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

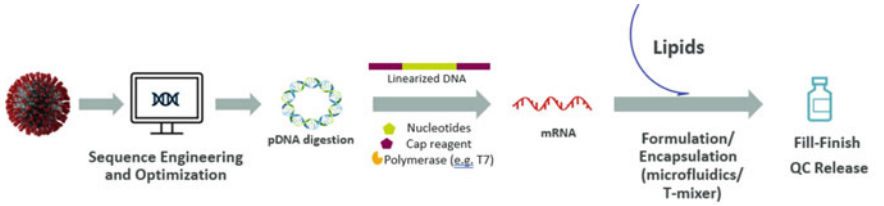
administered subcutaