetic mRNA for clinical use in a completed cell-free scenario, such as application in COVID-19 vaccines. This is one of the critical factors that attributes the Moderna and Pfizer–BioNTech vaccines to be approved by the FDA at an unprecedented pace (Corbett et al. 2020). Furthermore, the chemically modified nucleoside triphosphates (NTPs) are incorporated into the IVT process of mRNA transcript synthesis. For example, replacing 25% of uridine and cytidine in the original mRNA sequence with 2-thiouridine and 5-methyl-cytidine decreased mRNA binding to pattern recognition receptors synergistically, such as TLR3, TLR7, TLR8, and retinoic acid-inducible gene I (RIGI) in human peripheral blood mononuclear cells (PBMCs). This method was utilized to raise the average haematocrit and cure a lethal congenital lung disease in a mouse model using modified murine erythropoietin and surfactant protein B mRNA, respectively (Kormann et al. 2011). Other chemically modified NTPs applied on such as bone regeneration (Balmayor et al. 2016), 5′ cap structure (Wadhwa et al. 2020), alleviate the proinflammatory cytokine expression (Vaidyanathan et al. 2018), and improve translation efficiency (Hajj and Whitehead 2017) especially for the production of prophylactic proteins (Pardi et al. 2018). The Moderna and Pfizer–BioNTech COVID-19 vaccines, which produced > 94% efficacy in phase III clinical trials, contain chemically NTPs-modified mRNAs (Buschmann et al. 2021).

2.3 Capping

The mRNA generated from IVT must be further capped at the 5′ end to expand its full functions in mammalian cells. The 5′-cap of mRNA is a characteristic in eukaryotic cells, involved in numerous interactions required for a normal cellular function. The 5′-cap of mRNA consists of an inverted 7-methylguanosine connected to the head of the eukaryotic mRNA via a 5′–5′ triphosphate bridge, so-called cap 0 and served as quality control for correct mRNA processing and contributes to the stabilization of eukaryotic mRNA, splicing, nuclear export, initiation of translation and mRNA degradation. To initiate a normal translation process, the 5′-cap of mRNA would interact with the cap-binding complex (CBC) in the nucleus for nuclear export and the eukaryotic translation initiation factor 4E (eIF4E) in the cytoplasm for cap-dependent translation. Furthermore, capped RNA is a marker for the innate immune system to distinguish triphosphorylated infected viral RNAs from cellular RNAs. The cytosolic receptor RIG-I would be activated by short single and double-stranded triphosphorylated RNAs and collaborated with MDA-5 to initiate a

successful antiviral response. Moreover, the MDA-5 recognizes long triphosphorylated RNAs and RNAs lacking the 2′-OH methylation at the first nucleotide (cap1), a commonly observed modification in eukaryotes (Muttach et al. 2017).

A correct cap structure would be recognized by a cell to discriminate from pathogen infection. An uncapped mRNA transcript does not adequately represent a eukaryotic circumstance, and the preparation of correctly capped RNAs is essential to assess the function of mRNAs in the cellular context. Furthermore, altering the cap structure bears the potential to increase mRNA stability and translational efficiency. These two properties may dominate the critical factors of utilizing therapeutic mRNA shortly.

In the general lab-scaled mRNA synthesis, we usually have two methods for mRNA capping, including post-transcriptional capping and co-transcriptional capping during the IVT process. For co-transcriptional capping, many chemically synthesized cap analogs could be selected presently. One thing should be mentioned in the direction of mRNA synthesis after using cap analogs, and anti-reverse cap analog (ARCA) was developed to prevent the wrong direction of mRNA synthesis (Kwon et al. 2018). For post-transcriptional capping, the uncapped mRNA transcripts could usually be synthesized in large quantities and cost-efficient. Through Vaccinia virus Capping Enzyme (VCE) adds 7-methylguanylate cap structures (Cap 0) to the 5′ end of RNA generated by IVT, and Cap 0 is sufficient for efficient translation of the RNA in the eukaryotic systems.

Furthermore, the Cap 1 structure could be generated using Cap 0 RNA and 2′-O-methyltransferase. The Cap 1 RNA has been demonstrated to reduce the cellular innate immune response when the RNA is used in vivo. In our practical experience, post-transcriptional capping is convenient, high efficiency, and cost-saving, but the final concentration and weight of eluted mRNA would be slightly lower than that generated by the ARCA kit (Aini et al. 2016; Chan et al. 2019).

2.4 The 5′ and 3′ UTR, Including the PolyA Tail

Another critical factor that influences the mRNA stability and subsequent protein translation is the length of the polyA tail, which approximately 100–300 bp is considered sufficient for the binding of polyA-binding protein to cooperate with translation initiation factor proteins and initiate a successful translation (Linares-Fernandez et al. 2020). Another vital function of the polyA tail is interacting with the 5′ cap to protect the sequence from degradation by exonucleases and decapping enzymes (Wadhwa et al. 2020; Mugridge et al. 2018). Furthermore, the 5′ and 3′ UTRs, including the polyA tail, regulate mRNA translation, half-life, and subcellular localization.

Generally, two methods are used to incorporate the polyA tail into the mRNA sequence generated from the IVT process. They are co-transcriptional and post-transcriptional polyA tailing, respectively. Significant advances in the co-transcriptional polyA tailing have been made to streamline the large quantity of mRNA production. First, clinically used synthetic mRNA is transcribed in vitro

from a DNA plasmid template using the bacteriophage RNA polymerase, such as T7 RNA polymerase (T3 and SP6 RNA polymerases can also be used). Subsequently, co-transcriptionally capped with a 2′-*O*-methylated cap (termed as "CleanCap", developed by TriLink BioTechnologies company) and purified to remove double-stranded RNA (dsRNA) contaminants, reactants, and incomplete transcripts. The polyA tail encodes in the DNA template, eliminating reaction steps and reducing overall production time and material loss. Post-transcriptional polyA tailing using 2′-*O*-methyltransferase enzymes derived from the vaccinia virus.

For example, in our experiment prepared for the production of runx1 mRNA, the runx1 ORF was constructed in an SP73 vector containing a short 5′ UTR under T7 promoter control. For polyA tailing, we constructed both forms for comparison. One, the 3′ polyA tail was constructed in the vector for co-transcriptional polyA tailing (Fig. 1c). The other one, without polyA sequence in vector (Fig. 1d), mRNA generated from IVT synthesis will be further subjected to post-transcriptional polyadenylation through poly(A) polymerase reaction. Therefore, both typed mRNA transcripts were subjected to bioanalysis and gel electrophoresis, showing unique mRNA peaks and tailing-smeared forms, respectively (Fig. 1e, f, and g).

Incorporating the polyA tail in the DNA plasmid template also overcomes the tail length variability from enzymatic polyadenylation using polyA polymerase. PolyA tails of > 100 bp are optimal for therapeutic mRNAs. However, the DNA sequences encoding these long polyA stretches can destabilize the DNA plasmids template used for transcription. A solution to this stability issue is to include a short UGC linker in the polyA tail (Stadler et al. 2017; Eberle et al. 2020). The Pfizer–BioNTech vaccine BNT162b2 against SARS-CoV-2 uses this strategy and contains a ten bp UGC linker to produce the sequence A_{30}(10 bp UGC linker)A_{70}. Together, these innovations have overcome significant manufacturing bottlenecks and facilitated the development of a simple, cost-effective, and scalable one-step mRNA synthesis process.

2.5 Quality Analysis

The mRNA generated from IVT synthesis could be subjected to bioanalysis and gel electrophoresis to examine the quality of the transcript, which largely determines the following gene translation outcome and therapeutic efficacy. In general situation, the polyA sequence constructed in the vector was more suggested due to the higher concentration of mRNA and uniformed polyA length in final purified mRNA medicine.

3 Delivery Carrier Assembled as mRNA Medicine

mRNA medicine is tremendous large molecular therapeutics with negatively charged approximately 10^6 daltons, which cannot be diffused naturally into the cytosol

through the cell membrane with the anionic phospholipid bilayer. Besides, the mRNA medicine would be engulfed by immune cells and degraded by cytosolic nucleases. Many chemical and physical methods are currently developed to deliver mRNA medicine in vitro. However, few reports demonstrate the successful mRNA delivery in an in vivo circumstance, which requires low toxicity, minimizes unwanted immune responses, and controllable immunogenicity. To widely utilize mRNA medicine for clinical therapy requires the development of a safe and effective drug delivery vehicle (Hajj and Whitehead 2017).

The therapeutic mRNA ORF size range is approximately 300–1900 bp (~ 6.6 $\times$ 10^5 daltons) (https://origin-www.qiagen.com), and the mRNA is a single strand with a negative charge due to the phosphate group in the nucleotide chain. Therefore, the cationic polymer or lipids are usually used as the carrier vehicle for delivering therapeutic mRNA.

3.1 Lipid-Based Nanocarrier

Lipid-based nanoparticles are the most popular mRNA delivery vehicle and have gained the most advancement in use clinically. All mRNA-based vaccines against the SARS-CoV-2 in development or approved for clinical use before the end of 2021 employ lipid nanoparticles (LNPs) as delivery vehicles. LNPs possess numerous benefits for mRNA delivery, including simple formulation for manufacturing, modular recipe, biocompatibility, and large capacity for mRNA payload. The RNA medicine loaded and protected in the lipid-based nanoparticle, which typically includes four components: an ionizable lipid, cholesterol, a helper phospholipid, and a PEGylated lipid. All these formulae together encapsulate and protect the fragile mRNA core (Kim et al. 2021).

The lipid nanocarrier possesses a structure similar to the mammalian cell membrane, which would be fused with lipid nanocarrier, endocytosed mRNA-loaded lipid nanocarrier, and released the drug in the cytosol. Pfizer-BioNTech™ and Moderna™ SARS-CoV-2 vaccines utilize lipid-based mRNA carriers (Corbett et al. 2020).

3.2 Polymeric Nanocarrier

In addition to lipid-based nanocarrier, another nanoparticle with less clinical advancement than LNPs is polymeric nanocarrier. These polymers offer similar advantages to lipids that have been broadly applied for mRNA delivery (Kowalski et al. 2019). These polymers with cations condense, absorb nucleic acids into complexes, so-called polyplexes with various shapes and sizes, and can be engulfed into cells through endocytosis. Polyethylenimine (PEI) is the most widely explored polymer for nucleic acid delivery. Although its efficacy is excellent, its application is limited

due to its side-effected toxicity (Moghimi et al. 2005) attributed to its high charge density (Ulkoski et al. 2019). Although charge density is a critical consideration for mRNA complexation, an excessive charge could result in toxicity and serum aggregation (Mintzer and Simanek 2009).

Presently, the use of a low molecular weight form of the cationic polymer and incorporation of PEG into the formulation (Ke et al. 2020), conjugation to cyclodextrin (Li et al. 2016; Tan et al. 2020) and disulphide linkage (Breunig et al. 2007) can mitigate the toxicity of polyethyleneimine. Additionally, several alternative biodegradable polymers have been developed that are less toxic. For example, poly (β-amino ester)s excel at mRNA delivery, especially to the lung (Patel et al. 2019a). Furthermore, because they are easily synthesized by the Michael reaction (Lynn and Langer 2000), large poly(β-amino ester) libraries have been created that facilitate structure–function studies.

Furthermore, similar to the ionizable lipid-based composition in LNPs, pH-responsive polymers have also been examined for mRNA delivery. For example, poly (aspartamide)s conjugated with ionizable aminoethylene through aminolysis modified on the side chains. The mRNA-loaded nanomicelle composed by this block copolymer is protonated at the acidic pH inside endosomes, facilitating RNA delivery. The hydrophobicity and length of the aminoethylene side-chain tremendously influence the protonation and delivery efficacy of poly (aspartamide) (Kim et al. 2019). Furthermore, the pH-responsive charge-altering releasable nanocarriers have gained more attention due to their unique mRNA delivery mechanism. Despite the protonating scenarios after mRNA-loaded nanomicelles are engulfed by endosomes, these charge-altering, releasable nanocarriers self-degrade into neutral, nontoxic by-products at cytosolic pH, and rapid release of the mRNA into the cytoplasm (Haabeth et al. 2018; McKinlay et al. 2017).

One example, polyplex micelle-forming block copolymer modified from polyethylene glycol (PEG)-block-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) holds great promise for mRNA delivery and various medical applications (Kim et al. 2020) (Kataoka et al. 2000), such as applied on tissue engineering and regenerative medicine in our lab studies. We prepared the polyplex nanomicelles to complex with the Luc2 mRNA. We first synthesized the PEG-*b*-PBLA block copolymer (MW $\approx$ 28,000 with a unimodal molecular weight distribution of MW/Mn (weight-average molecular weight/number-average molecular weight) $\approx$ 1.1) and generated *N*-substituted polyaspartamides (designated as PEG-PAsp(TEP)) bearing four repeating units of tetraethylenepentamine (TEP) in the side chain to increase the positive charge. One exciting improvement upon such a model of mRNA delivery would be incorporating PEG chains to the modified polyaspartamide framework as PEG can help confer "stealth-like" properties to the overall polyplex nanocomposite (Amoozgar and Yeo 2012; Gref et al. 2000; Jokerst et al. 2011). This PEG installation is an essential step in this synergistic model, dominates the immunogenic response after in vivo administration, and may further help improve the overall stability of this mRNA nanomicelle delivery system.

The literature previously reported that the critical function of the polyamine in this complex would condense and adsorb mRNA via electrostatic interaction

and hydrogen bonding. Therefore, under a typical physiological environment, it is hypothesized that these polymeric block copolymers would organize to condense the mRNA load in the interior core, simultaneously exposing the more hydrophilic PEG domains to the peripheral region, ultimately organizing itself into the form of polyplex nanomicelle as nanoarchitecture as shown in Fig. 2a. Furthermore, the addition of PEG was also intended to assist in shielding the interiorly trapped mRNA against potential nuclease attack from physiological circumstances. Therefore, we performed both transmission electron microscopy (TEM) and dynamic light scattering (DLS) to evaluate the precise sizing of these particles in our previous study (Chan et al. 2019), revealed the micelles' sizing ranged between 31 and 35 nm in general (Fig. 2b, c).

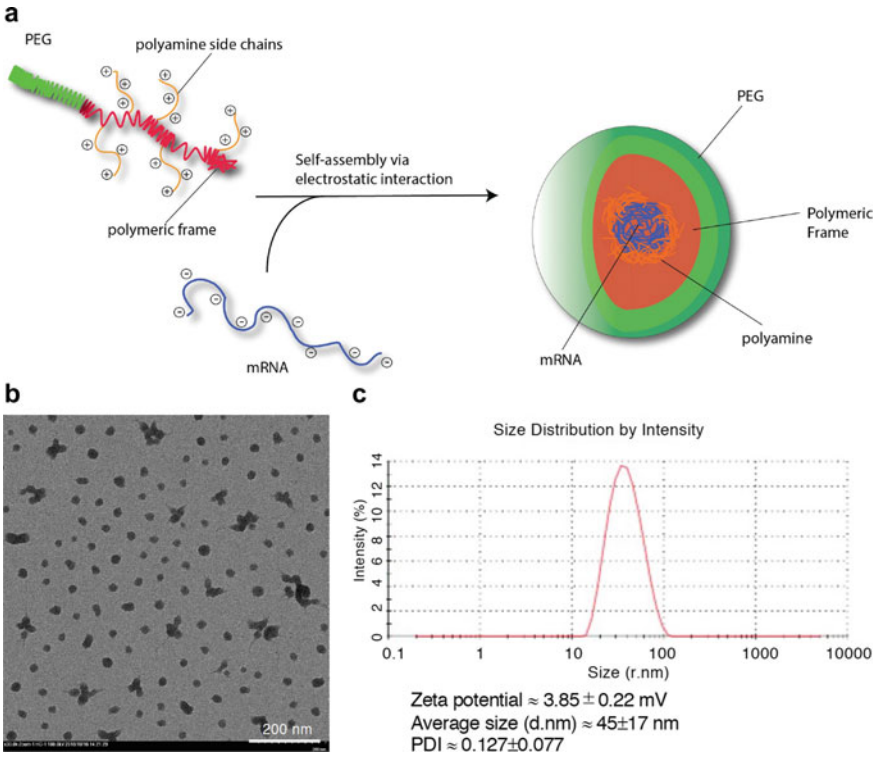

Fig. 2 Self-assembly mRNA-loaded polyplex nanomicelle and physicochemical properties. a Scheme of PEGylated polyamine-based block copolymer electrostatically interacts with mRNA to form self-assembly polyplex nanomicelles. **b** Transmission electron microscopy (TEM) examines the Luc2 mRNA-loaded self-assembly polyplex nanomicelles. **c** Physicochemical properties of Luc2 mRNA-loaded self-assembly polyplex nanomicelles through dynamic light scattering (DLS) measurement. (Figures adapted from Nanomaterials (Basel). 2019 Jan 5:9(1):67 and has gained permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

These PEGylated polyaspartate block copolymer modified with aminoethylene repeats has demonstrated the mRNA delivery capability in the liver (Matsui et al. 2015, 2018), CSF (Uchida et al. 2013; Uchida and Kataoka 2019), pancreatic cancer (Uchida et al. 2015), lung (Yoshinaga et al. 2019), brain (Lin et al. 2016; Federico et al. 2017; Fukushima et al. 2021; Abbasi et al. 2021), spinal cord (Crowley et al. 2019), spinal disc (Lin et al. 2019), knee joint osteoarthritis (Aini et al. 2016), and olfactory nerve malfunction (Baba et al. 2015). All these applications will be discussed in the following sections.

3.3 Peptide-Related Carrier

In addition to the previously described lipid and polymer-based gene carriers, peptides could also be served as gene delivery vehicles of therapeutic mRNA. The peptide sequence comprises the charged amine, either cationic or amphipathic amine groups (for example, arginine) in their backbone and side chains, which could electrostatically bound with mRNA to form the nanocomplexes. For instance, a fusogenic cell-penetrating peptide described in previous publish contains repetitive arginine-alanine-leucine-alanine (RALA) motifs, leading the nanocomplexes to change the conformation at endosomal pH, facilitating pore formation in the endosomal membrane and ameliorating the endosomal escape capability (McCarthy et al. 2014). Another paper demonstrated that the glutamic-alanine-leucine-alanine (EALA) motifs and lysine-alanine-leucine-alanine (KALA) motifs also have the function to interact with nucleic acids and perturb the cell membranes (Li et al. 2004). In addition, RALA was used to deliver mRNA to dendritic cells, particularly antigen-presenting cells (APCs) of the immune system, subsequently eliciting T cell-mediated immunity (Udhayakumar et al. 2017).

Recently, some commercially available cell-penetrating peptides have been launched. One of them, PepFect14, delivers mRNA to an ovarian cancer cell xenografted model established in mice (van den Brand et al. 2019). Another product, arginine-rich protamine peptides (approximately four kDa), turned to cationic property at neutral pH, which could be used to condense, absorb mRNA and facilitate delivery of the peptide-mRNA complexes (Kauffman et al. 2016). In addition, for protamine-based peptides complexed with mRNA, the complexes could activate Toll-like receptor (TLR7, TLR8) pathways that recognize single-stranded mRNA (Kallen et al. 2013). Therefore, the complexes could be served as an adjuvant for vaccine boost or immunotherapy applications. Some well-known applications, CureVac AG company, have been launched trials to evaluate a protamine-containing delivery platform termed as "RNActive" in clinical trials for melanoma (Weide et al. 2009), prostate cancer (Kubler et al. 2015), and non-small-cell lung cancer (Papachristofilou et al. 2019).

3.4 Other Typed Carrier

Other carrier types are used for the mRNA delivery, including the squalene-based cationic nanoemulsions, gene guns (Tavernier et al. 2011), transcript activated matrices (TAMs), or so-called gene activated matrix (GAM). Squalene-based cationic nanoemulsion consists of oil-typed squalene in the nanomicelle core and a lipid shell used to stabilize the squalene and adsorb the therapeutic mRNA on its out shell (Brito et al. 2014). Part of squalene formulations, such as Novartis's MF59, served as adjuvants in a series of FDA-approved influenza vaccines (Tsai 2013). MF59 formulation causes cells surrounding the injection site to secrete chemokines, which recruit antigen-presenting cells (APCs), induce differentiation of monocytes into dendritic cells, and enhance the invaded pathogen-related antigens uptake by APCs (O'Hagan et al. 2012). Another study, human bone morphogenic protein (BMP)-2-encoding mRNA prepared as TAMs, has demonstrated the capability to induce the osteogenic differentiation of MC3T3-E1 cells in vitro and bone regeneration in a non-critical sized femoral bone defect in a rat model (Badieyan et al. 2016) and furthermore combined with stem cells transplantation also induced satisfactory hone healing (Balmayor et al. 2017).

Gold and tungsten are commonly used in gene guns for mRNA delivery because they are readily available and could be coated with nucleic acids. However, numerous other materials have been tested, including platinum, iron, iridium, uranium, and glass. Improvements in the gun itself and the microprojectiles present the potential for this technology to expand in utility. The mRNA-coated microprojectiles were launched into mouse ears with the hand-held gun (Johnston and Tang 1994). Also, a gene gun-mediated gene delivery has effectively transported RNA molecules into several mammalian somatic tissue types. A study using gene gun treatment in mouse epidermis in vivo with messenger RNA expressing the human alpha-1 antitrypsin elicits a robust and consistent antibody response. Furthermore, the gene expression showed an increased titre with subsequent boosts. This study points to a future opportunity of applying RNA delivery methodologies for transgenic studies, genetic vaccination, and gene therapy (Qiu et al. 1996).

3.5 Cation-Free Administration

The last method proposed to deliver mRNA is a cationic material-free administration scenario. The significant toxicity of using cationic polymer to interact with biomolecules with anionic properties such as protein or nucleic acids is derived from the non-specific interaction with the cell membrane. Therefore, an experiment using PEGylated RNA oligonucleotides for mRNA delivery was conducted, showing improved serum nuclease resistance (Yoshinaga et al. 2021). The therapeutic mRNA was hybridized with sophisticatedly designed complementary oligonucleotide RNA, providing a shielding effect. This group used this PEGylated oligonucleotide RNA

to achieve a cationic polymer-free scenario for mRNA delivery. The PEG strands covering the mRNA were proposed to reduce non-specific interaction with charged biomolecules in physiological utilization. Furthermore, to achieve a more efficient mRNA delivery in clinical usages, the PEG strand is easy to modify further to bear other specific ligands, such as tissue or organ-specific homing ligand.

4 Release Profile of mRNA Medicine

4.1 Endosomal Escape

The exact mechanisms of how the polyplex nanomicelle escape from the endosomes is unclear. One popular hypothesis is the "proton sponge theory"; the proton buffering characteristic of the polyplex nanomicelle leads to osmotic swelling and rupture of the endosomes' lipid bilayer membranes (Bus et al. 2018). The other scenario of endosomal escape based on previous data is proposed (Fig. 3) (Chang et al. 2022). The mRNA polyplex nanomicelle comprising N-substituted polyaspartamides with aminoethylene side-chain residues were examined in rat-derived bone

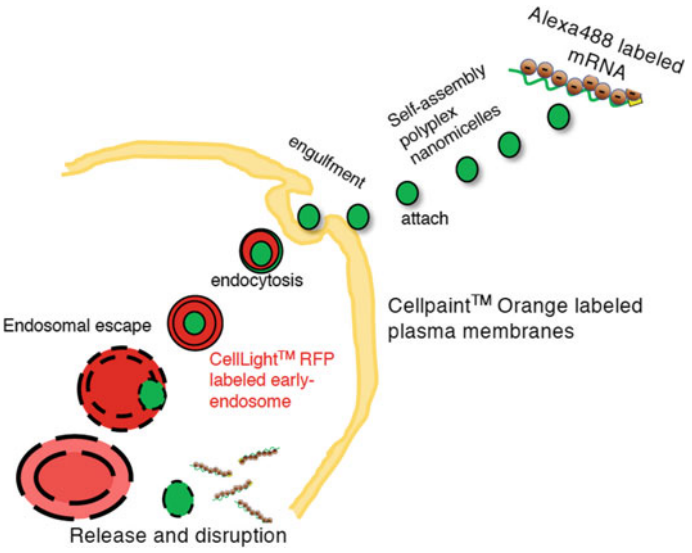

Fig. 3 Endocytosis of Luc2 mRNA-loaded polyplex nanomicelle. The proposed self-assembly polyplex nanomicelle delivers mRNA medicine into cells via endocytosis and the fluorescent dyes labelled mRNA, early endosome, and cytosol for 2-photonic microscopy. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

marrow stem cells. Endosomal escape capability and nuclease resistance capability are correlated with the modulated protonation behaviour of aminoethylene repeats due to different pH circumstances, revealing the critical importance of medicinal chemistry to design polycation structures for promoted mRNA transfection (Uchida et al. 2014). Besides, the odd–even effect also mainly dominates the endosomal escape behaviour of polyplex nanomicelle (Uchida et al. 2014).

In our study, the recombinant therapeutic protein expressed from mRNA delivery is critically attributed to mRNA-loaded nanomicelles' efficient endosomal escape capability at post-endocytosis. TEP N-substituted PEG-polyaspartate block copolymer holds a Gauche conformational transition effect (Tanaka et al. 2020; Cabral and Kataoka 2009), which would be protonated at the acidic circumstance in the endocytosis pathway when the nanomicelles reached late endosome, approximately pH 5.5, attracted apparent anion influx from the cytosol, such as Cl^-, leading to elevated osmotic pressure in the endosome (Uchida et al. 2011; Fröhlich 2012), meanwhile directly interacted with endosomal membrane, resulted in endosomal membrane disruption and nanomicelle escape and consequential mRNA release, achieved high-efficient antigen expression at a short post-transfection time.

4.2 Sustained Expression and Resistance to Nuclease Attack

To achieve the sustained expression of mRNA medicine, one can use a UGC linker between two transcripts, such as the application in manufacturing the Pfizer–BioNTech vaccine BNT162b2 (Stadler et al. 2017; Eberle et al. 2020). Besides, the endosomal escape capability of mRNA-loaded nanoparticles would also influence the mRNA transcript expression scenario and dominate the optimal application fields. For example, the PEG-PAsp(DET) and PEG-PAsp(TEP) held the faster endosomal escaping efficiency and showed superior mRNA medicine therapeutic efficacy in the brain (Lin et al. 2016; Federico et al. 2017; Fukushima et al. 2021; Abbasi et al. 2021). However, PEG-PAsp(TET) polyplex block copolymer encapsulated mRNA medicine expressed superior anabolism altering capability in osteoarthritis animal models (Aini et al. 2016).

An increase in mRNA stability by adding a poly(A) tail of 120 nucleotides in length has been demonstrated (Holtkamp et al. 2006). The optimization UTRs to achieve stability and translational efficiency is also very satisfactory. One strategy combined optimized UTR and chemically modified nucleotide, simultaneously achieving low immunogenicity and higher stability. The uridine and cytidine in the mRNA sequence were replaced by 25% chemically modified 2- thiouridine and 5-methyl-cytidine, respectively (Kormann et al. 2011). This approach was successfully applied for stem cell engineering and bone healing in the rat critical bone defect model (Balmayor et al. 2016).

In addition to the chemically modified nucleotide, stabilizing the mRNA structure, lowering the immunogenicity, and prolonging the mRNA medicine expression

time in vivo was observed. Other approaches directly modified the nucleoside structure, enhancing the resistance to nuclease attack and achieving some milestones. To date, among the 2′-ribose modifications, 2′deoxy-2′-fluoro-RNA (2′-F-RNA) shows remarkable properties for RNA interference (RNAi) applications. It enhanced nuclease resistance and improved siRNA delivery efficacy in vitro and in vivo (Pallan et al. 2011), and RNA oligomers delivery (Shu et al. 2011).

2′-O-methyladenosine-5′-triphosphate increased stability of the resulting RNA strand against nucleases. Thus, this methylation makes it a useful NTP for aptamer synthesis where increased nucleotide composition variation and increased nuclease resistance are desired (Xiao et al. 2012). Another chemically modified nucleotide, 2′,3′-dideoxyguanosine-5′-O-(1-thiotriphosphate), and other ddNTP analog contains a nuclease-resistant phosphorothioate linkage, 1-thiotriphosphate, causes chain termination during polymerase-mediated polymerization. These modified ddNTPs are helpful in antiviral research and many biotechnology applications (Adhikary et al. 2013).

Methylation at cytidine's number 5 atom position is a common, post-transcriptional modification in many RNA species, such as mRNA, miRNA, and tRNA, due to RNA methylation dominating many cellular functions, such as RNA stability, immune response, resistance to antibiotics, mRNA reading frame maintenance, and splicing. One of the modifications, 5-methyl-cytidine-5′-triphosphate (5-Methyl-CTP), is a commonly modified nucleoside triphosphate utilized to impart desirable biotechnical functions, such as increased nuclease stability, increased translation, or reduced interaction of innate immune receptors with in vitro transcribed RNA (Kariko et al. 2011).

Pseudouridine (5-ribosyluracil) was the first modified ribonucleoside discovered and is the most abundant natural modified RNA base, which could be found in structural RNAs, such as transfer, ribosomal and small nuclear RNA. Pseudouridine has been found to enhance base stacking and translation. Pseudouridine-5′-triphosphate (Pseudo-UTP) is used to increase the nuclease stability, translation of mRNA, or alter the interaction of innate immune receptors with in vitro transcribed RNA (Kariko et al. 2008). Totally, 5-Methyl-CTP and Pseudo-UTP and 2-Thio-UTP have been demonstrated to reduce innate immune response, enhance translation, longevity, and improve the therapeutic efficacy of mRNA (Vaidyanathan et al. 2018; Kormann et al. 2011). Another demonstration of 5-Methyl-CTP, Pseudo-UTP, and ARCA was used to prepare in vitro transcribed mRNA, which was successfully applied on inducing pluripotency stem cells (iPSCs) (Warren et al. 2012).

5 Application in Regenerative Medicine

5.1 Brain and CSF

mRNA medicine has been demonstrated in several studies of brain diseases. The feasibility of using polymeric nanomicelle to co-encapsulate mRNA expressing Cas9 nuclease and single-guide RNA (sgRNA) for genomic editing in vivo has been explored (Abbasi et al. 2021). This study revealed that packaging sgRNA and Cas9 expressing mRNA in the identical polymeric nanomicelle particles prevent the sgRNA from being diluted by the body fluid, meanwhile ameliorating the tolerability of sgRNA against enzymatic degradation in physiological conditions. Furthermore, this study demonstrated the sgRNA and Cas9 expressing mRNA co-packaged polymeric nanomicelle-induced genomic editing in parenchymal cells in the mouse brain, including neurons, astrocytes, and microglia using intraparenchymal injection. Totally, the genomic editing and gene expressing efficiency using sgRNA and Cas9 expressing mRNA co-packaged polymeric nanomicelle, apparently superior to that of the sgRNA and Cas9 expressing mRNA packaged in an individual polymeric nanomicelle particle separately. Another study addressed ischaemic neuronal death using mRNA therapeutics. The ischaemic neuronal death causes lifelong severe neurological disorders; currently, no proven effective treatment can prevent ischemia elicited neuronal death. They investigated the brain-derived neurotrophic factor (BDNF) expressing mRNA therapeutics for preventing neuronal death in a rat model of transient global ischemia (TGI) (Fukushima et al. 2021). Their data showed that the BDNF mRNA significantly increased the survival rate of hippocampal neurons after TGI as well as increased the BDNF recombinant protein expression in the rat hippocampus.

Another study addressed mRNA-loaded polymeric nanomicelles administration in CNS through intrathecal injection in the cisterna magna of mice. Successfully observed the gene expression in the brain stem and surrounding neural tissue (Uchida et al. 2013). Furthermore, the same group gets one more step close to the neural disease clinically; Alzheimer's disease (AD) was studied using mRNA medicine (Lin et al. 2016). They studied an mRNA expressing neprilysin (NEP), a protease that degrades Aβ and is a good candidate for this purpose. NEP is a membrane protein composed of ~750 residues (~110 kDa), capable of degrading Aβ monomers and oligomers (Iwata et al. 2001). Principally, the degradation of Aβ by NEP would effectively regulate the initiation and progression of the early stage of AD, resulting in alleviated Aβ deposition and preventing the pathogenic progression in the brain (Iwata et al. 2013; El-Amouri et al. 2008). The strategy of breaking the balance between the monomer or oligomer form of soluble Aβ and the fibril form of insoluble Aβ is potentially to alleviate or prevent the pathogenic change of AD. The exciting characteristic of mRNA medicine is the diffuse scenario in the target tissue. Their study administered GFP-NEP mRNA through intracerebroventricular (i.c.v.) infusion demonstrated that the GFP fused NEP recombinant protein expression broadly diffused in the tissues surrounding the brain ventricle (Fig. 4). This phenomenon

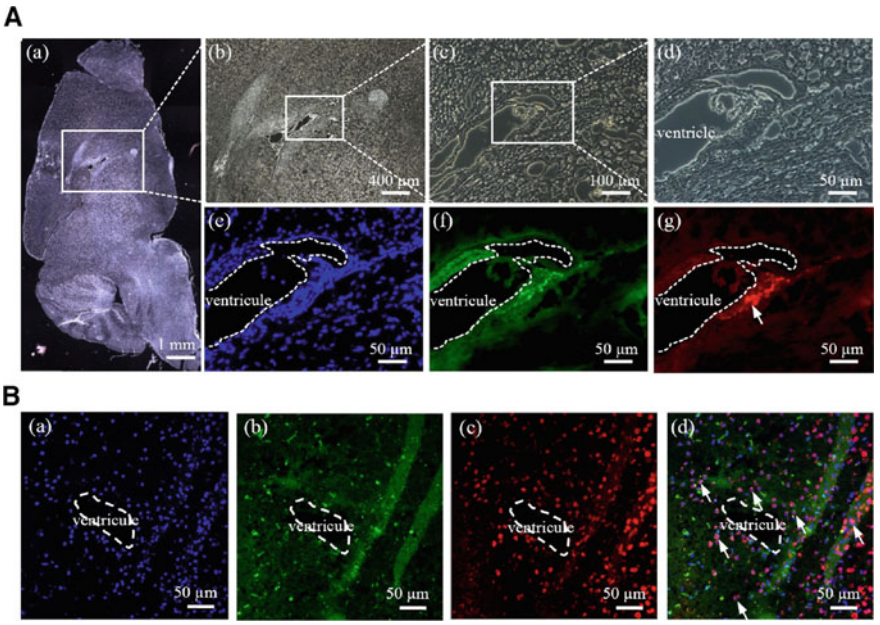

Fig. 4 mRNA-loaded polyplex nanomicelle medicine for brain infusion. Histological evaluation of GFP-neprilysin (GFP-NEP) mRNA-loaded polyplex nanomicelles intracerebroventricular (i.c.v.) infusion in mouse brain. **A** The Cy5 labelled GFP-NEP mRNA was mixed with PEG-PAsp(DET) to form polyplex nanomicelles and injected by i.c.v. infusion. At 24 h post-infusion, the brain was removed for histological examination. **a-d** were bright views of serial magnification showing the infused ventricles, and an identical slide was used for **e** DAPI staining, **f** IHC staining of the GFP-NEP fusion protein, and **g** tracking of Cy5 fluorescence, indicated by a white arrow. **B** Section from GFP-NEP mRNA i.c.v. infusion at 24 h post-infusion further proceeded with **a** DAPI staining, **b** IHC staining of the GFP-NEP fusion protein, and **c** IHC staining of NeuN marker. **d** shows the merged photo, the white arrows indicate widespread expression of both GFP and NeuN in neurons. (Figures adapted from Journal of controlled release. 2016 Aug 10, 235:268 and has gained the permission to reuse through RightsLink®)

was also similarly observed in an mRNA-loaded polymeric nanomicelle hydrodynamically injection in the liver, where the recombinant protein encoded by mRNA administration was detected in almost all liver cells after injection (Matsui et al. 2015). This characteristic is essential for administering a therapeutic protein that is not secreted or membrane-bound; in this case, transfecting more cells becomes necessary.

Furthermore, the gene expression efficiency of mRNA-loaded polymeric nanomicelle administration in the brain was quantitatively compared to the same polymeric nanomicelle loaded with plasmid DNA. A firefly luciferase-expressed mRNA (Luc2 mRNA) and plasmid DNA (Luc2 pDNA)-loaded polymeric nanomicelles were prepared for intracerebroventricular injection, followed by the in vivo imaging system (IVIS) examination. Luc2 mRNA nanomicelle provided apparent luciferase expression in the brain than the Luc2 pDNA or naked Luc2 mRNA (Fig. 5) (Lin et al.

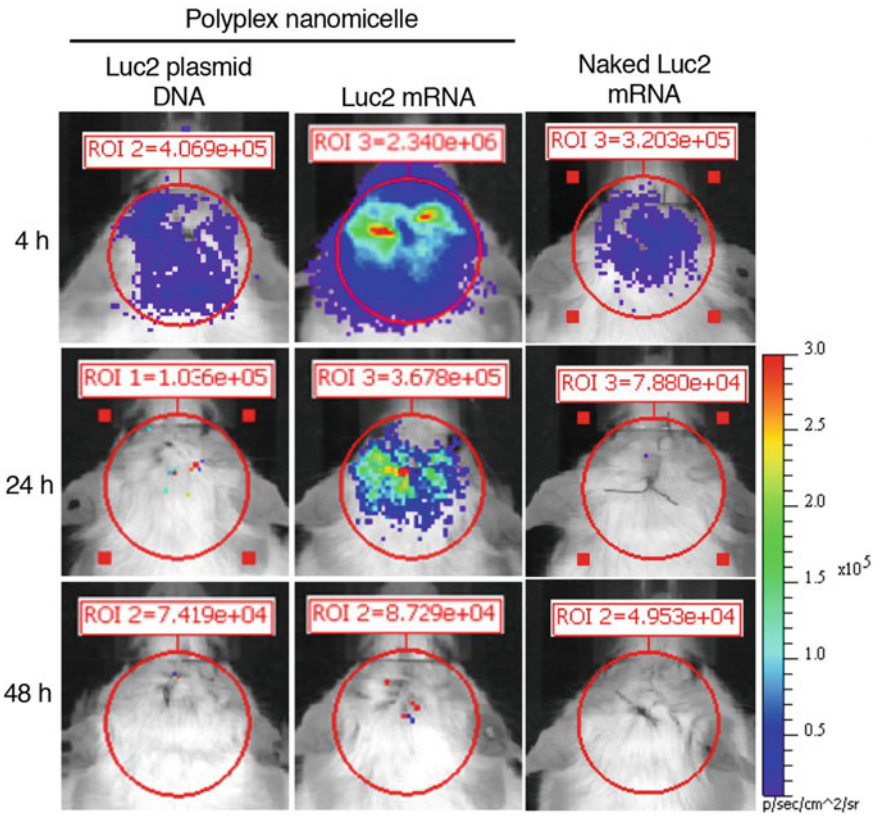

Fig. 5 mRNA-loaded polyplex nanomicelles resulted in superior gene expression in brain tissues after i.c.v. infusion. IVIS images of mice i.c.v. injected with polyplex nanomicelles containing Luc2 plasmid DNA or Luc2 mRNA, compared with infusion using non-protected, naked Luc2 mRNA. (Figures adapted from Journal of controlled release. 2016 Aug 10, 235:268 and has gained the permission to reuse through RightsLink®)

2016). Another study using mRNA encoding Aβ-targeting single-chain variable fragment (scFV) antibody with a secretion signal for passive immunotherapy showed the amyloid burden decreased in an acute amyloidosis model in mice, indicating that the therapeutic scFv encoded by mRNA medicine, an innovative polymeric nanomicelle delivery vehicle, and a suitable secretion signal are necessary for a successful therapeutic formulation (Federico et al. 2017).

5.2 Cartilage

The usage of mRNA medicine in the therapy of cartilage-related disorders, such as osteoarthritis (OA), has been demonstrated. OA is a chronic degenerative joint disease

that broadly happens in the elderly. Unfortunately, presently, no disease-modifying osteoarthritis drug (DMOAD) has been proved for clinical use. A team raised a disease-modifying strategy for OA treatment using anabolic factor mRNA medicine administrated by polymeric nanomicelles (Aini et al. 2016). They developed a polyplex nanomicelle loaded with mRNA expressing runt-related transcription factor 1 (runx1), followed by intraarticular injection in OA knee in a mouse model established by tendon resection. Runx1 is demonstrated to dominate the anabolism effect in the extracellular matrix (ECM) in cartilage, meanwhile playing a direct role in kartogenin-mediated cartilage repair using MSCs (Johnson et al. 2012). Moreover, runx1 overexpression enhanced collagen type II expression and GAG content in OA joint (Yano et al. 2013). Their data demonstrated that the OA progression was significantly alleviated in the runx1 mRNA nanomicelle administration group compared to the non-treatment control (Aini et al. 2016).

5.3 Bone

As previously described, so-called TAM or GAM has been successfully merged with mRNA medicine and applied to critical bone defect healing was demonstrated. TAM loaded with human BMP2 expressing mRNA can efficiently promote bone regeneration in a femoral bone defect model established in the rat (Badieyan et al. 2016). They prepared a collagen sponge pre-loaded with chemically modified mRNA and vacuum-dried to establish a TAM. Subsequently, the sustained delivery, bioavailability, safety, suitability for long-term storage, and efficacy of stimulating bone regeneration in rats' femoral bone defect model were examined. In addition, another matrix manufactured by fibrin to examine the efficacy for stimulating bone regeneration in vivo was also explored (Balmayor et al. 2016). Almost identical experimental procedures, chemically modified human BMP2 encoding mRNA, except for the matrix basal material. Their data showed the improved bio-efficiency of recombinant BMP-2 growth factor in bone tissue repair in a rat model.

Furthermore, the same group manufactured micro–macro biphasic calcium phosphate (MBCP) granules, loaded with chemically modified mRNA medicine, and examined the capability to induce the osteogenesis of stem cells. Their data proved the concept of developing efficient TAMs for bone regeneration by combining chemically modified mRNA (cmRNA) and optimized biomaterials. Besides, the chemically modified mRNA expressing the recombinant human BMP-2 loaded in MBCP granules induced robust collagen I and osteocalcin gene expression in the stem cell culture circumstance (Balmayor et al. 2017). Furthermore, Utzinger et al. developed a bioactive microsphere containing cmRNA bearing lipoplexes to incorporate a therapeutic component to prepare as injectable calcium phosphate types of cement (CPC). Their study demonstrated a methodology to incorporate cmRNA bearing lipoplexes into CPC without loss of function, holding the property to release and transfect immediately after administration in vivo during the cement degradation and cells infiltration (Utzinger et al. 2017). Another study incorporating chemically modified RNA in the

translation initiator of short untranslated regions (TISU) in BMP2 mRNA proposes to extend the mRNA biostability to induce robust osteogenesis. Their study removed some undesirable sequences in BMP2 mRNA, such as the upstream open reading frame in the 5′-untranslated region (UTR) and polyadenylation sequence with an AU-rich tract in the 3′UTR. Subsequently, a translation initiator of short UTRs (TISU) was incorporated with 5-iodo-modified pyrimidine nucleotides. Their data showed superior bone formation, endochondral ossification, and improved vascularization in a critical-sized femoral defect in rats (Zhang et al. 2019).

5.4 Intervertebral Disc (IVD)

We demonstrated mRNA medicine's utilization to mitigate the intervertebral disc (IVD) degeneration scenario (Lin et al. 2019). The IVD is a dynamic tissue, the ECM undergoes continuous remodelling as IVD cells produce new ECM material and proteases degrade old material (Urban and Roberts 2003). Therefore, disc degeneration is not only an ageing disorder but is also related to the shortage of critical factors involved in the homeostasis of the disc matrix (Chan et al. 2006). Since disc degeneration is defined as the disruption of the equilibrium between the anabolism and catabolism of the disc matrix (Fontana et al. 2015). Similarly, disrupted homeostasis exists in chondrocytes in osteoarthritis, and current therapeutic strategies aim to alter this misbalanced homeostasis (Blanco and Ruiz-Romero 2013). Therefore, we hypothesize that similar therapeutic strategies and anabolic factors can be applied to treat disc degeneration, significantly induced by traumatic injury, which needs the excellent and real-time supplement of disc ECM to support the disc structure.

We prove this hypothesis in a rat coccygeal disc model of traumatic injury-induced disc degeneration (Liao 2016; Inoue et al. 2015; Grunert et al. 2014), and utilized a polyethylene glycol (PEG)-polyamino acid nanocarrier to encapsulate a therapeutic mRNA encoding Runx-1, cartilage-anabolic transcription factor (Aini et al. 2016; Baba et al. 2015; Lin et al. 2016; Uchida et al. 2013, 2016).

The PEGylated nanocarrier protection group exhibited superior reporter mRNA expression efficiency than the naked mRNA injection in the rat coccygeal disc injection model and persisted to at least seven days post-injection (Fig. 6). However, the naked mRNA administration only emitted very sparse luminescence and could not be detected by IVIS. Based on this result, then, we triggered traumatic disc degeneration in rat coccygeal discs using a needle puncture and subsequently administrated mRNA therapeutics to examine the regeneration capability.

Magnetic resonance imaging (MRI) data revealed that Runx-1 mRNA delivered by nanocarriers resulted in a higher intensity of MRI-T2 signals, mitigating the loss rate of hydration content inside the disc and slowing the speed of disc degeneration (Fig. 7) for at least four weeks post-injury. This strategy may be helpful to compete with the degeneration process and slow the gradual worsening of disc damage over time. We hypothesize that mucoid materials loss accompanied by decreased hydration content in the acutely traumatized disc eventually leads to complete disc degeneration. Data

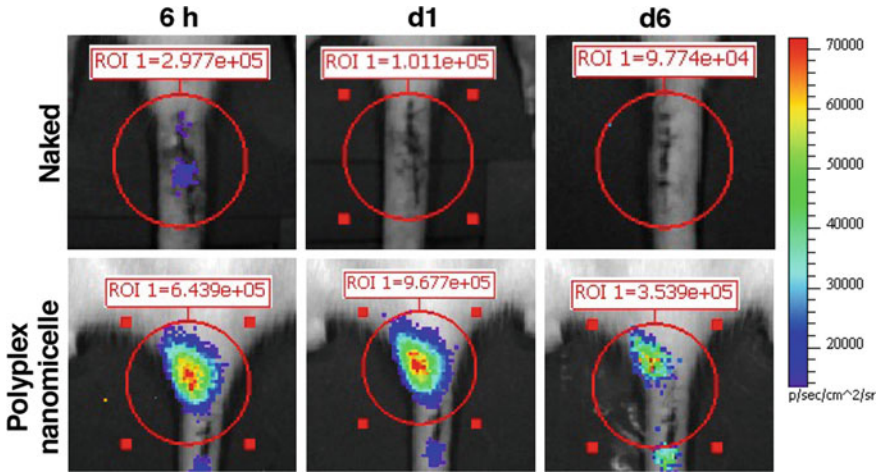

Fig. 6 Luc2 mRNA delivery in the coccygeal disc defect and IVIS observation to detect the mRNA expression duration. Representative images show the luciferase expression in the coccygeal disc in rats. Luc2 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form. Images were captured by IVIS examination at 6 h to six days post-injection. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

shows the use of nanocarrier developed in our group for mRNA therapeutics delivery, which effectively prevented the inflammation elicited by the naked mRNA administration (Fig. 8) and promoted more apparent collagen, GAG, and aggrecan secretion (Fig. 8) (Lin et al. 2019).

5.5 Olfactory Neuron

mRNA medicine for treating a temporarily olfactory neuron malfunction has been explored in an exciting study (Baba et al. 2015). They temporarily induced an olfactory dysfunction in a mouse model, followed by intranasal administration of polymeric nanomicelle loaded with a therapeutic mRNA, brain-derived neurotrophic factor (BDNF)-expressing mRNA, providing an efficient and persistent recombinant protein expression in the mouse nasal tissues. Remarkably, the recombinant protein expressed mainly in the lamina propria in the nasal cavity primarily contains olfactory nerve fibres, which effectively regulate the immunogenicity of mRNA. Their data showed remarkably ameliorated recovery of olfactory function and repairing the olfactory epithelium to nearly intact tissue. Thus, they showed the therapeutic potential of introducing exogenous mRNA to treat neurological disorders (Baba et al. 2015).

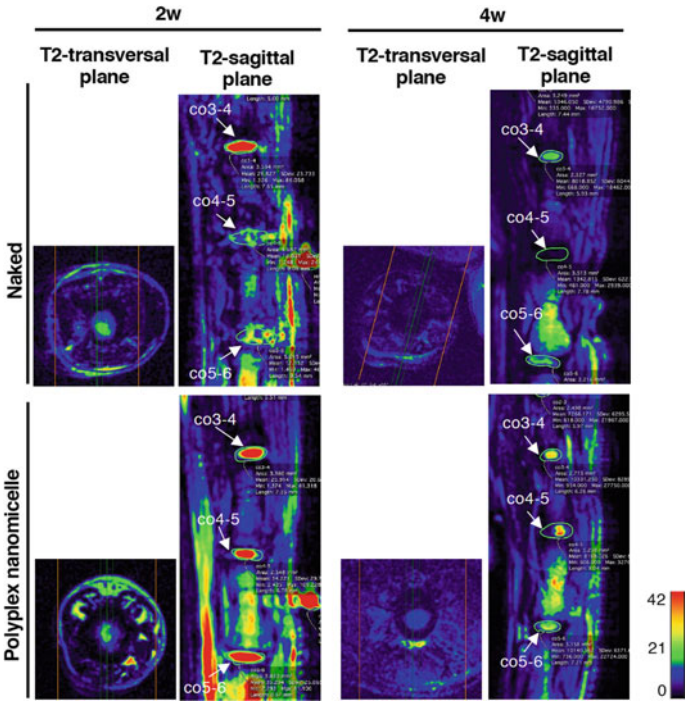

Fig. 7 Runx-1 mRNA delivery in the coccygeal disc defect and MRI examination to detect the hydration change of punctured disc in rats. **a** Runx-1 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form, and MRI was carried out at 2- and 4-weeks post-injection. Representative images show the T1- and T2-weighted MRIs, which were converted to colour histograms based on the CLUT in the OsiriX MD software. Due to the disc defect being created in the co4-5, the T2-transversal plane shows the T2-weighted image retrieved from the co4-5 in the sagittal plane to directly present the damaged disc's hydration content. Region of interest (ROI) was circled at co3-4, co4-5, and co 5–6 at T2-weighted image from sagittal plane for subsequent CLUT colour intensity calculation. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

5.6 Liver

Fulminant hepatitis in a mouse model treated with B-cell lymphoma-2 (BCL-2) protein-expressing mRNA medicine was described (Matsui et al. 2015). Bcl-2 has been demonstrated to possess the anti-apoptotic effect, which was considered a strategy to combat fulminant hepatitis. The authors manufactured a polymeric nanomicelle loaded with BCL-2 expressing mRNA for hydrodynamically intravenous injection. Their data revealed that the BCL-2 drug delivery in an mRNA form was more effective in reducing fulminant hepatitis-induced apoptosis in the liver than the BCL-2 drug delivery in a plasmid DNA form. They concluded that the

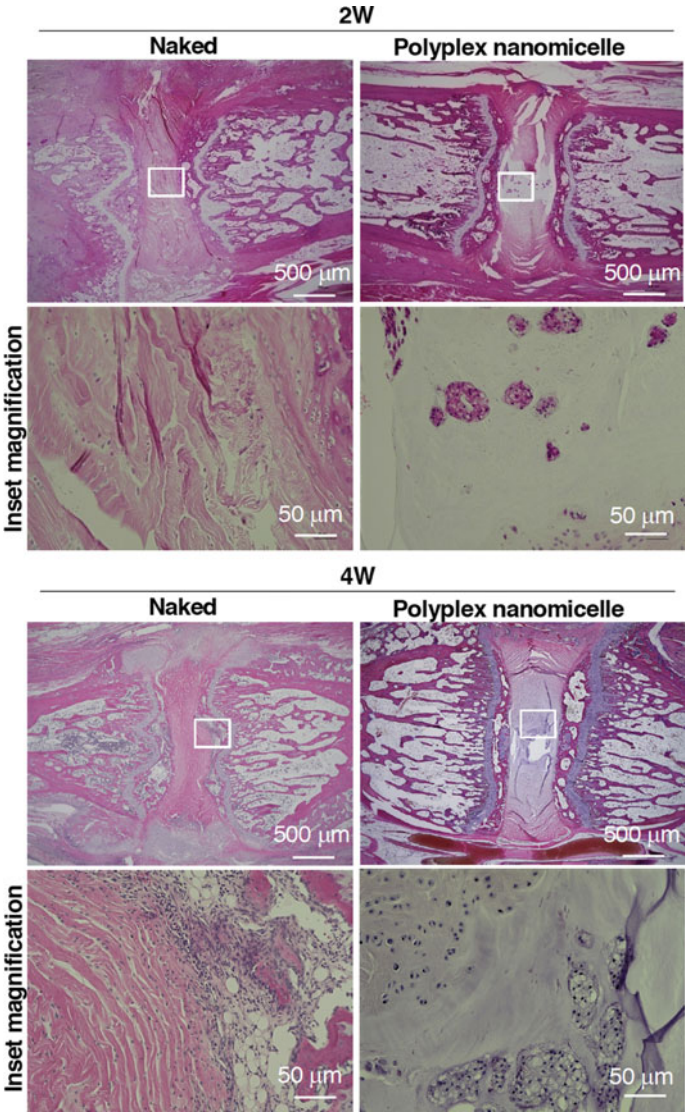

Fig. 8 Hematoxylin and Eosin (H&E) staining to examine the disc's fibrous tissue and jelly-like material. Representative images from the post-punctured animal. Runx-1 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form. The punctured disc was collected at 2- and 4-weeks post-injection and subjected to H&E staining. The solid line boxed area was magnified to observe the detailed fibrous tissue infiltration or jelly-like material used to represent the hydration content. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

mRNA-based therapeutics combined with an effective gene delivery carrier could be served as a promising treatment for the intractable disease associated with excessive apoptosis (Matsui et al. 2015).

Furthermore, the same group also applied the BCL-2 mRNA technology to improve the hepatocyte engraftment efficiency (Matsui et al. 2018). Their data revealed that transfection of a BCL-2 expressing mRNA in an ex vivo scenario led to an enhanced survival rate of engrafted hepatocytes in both standard and diseased mouse models with liver malfunction. Meanwhile, the engraftment of BCL-2 mRNA medicine administrated hepatocyte also recovers the liver function from chronic hepatitis. Furthermore, the engrafted hepatocytes that survived for at least one month maintained their hepatic function in the recipient animal. This study demonstrated that even a transient BCL-2 protein expression, sufficient to prevent the transplanted hepatocyte death, is a critical bottleneck and obstacle in the allogeneic cell transplantation. Remarkably, the temporally controlled pro-survival factor protein expression mediated by mRNA administration is a good candidate for a further clinical setting, fitting the maximum demand for safety concerns (Matsui et al. 2018).

5.7 Cardiac and Skeletal Muscle Cells

In the field of cardiac and skeletal muscle gene transfection, fewer studies using mRNA medicine were described, which may be due to the gene transfection in cardiac muscle using naked plasmid DNA showing a surprising effect. Recently, a report described a time- and dose-dependent manner of the therapeutic protein expression in vivo using chemically modified mRNA in an intracardiac and intramuscular injection (Chien et al. 2014). Their data suggested that muscle tissue may also possess a particular property of taking up modified and un-modified mRNA medicine, leading to a tremendous therapeutic protein expression on-site. Even the fully differentiated adult rod-shaped cardiac muscle cells or fused skeletal muscle myotubes could be transfected by mRNA, leading to a high-efficient recombinant protein expression. Besides, this group also developed a time- and dose-dependent, efficient protein expression protocol led by therapeutic mRNA administration in vivo in intracardiac and intramuscular injection, demonstrating that chemically modified mRNA holds a promising capability to direct the expression of any protein of interest.

5.8 Spinal Cord

An mRNA medicine therapy for spinal cord injury treatment was also reported (Crowley et al. 2019). This group developed a polymeric nanomicelle used to carry brain-derived neurotrophic factor (BDNF) mRNA medicine to treat spinal cord injury (SCI) in a mouse model. SCI affects nearly 300,000 people per year globally, especially the young men population, which is the majority of this disease,

usually resulting from sports injuries, car and bike accidents, falls, and violence. Recovery from SCI is tremendously tricky due to the physiological changes and loss of neural functions resulting from the initial spine injury. Besides, the injured tissue emerged as inflammation, apoptosis, loss of myelin, and formation of a glial scar that retards the new axon ingrowth in the days to weeks following SCI, the so-called secondary injury that debilitates the recovery of neural motor function. This group proposed using an mRNA medicine expressing BDNF to prevent secondary injury, improving the long-termed neuron recovery by keeping the neural tissue alive during this critical period.

5.9 Stem Cells Engineering

More and more studies demonstrated that the biological modification of MSCs could be safely engineered by mRNA therapeutics transfection, transiently achieves the intended function, or leads to a targeted differentiation (Andrzejewska et al. 2020; Kim et al. 2018; Van Pham et al. 2014; Zhang et al. 2021). One exciting study using mRNA transfection to engineer MSCs to possess a specific target homing property was shown (Kwon et al. 2018). Due to transfect mRNA into MSCs with a facile, prompt, and transient expression of homing ligand, modified on the surface of MSCs is considered safer than the permanent genetic modification for MSCs. MSCs modified by other cell surface modification methods may disturb the unique differentiation commitment due to the permanent changes of the cellular membrane. Although MSCs possess their intrinsic and unique homing or migration properties, the protein ligands involved in regulating migration are principally expressed on the cellular membrane of MSCs. However, these particular tissue homing-assisted ligands may lose during the MSC expansion in vitro, leading to only a tiny portion of MSCs eventually reaching the target tissue. In principle, applying naïve MSCs for allogenic cell-based regenerative medicine through bolus injection is difficult to achieve the therapeutic effect. They concluded that these unique mRNA transfections, such as homing protein–ligand expression, make MSCs engineered modification safer and more powerful (Kwon et al. 2018).

6 Conclusion

By understanding how the mRNA transcript interacts with the immune system and advancing nucleoside chemistry and manufacturing, scientists have improved the therapeutic and prophylactic application of mRNA medicine for many medical purposes. Primarily, it gained much attention and success in vaccine development combating the urgent and severe COVID-19 pandemic. The mRNA medicine holds many advantages, no chromosome insertion concern, no DNA backbone remnant, facial to produce in large quantity and mix as a therapeutic cocktail, promptly adapt

the transcript to meet the distinct disease requirement, and a bunch of carrier and modified nucleotide choice to alleviate the immune complication and sustain the expression. From the DNA template preparation, IVT, capping, quality analysis to the final of nanoparticle encapsulation or carrier loading, mRNA medicine has successfully demonstrated its therapeutic capability in many fields of regenerative medicine, which would disclose more and more successful examples shortly.

7 Perspective

For the consideration of mRNA medicine design, we propose the carrier should comply with (I) low cytotoxicity, excellent biocompatibility, (II) efficient endosomal escape capability, (III) higher capacity for mRNA payload, and (IV) holds excellent stealth properties in the immunocompetent surveillance. Besides attempting to achieve a satisfactory tissue healing outcome, the utilization of mRNA medicine should combine with optimal scaffolds, holding suitable biomechanical properties in compliance with the grafted site. For specific disease therapy, the utilization of mRNA medicine should be considered more comprehensively, such as the application in vaccine development. Some pathogens may need the attenuated or subunit vaccine rather than the mRNA vaccine. In regenerative medicine, whether the disease scenario needs the recombinant protein administration, viral vector gene delivery, or therapy of mRNA medicine, which needs more thorough studies to demonstrate.

Acknowledgements The authors thank Ms. Zhu-Yin Chen and the Medical Research Core Facilities, Office of Research & Development at China Medical University, Taichung, Taiwan, R.O.C., for their assistance in experiments and data analysis.

Grant Support
This work was financially supported by the Minister of Science and Technology (MOST 108–2221-E-039–006-MY3, MOST 107–2314-B-039–023, MOST 107–2622-E-039–003-CC1 and MOST 108–2622-E-039–002-CC1) and China Medical University (CMU110-MF-83). However, the funding sources had no involvement in study design, the collection, analysis, and interpretation of data, the report's writing, and the decision to submit the article for publication.

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Nonsequential Pre-mRNA Splicing: From Basic Understanding to Impacts on Splice-Manipulating Therapies

Kristin A. Ham, Steve D. Wilton, and May T. Aung-Htut

Contents

Abstract The pre-mRNA splicing process is an essential aspect of gene expression and function and plays a substantial role in the complexity of higher eukaryotes. The development of antisense oligonucleotides (AOs) to harness the splicing process and manipulate it to treat various inherited and acquired diseases has been boosted by its flexibility and customisation capability. As the amount of research in this space increases, certain aspects need to be considered, in particular, how nonsequential splicing of pre-mRNA can impact AO-mediated splicing manipulation. In this chapter, we reviewed literature discussing intron removal order and several examples of disease-causing mutations impacted by this phenomenon. We also compared two strategies used to study intron removal order and the occasions that they are best suited. Finally, we discuss how nonsequential splicing could facilitate or impede the

K. A. Ham · S. D. Wilton · M. T. Aung-Htut (✉)
Centre for Molecular Medicine and Innovative Therapeutics, Health Futures Institute, Murdoch University, Perth, WA 6150, Australia
e-mail: M.Aung-Htut@murdoch.edu.au

Centre for Neuromuscular and Neurological Disorders, Perron Institute for Neurological and Translational Science, The University of Western Australia, Perth, WA 6009, Australia

S. Jurga and J. Barciszewski (eds.), *Messenger RNA Therapeutics*, RNA Technologies 13,
https://doi.org/10.1007/978-3-031-08415-7_19

development of splice-manipulating AOs and aspects to consider when analysing AO effectiveness.

Keywords Antisense oligonucleotides · Nonsequential intron removal order · Pre-mRNA splicing · Splice-manipulating therapy · Multi-exon skipping · Exon blocks

1 Introduction

During pre-mRNA splicing, exons are retained and introns are excised to generate a mature mRNA ready to be translated into a protein, or in some cases, to serve other functions as a non-coding RNA. The pre-mRNA splicing process is a fundamental aspect of gene expression and function, highlighted by the fact that an estimated 94% of human genes contain non-coding intronic sequences (Ward and Cooper 2010). Pre-mRNA splicing involves a plethora of *trans*-splicing factors that interact with numerous *cis*-splicing motifs on the transcript in a highly orchestrated and coordinated fashion (Fig. 1). A large multi-protein complex called the spliceosome is responsible for the coordination of this process. The major spliceosome, along with hundreds of associated splicing factors, is responsible for over 95% of all splicing reactions, including alternative splicing (Wahl et al. 2009; Kelemen et al. 2013; Lee and Rio 2015; Baralle and Giudice 2017; Park et al. 2018). While in vitro models suggest that a single spliceosome assembles on each intron, a supraspliceosome model has been proposed consistent with the model of co-transcriptional splicing (Fig. 2). The supraspliceosome, a multi-processor machine composed of four native spliceosome units, can splice four introns, although not always sequentially. Once completed, the pre-mRNA moves through the supraspliceosome unit in a 'rolling model' manner (Sperling 2017).

The use of antisense oligonucleotides (AOs) to manipulate splicing is gaining increasing interest and traction as therapeutic options for various inherited and acquired diseases (Havens and Hastings 2016; Rodrigues and Yokota 2018; Li et al. 2021). Since the recent approvals of four antisense drugs to treat Duchenne muscular dystrophy, one for spinal muscular atrophy and one for Batton disease, many more splice-modulating therapeutics are in various stages of clinical development (Crooke et al. 2021). Exon exclusion strategies, exploited for the treatment of Duchenne muscular dystrophy, rely on disrupting the balance of *cis*-acting splicing motifs within pre-mRNAs and *trans*-acting binding proteins and the subsequent interactions of these complexes (Havens and Hastings 2016). Interestingly, on several occasions, we observed that a block is consistently excised from the mature mRNA after treating cells with an AO designed to a single exon for skipping that specific target (Table 1). These results prompted us to investigate whether the order of exon splicing/intron removal influences AO-mediated modulation of pre-mRNA processing.

Several studies have shown that intron removal has a defined order or preference, and this does not have to follow the sequential order of the exons and introns as

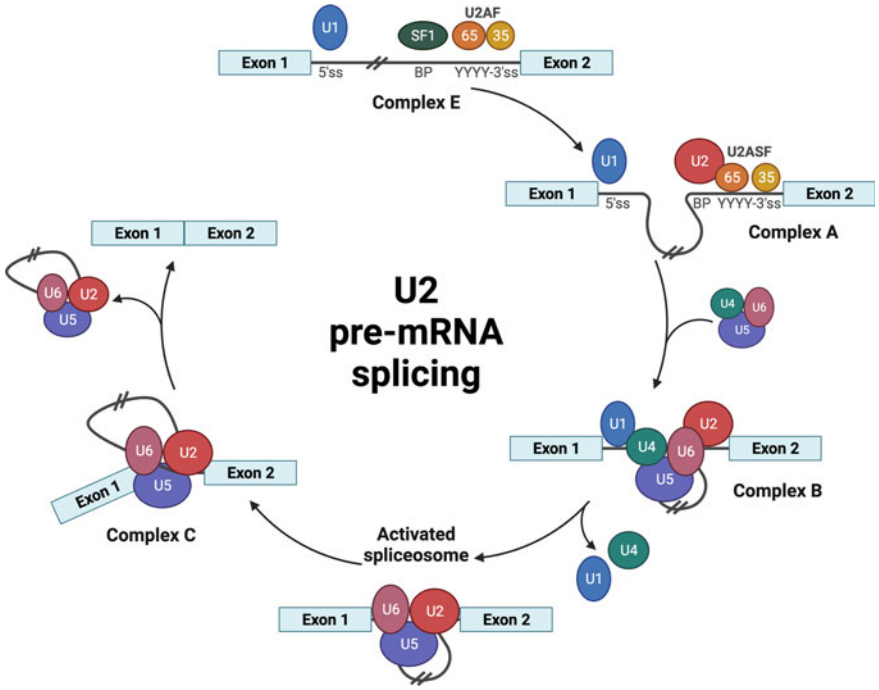

Fig. 1 The pre-mRNA splicing process and stepwise assembly of the major spliceosome. Complex E assembly involves binding of the U1 small nuclear ribonucleoprotein (snRNP) (U1) to the 5′ splice site (ss); non-snRNP splicing factor 1 (SF1) binds to the branchpoint sequence (BP), and U2 auxiliary factor (U2AF) subunits 65 and 35 bind to the polypyrimidine tract (YYYY). U2 snRNP then recognises the BP to generate the complex A. The pre-assembled U4/U6-U5 tri-snRNP is recruited to the spliceosome and forms the pre-catalytic complex B. Subsequently, U6 replaces U1 at the 5′ss, and both U1 and U4 dissociate from the complex, forming the activated spliceosome. In complex C, the 5′ss is cleaved and joins the BP to form a lariat within the intron. The 3′ss is then cleaved, the lariat is released, and the two exons are ligated together. The spliceosomal snRNPs are recycled, and this process is repeated until no introns remain in the mRNA, thus producing a mature mRNA transcript. Adapted from Wahl et al. (2009). Created with BioRender.com

they are transcribed by RNA polymerase II (RNA Pol II) (Tsai et al. 1980; Zeitlin and Efstratiadis 1984; Noteborn et al. 1986; Gudas et al. 1990; Kessler et al. 1993; Yang et al. 2012; Gazzoli et al. 2016; Gumińska et al. 2018; Ham et al. 2020). A single nucleotide change can disrupt this order and thus alter the pre-mRNA splicing process, leading to alternative and cryptic splicing events (Schwarze et al. 1999; Takahara et al. 2002; Attanasio et al. 2003). Complex genes with many exons and short introns, including the collagen genes, appear to be particularly vulnerable to these changes. Interestingly, in the massive dystrophin (*DMD*) gene with average-sized exons generally separated by large introns, 40% of its introns are not removed sequentially, and blocks of exons are processed together (Gazzoli et al. 2016). With the recent explosion of publications in the last decade on the development of splice

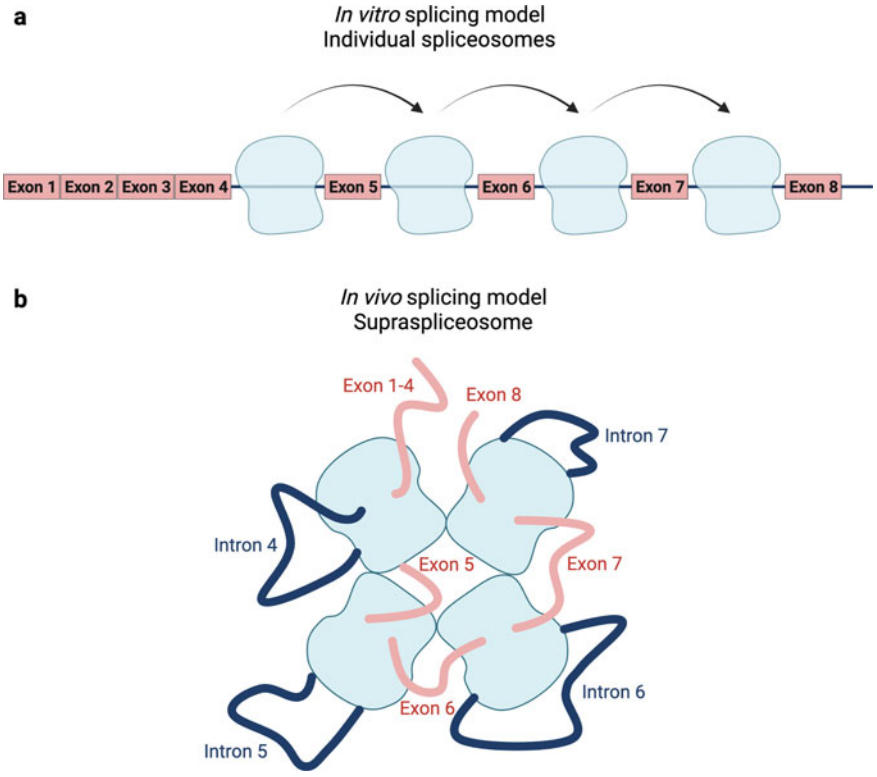

Fig. 2 In vitro and in vivo spliceosome models. **a** In vitro splicing model depicts that the spliceosome assembles on individual introns in a stepwise fashion and the introns are processed in the order in which they are transcribed. **b** In vivo splicing model describes a supraspliceosome structure that consists of four native spliceosome subunits held together on a single pre-mRNA that can process four introns concurrently but not always serially. Adapted from Sperling (2017). Created with BioRender.com

manipulation therapy to treat various diseases, the implication of intron removal order from a pre-mRNA transcript has largely been overlooked.

This chapter will summarise previous knowledge on intron removal order and the impacts on disease-causing mutations. We will also compare the various strategies used to study intron removal order. Finally, we will discuss how nonsequential splicing can either facilitate or impede the development of splice-manipulating AOs.

2 What Influences Intron Removal Order?

Splicing is a complex and delicately balanced process involving multiple *cis* and *trans* elements crucial for tissue-specific splicing fidelity. While disagreement exists

Table 1 Examples from our laboratory where an AO designed to induce single exon skipping led to the skipping of multiple exons from the mature mRNA

Gene target (species)	Target exon(s)	Exons skipped after treatment	References
Dmd (mouse)	23	Δ23, Δ21–23, Δ22–23	(Mann et al. 2001)
Dmd (canine) missing exon 7	6	Δ6, Δ9, Δ6 + 9	(McClorey et al. 2006)
	8	Δ9, Δ8 + 9	
	6, 8	Δ9, Δ6 + 9, Δ8 + 9, Δ6 + 8 + 9, Δ5–9, Δ5–10	
Dmd (mouse)	23, 53	Δ22–23, Δ53–54	(Mitrpant et al. 2009)
DMD (human)	24	Δ24–25	(Mitrpant et al. 2009)
DMD (human)	20	Δ20, Δ20–21	(Adkin et al. 2012)
DMD (human)	8	Δ8, Δ9, Δ8–9	(Fletcher et al. 2012)
ITGA4 (human)	3	Δ3–4	(Aung-Htut et al. 2019)
COL7A1 (human)	73	Δ73, Δ73–74	(Ham et al. 2020)

as to the strongest predictors of splicing order, there is a consensus that a combination of multiple factors regulates the splicing pathway.

Chromatin structure can affect the rate of RNA Pol II transcription elongation, which in turn affects splicing regulation and intron removal order as splicing occurs mainly co-transcriptionally. This is known as the 'kinetic coupling' model (Schor et al. 2013). Weak exons (exons with weak splice sites) and larger exons flanked by short introns are more likely to be excluded from the mature transcript with a fast elongation rate (De La Mata et al. 2003; Fong et al. 2014). Chromatin modifications and structure may assist the spliceosome in exon definition by slowing down the transcription elongation rate and allowing time to recruit the necessary or appropriate splicing factors (Shukla and Oberdoerffer 2012; Haque and Oberdoerffer 2014).

For example, the fibronectin transcript contains cassette exons that are alternatively spliced. Exon 33 has a predicted weak 3′ splice site that is normally outcompeted by a 3′ splice site in a downstream exon (Fig. 3). The relative abundance of fibronectin transcripts, either missing or retaining exon 33, could be manipulated by the addition of RNA Pol II activating transcriptional regulatory elements (Kadener et al. 2002) or by slowing down RNA Pol II elongation (De La Mata et al. 2003; Nogues et al. 2003), respectively. Exon 33 skipping is favoured by a rapid RNA Pol II elongation rate as both 3′ splice sites are accessible to the spliceosome, and the downstream splice site would be selected as it is a stronger sequence (Fig. 3a). Conversely, exon 33 inclusion is favoured by a slow RNA Pol II elongation rate as the superior downstream 3' splice site would not yet be transcribed and therefore not accessible to the spliceosome at the time of splicing. In this case, the weaker splice site would appear to be the only choice by the spliceosome, and thus, exon 33 would be included in the mature mRNA (Fig. 3b).

The involvement of intron and exon length in intron removal is still under debate. In one study, intron length was observed to be associated with the order of intron

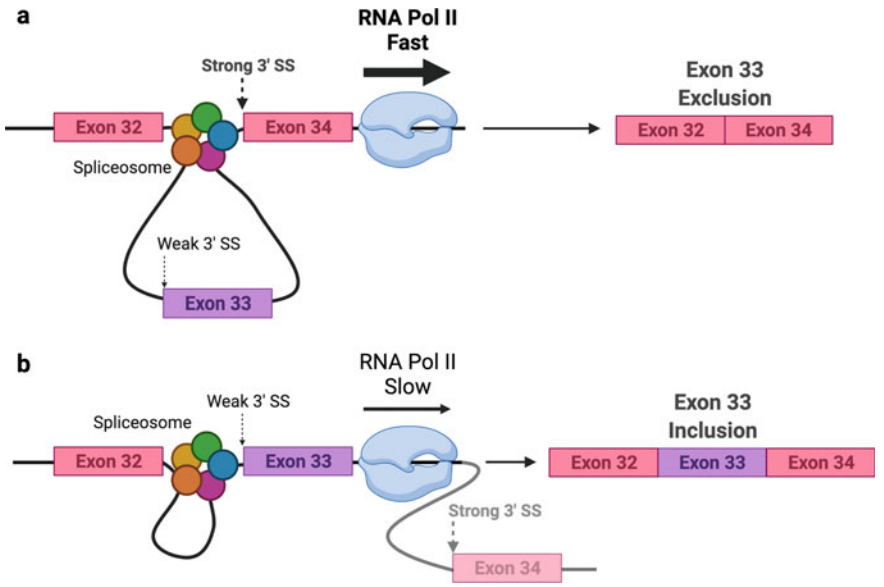

Fig. 3 Fibronectin exon 33 exclusion and inclusion models. **a** Fast RNA polymerase II (RNA Pol II) elongation promotes exon 33 exclusion by exposing both the weak and strong 3′ splice sites (ss) to the spliceosome. **b** Slow RNA pol II elongation promotes exon 33 inclusion by only presenting the weaker 3′ ss to the spliceosome. Created with BioRender.com

removal, where longer introns tend to be removed after shorter introns (Kim et al. 2017). This report contradicts the study conducted by Gazzoli et al (2016), where no correlation between intron length and intron removal order was reported. On the other hand, alternative splice site activation does seem to be influenced by intron length as alternative splice sites flanked by larger introns tend to be activated less often (Fox-Walsh et al. 2005). It is clear that simple modelling through kinetic probability, where longer introns result in a lower likelihood of two exons coming into contact compared to small introns, does not fit the splicing order for all transcripts (Kim et al. 2017).

In addition, it has been found that the splicing consensus sequence did not influence intron removal order as introns with similar strengths were observed in introns that spliced first and last. The study also identified 17 splicing factor binding motifs which influenced splicing. Of these, serine and arginine-rich splicing factor 3 binding was associated with introns that spliced last, and the remaining motifs were uracil rich and primarily found in introns that spliced first (Kim et al. 2017).

3 Genes Studied for the Order of Intron Removal

Several examples of intron removal order investigation exist in the literature. This phenomenon is essential to help understand alternative splicing, cassette exons, and how some mutations can result in numerous splice isoforms. Comparing the intron removal order of large genes with numerous introns is complex. For example, the *DMD* gene contains 79 exons spread across some 2.3 megabases, and transcription of the *DMD* transcript takes approximately 16 h (Tennyson et al. 1995). Thus, comparing the order of intron removal of the most downstream introns to upstream introns is impossible as RT-PCR could not efficiently amplify the majority of introns. Therefore, the *DMD* transcript was analysed in smaller blocks of five introns using a shifting frame of three (Gazzoli et al. 2016). Collagen gene architecture is completely different, some with an even higher number of introns and exons spread across an area smaller than most dystrophin introns. The *COL7A1* gene contains 118 exons spread across 30 kilobases, drastically smaller than the *DMD* gene; thus, *COL7A1* transcription is likely to be completed in minutes rather than hours (Ham et al. 2020). To date, only small sections of some collagen genes have been analysed to confirm the intron removal order.

3.1 Dystrophin

Intron removal order in the *DMD* gene using a capture-pre-mRNA-sequencing method and RNA collected from the nuclei of three differentiated human muscle cell lines was investigated (Gazzoli et al. 2016). Not only did the authors uncover that 40% of *DMD* introns were removed non-sequentially, but they also reported that the *DMD* transcript is grouped into exon blocks that have slow-processed introns on either side. Another feature of the *DMD* gene is the presence of large introns. Several introns are larger than 50 kilobases and are predicted to require multi-step processing, such as recursive and nested splicing, to remove them (Gazzoli et al. 2016). Pseudoexons, intronic sequences with exonic characteristics but normally ignored by the splicing machinery, have been discovered within these larger introns. Interestingly, pseudoexon splice sites commonly coincide with many predicted recursive splice sites (Keegan 2020).

3.2 Collagen Type I Alpha 1

The effects of an intron 8 donor splice site mutation in collagen type I alpha 1 (*COL1A1*) on the production of various splice isoforms, including the order of intron removal in the region of the mutation from exon 5 to intron 10, were examined

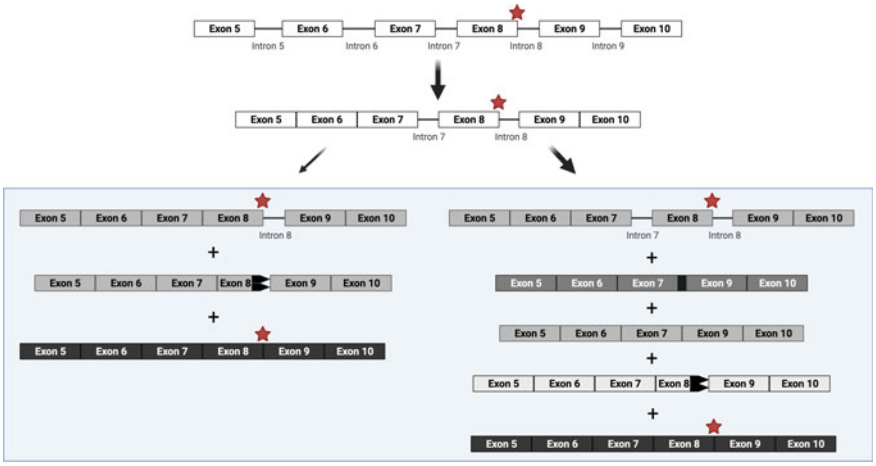

Fig. 4 The production of various splice isoforms from *COL1A1* as a result of an exon 8 donor site mutation (indicated with a star). The predicted major and minor pathways of intron removal order are indicated with a thick and thin arrow, respectively. Transcripts with white exons represent splice intermediates. Amplification products, located within the box, and their relative proportions are indicated by a gradient (darker transcript = larger proportion; lighter transcript = smaller proportion). The black box after exon 7 represents the extension of exon 7. The black points after exon 8 represent a cryptic donor splice site activation within exon 8. Adapted from Schwarze et al. (1999). Created with BioRender.com

(Schwarze et al. 1999). This donor site mutation resulted in several mutant transcripts: extension of exon 7, intron 8 retention, retention of introns 7 and 8, skipping of exon 8, and the use of a cryptic donor splice site within exon 8. In processing the normal transcript, introns 5, 6, and 9 are rapidly removed first, followed predominantly by intron 8 before intron 7 removal, however, this order is not absolute (Fig. 4). The mutation at the donor site of intron 8 leads to 'splice paralysis', including dysregulation of the rapid splicing pathway and accumulation of splice intermediates in the nucleus and caused an osteogenesis imperfecta type IV phenotype in the patient (Schwarze et al. 1999).

3.3 Collagen Type V Alpha 1

A novel splice site mutation was identified within the intron 4 acceptor site in collagen type V alpha 1 (*COL5A1*), leading to an Ehlers-Danlos syndrome type I phenotype. Both exons 5 and 6 were skipped in the major product, along with several minor isoforms resulting from exon 5 skipping as well as activation of cryptic acceptor sites in exon 5. The rapid removal of intron 5 is the preferred order, which leads to exon 5 and 6 double skipping and a small amount of cryptic splice site activation in exon 5. However, in the minor pathway, where intron 6 is removed rapidly, both exon

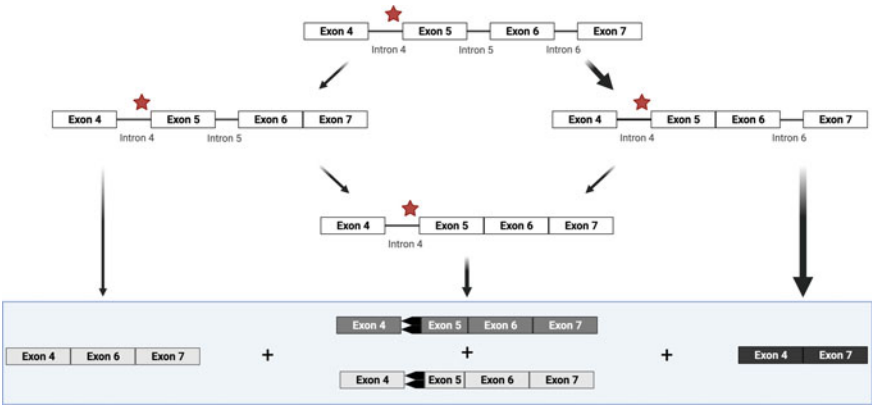

Fig. 5 The production of various splice isoforms from *COL5A1* as a result of a mutation within intron 4 (indicated with a star). The predicted major and minor pathways of intron removal order are indicated with a thick and thin arrow, respectively. Transcripts with white exons represent splice intermediates. Amplification products, located within the box, and their relative proportions are indicated by a gradient (darker transcripts = larger proportion; lighter transcripts = smaller proportion). The black points at the start of exon 5 represent the cryptic acceptor splice sites activated within exon 5. Adapted from Takahara et al. (2002). Created with BioRender.com

5 skipping and cryptic splice site activation in exon 5 occur (Fig. 5). By studying the splicing order of complex and exon dense genes such as the collagens, models could be developed to predict the effects and consequences of splice site mutations for genetic counselling or therapy design (Takahara et al. 2002).

3.4 Collagen Type VII Alpha 1

While targeting collagen type VII alpha 1 (*COL7A1*) exon 73 for excision from the mature mRNA in normal fibroblast cells, we discovered that AOs targeting exon 73 induced various spliced transcripts, including excision of adjacent non-targeted exons and/or retention of nearby introns in some transcripts (Ham et al. 2020). These products resulting from targeting exon 73 for skipping include: excising exon 73 alone, removing exon 73 and retaining intron 76, missing exons 73 and 74, or skipping exons 73 and 74, and including intron 76. We found that the nonsequential splicing of certain introns may alter pre-mRNA processing during AO-mediated exon skipping. From introns 72 to 76, it was determined that introns 72 and 75 were removed prior to introns 73 and 74, which were, in turn, removed before intron 76.

4 Strategies Used to Study Intron Removal Order

Various methods have been used to study the order of intron removal in an assortment of genes. Prior to next-generation sequencing technology, many earlier studies utilised an innovative RT-PCR method to investigate the splicing pathways of pre-mRNAs.

4.1 Reverse Transcription Polymerase Chain Reaction Method

An innovative RT-PCR method is still utilised to compare pre-mRNA splicing of small portions of genes (Kessler et al. 1993). In this method, primer sets are strategically designed to anneal within introns and exons to detect pre-mRNA using RT-PCR analysis (Fig. 6). The intronic and exonic primer pairs provide adequate sensitivity for detecting low levels of splicing intermediates even from total RNA after DNase treatment. A gene-specific primer designed to anneal to the last exon of the target gene, not random hexamers, should be used for the RT step to ensure that the RT-PCR

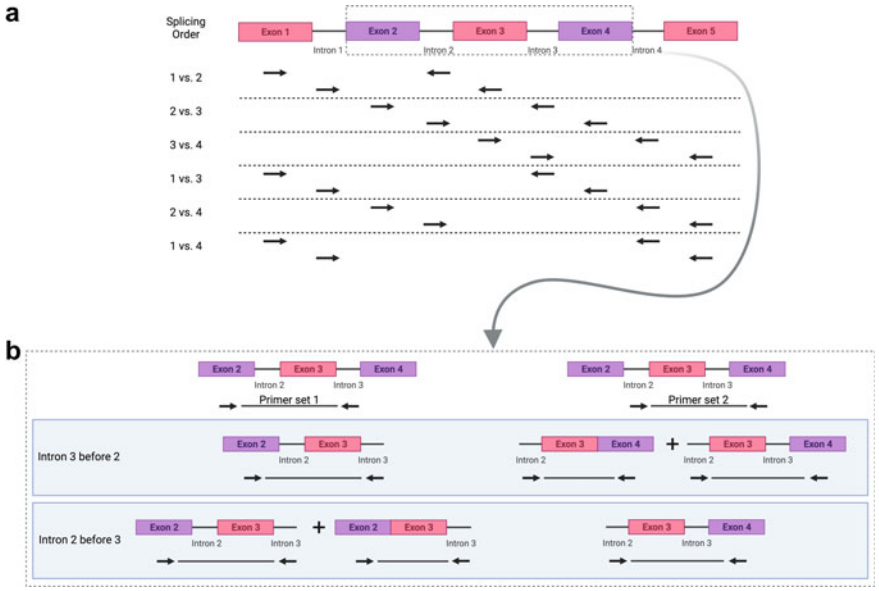

Fig. 6 **a** Example of the primer sets required to determine the order of intron removal in a section consisting of five exons and four introns. **b** The diagram shows the two different primer sets required to determine the splicing order of introns 2 and 3. The two panels below illustrate the different PCR products expected if one intron is spliced before the other. Arrows represent forward and reverse primers located either within an exon or intron. Created with BioRender.com

products come from genuine splice intermediates. A gene-specific primer-mediated RT may not be feasible to study the whole gene as most RT synthesis systems have an upper limit for elongation of 12 kilobases.

There are some limitations to this RT-PCR method of analysis. This method can analyse genes with small introns and exons as manageable product sizes are generated. Genes with large exons and large introns would prove more difficult as current PCR systems have a limitation on the size of the product. Additionally, as preferential amplification of the smaller transcripts is common with some RT-PCR systems (Walsh et al. 1992), a long-range PCR system should be considered. While the RT-PCR method is economical and can be performed using standard laboratory techniques and reagents, it can also be laborious. For example, analysing a section of four introns and five exons requires a minimum of 12 primers and 12 RT-PCR reactions (Fig. 6a).

4.2 Next-Generation RNA Sequencing

Genes with a larger exon and intron structure require a more complex method of analysis. Next-generation RNA sequencing has been used to study pre-mRNA splicing in the dystrophin gene (Pulyakhina et al. 2015; Gazzoli et al. 2016). Due to the low level of dystrophin expression, the pre-mRNA was enriched for dystrophin as well as three control genes. Enrichment was achieved by 'capturing' *DMD* pre-mRNA using a customised library consisting of probes covering the entire pre-mRNA transcript except for repeat masked areas (Gazzoli et al. 2016). The probes included exons, introns, the promoters, and untranslated regions of the *DMD* gene. Ultra-deep transcript sequencing is required to increase the number of reads. The authors performed Illumina HiSeq paired-end sequencing and postulated that the relative speed of intron removal would correlate with intronic read coverage, regardless of the size of the intron. Total RNA sequencing does not provide enough intron coverage. Therefore, pre-mRNA extracted exclusively from the nuclei is recommended.

A novel programme called SplicePie was developed to analyse the pre-mRNA-sequencing dataset and determine the splicing order (Pulyakhina et al. 2015). SplicePie is a computational pipeline that can detect non-sequentially and recursively spliced introns along with alternative splicing events such as exon skipping, intron retention and novel exons (Pulyakhina et al. 2015). Two approaches were used to evaluate the RNA-seq data (Fig. 7). The first, a coverage-based approach, relies on the assumption that introns that have a higher coverage are present in the transcript for longer and thus spliced later (Fig. 7a). The second, a read-based approach, compares the number of read pairs mapped from an exon/exon junction to the downstream intron with the upstream intron (Fig. 7b).

Much like the RT-PCR analysis method, RNA-seq cannot determine the order of splicing for the entire transcript. The first introns are generally the largest in a gene. In addition to standard intron structure, first introns possess functional elements that may assist with gene expression regulation (Bradnam and Korf 2008). Since splicing

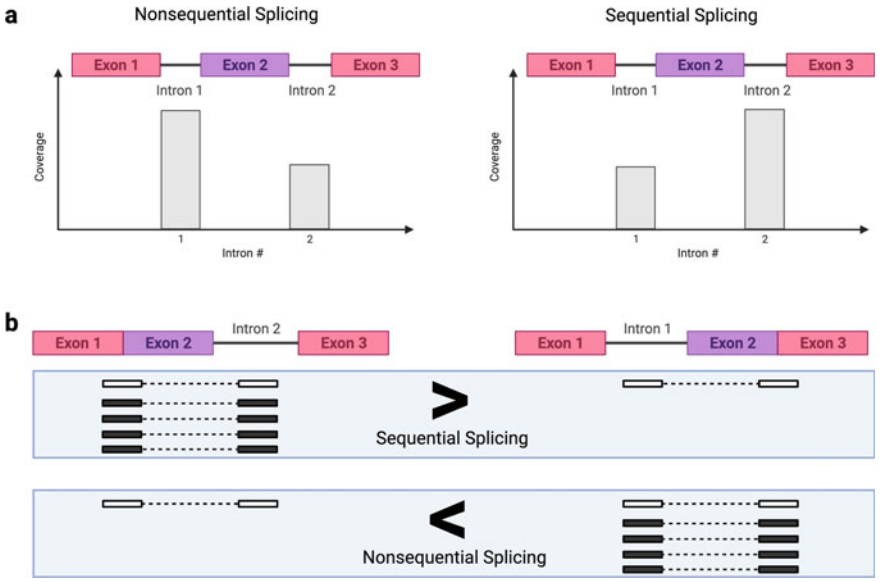

Fig. 7 RNA sequencing data is evaluated by two methods to determine the intron removal order. **a** Coverage-based approach compares the coverage of two adjacent introns and determines the order of removal. The intron with a higher coverage is assumed to be present in the samples for the longer and thus spliced later. More coverage of the upstream intron indicates nonsequential splicing, where higher coverage of the downstream intron indicates sequential splicing. The exon coverage is not shown in this illustration. **b** Read-based approach compares the number of read pairs mapped from the exon 1/exon 2 junction to the downstream intron 2 with the exon 2/exon 3 junction to the upstream intron 1. The white boxes show the anticipated read pairs, and the black boxes show the read pair abundance. Created with BioRender.com

occurs co-transcriptionally, the first intron is likely removed before the last intron is even transcribed. For this reason, smaller groups of five introns were explored at a time using a shifting frame of three (Gazzoli et al. 2016). In addition, the RNA-seq data were obtained from a pool of cells in various transcriptional stages, making the interpretations and conclusions tricky. Single-cell RNA sequencing technology, although still in its infancy, may in future provide more insight into the overall pre-mRNA splicing process for an entire transcript.

Capture-pre-mRNA-seq is more sensitive than RT-PCR analysis as it can enrich for lowly expressed genes, but this technology is not readily accessible to every laboratory. Exploring the splicing outcomes of a mutation or the use of a splice-modulating AO can be performed with the simpler method of RT-PCR if the correct parameters are used (Ham et al. 2020).

5 Pre-mRNA Splicing Order Can Impact Exon Skipping Antisense Oligonucleotide Design

The first AO-mediated splice correction in a cell-free model system studying abnormal β-globin gene expression was reported in 1993. It was found that the cryptic splice sites induced by mutations in introns 1 and 2 of the β-globin gene could be masked using AOs in this pivotal in vitro experiment (Dominski and Kole 1993). There are now six splice-modulating AOs approved by the United States Food and Drug Administration (FDA) to treat various diseases, four of which induce skipping of different exons from dystrophin gene transcript. However, we still have much to learn to improve splice-modulating AOs as no single set of rules can be applied to all transcripts.

EXONDYS 51 is the first exon skipping AO granted accelerated approval by the FDA to treat Duchenne muscular dystrophy. This drug alone can treat the largest cohort of patients (13%) by removing exon 51 from the *DMD* transcript (Young and Pyle 2016). Since its approval, the FDA has approved three additional drugs targeting two exons, 45 and 53, for removal from the *DMD* transcript. These four drugs can collectively treat 28.8% of all Duchenne muscular dystrophy patients (Aartsma-Rus et al. 2009).

In the *DMD* transcript, it is hypothesised that smaller blocks of exons are removed together and flanked by slower processed introns on either side (Fig. 8a). These small exon blocks could be more easily removed together, and exon skipping efficiency can be improved if the block is targeted as a whole. The area between exons 45 and 55 is a genomic deletion hotspot in the *DMD* gene (Dunnen et al. 1989; Koenig et al. 1989; Nobile et al. 1997). Removing these 11 exons from the *DMD* transcript would retain the reading frame and hypothetically benefit 40% of all Duchenne muscular dystrophy patients (Aartsma-Rus et al. 2009). Most significantly, this particular deletion has been identified in many cases of Becker muscular dystrophy and is much less severe (Ferreiro et al. 2009).

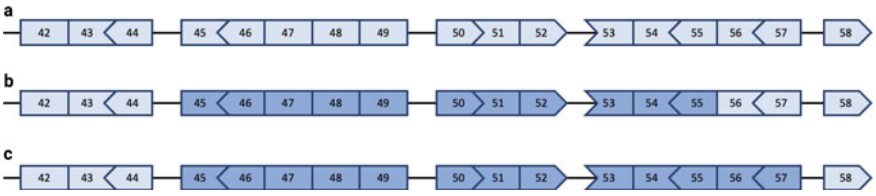

Fig. 8 Representation of the exon blocks within the *DMD* transcript from exons 42 to 58. The exon shape indicates the exon reading frame. Codons disrupted by exon junctions are indicated by <or>, while a 'I' indicates an exon ending at the end of a codon. **a** *DMD* gene deletion hotspot region from exons 45 to 55, including flanking exons. **b** Removing the deletion hotspot from exons 45 to 55 (indicated by dark shading) would create an in-frame transcript but fragment the fourth exon block. **c** Removing exons 45 to 57 (indicated by dark shading) would create an in-frame transcript and remove three exon block sections entirely. Created with BioRender.com

Initial studies attempting to remove exons 45–55 were unsuccessful, and the strategy was deemed unfeasible (Van Vliet et al. 2008). A combination of AOs targeting each exon and two AOs targeting exons 45 and 55 linked together by a chain of uracil bases was trialled. Exon skipping efficiency was variable, and the presence of revertant fibres could account for the low levels. Over a decade later, exons 45–55 were successfully removed from the *DMD* transcript in three immortalised *DMD* patient cell lines: exon 45–52 deletion, exon 48–50 deletion, and exon 52 deletion (Echigoya et al. 2019). For each patient cell line, a cocktail of AOs targeting the required exons for removal was needed. These AO cocktails must bind to the same pre-mRNA molecule simultaneously to have the desired effect.

It has been suggested that co-transcriptional splicing and intron removal order render removing exons 45–55 from the *DMD* gene transcript very challenging (Gazzoli et al. 2016). Targeting exons 45 to 55 for removal would split an exon block (Fig. 8b) and perhaps targeting entire blocks of exons at once; for example, exons 45–57 would require fewer AOs (Fig. 8c). Dystrophin isoform mapping suggests that transcripts missing exons 56 and 57 encode for a protein that appears to be functional (Li et al. 2020), highlighting that removal of the larger portion of exons from 45 to 57 is a potential therapeutic strategy.

Alternative transcripts missing multiple exons in various regions of the *DMD* transcript; exon 2–7, 3–7, 28–29, and 27–29 have been observed at low levels even in unaffected human myotubes (Chelly et al. 1990; Aartsma-Rus et al. 2002). Hence, efficient exon skipping is likely to be achieved if AOs are targeted to those regions, and the exons might be removed as a block. For example, when exon 8 was targeted with a single AO, exon 9 was also excised in human and canine myotubes (McClorey et al. 2006; Saito et al. 2009). It is interesting to note that exon 9 was always skipped when exon 8 was targeted for removal, but exon 9 could be specifically targeted for removal without also affecting exon 8 (McClorey et al. 2006). This suggests that exon 9 must proceed exon 8 splicing during dystrophin mRNA maturation.

One caveat is that the excision of these exon blocks should produce semi-functional dystrophin protein in order to be therapeutic. Removing exons 8 and 9 together led to an out-of-frame transcript, and hence, additional AOs to induce exon 6 and 7 skipping would be required to reframe the *DMD* transcript. Exon 8 skipping could be applied to frame-shifting deletions missing either exons 3–7, 4–7, 5–7, or 6–7, the minor deletion hotspot of the *DMD* gene (Neri et al. 2020). It is possible that mis-splicing could be linked to either unstable DNA regions prone to deletions or the massive length of introns in this region.

Next to *DMD*, the most studied gene transcripts for the order of intron removal are collagen genes. In our laboratory, we observe multi-exon excision in several regions of the collagen gene transcripts after treating cells with a single AO targeting an exon (unpublished data). Recently, we reported nonsequential processing of *COL7A1* pre-mRNA and the impact on AO-induced exon 73 skipping (Ham et al. 2020). It appeared that some AOs targeting exon 73 for removal influence the pre-mRNA splicing of intron 76 since intron 76 is removed serially after introns 73 and 74. However, the intron removal order is not the only mechanism responsible for the retention of intron 76. Otherwise, any AO targeting exon 73 should result in intron 76 inclusion

in the transcript, which was not the case. Other factors, such as pre-mRNA secondary structure, AO sequence and chemistry, exonic splicing enhancer and silencer profiles, and cell type, likely contribute to aberrant splicing patterns. Nonetheless, the results could be different if the introns were removed sequentially.

In addition to dystrophin and collagen genes, we also observed multi-exon skipping in other genes, including integrin alpha 4 (Aung-Htut et al. 2019). Hence, it is recommended to begin with an analysis of the sequence of the removal of the nearby introns for a chosen target exon in the transcript as this could assist in designing an appropriate RT-PCR assay. Placing RT-PCR primers too close to an exon target may miss multi-exon skipping or other unwanted splicing events. Additionally, aberrant splicing events will not be captured if real-time RT-PCR is the sole method for analysis. We also encourage the use of patient-derived cells or cell lines expressing the target gene rather than an overexpression mini-gene constructs in a plasmid because splicing of some introns can rely on the presence (or absence) of the distant introns. This is evident in the study of neurofibromin 1 mutations in exon 37, where the exon 36 and 37 skipping was only recapitulated after exon 31 to 38 was included in the study (Baralle et al. 2006).

6 Conclusion

Nonsequential splicing was reported in 1980 (Tsai et al. 1980), only a few years after introns were first discovered (Berk and Sharp 1977; Chow et al. 1977), yet it is not entirely understood what factors influence the order of splicing and why it is important. We have learned that intron splicing order does have an impact on splicing defects caused by pathogenic mutations. Here, we highlight that the order of intron removal should be carefully considered during splice-manipulating AO design to achieve precise exon skipping. As the number of research groups attempting AO-mediated splice manipulation to treat numerous disorders intensifies, certain aspects, including how nonsequential splicing of pre-mRNA can impact AO-mediated splicing manipulation, need to be considered.

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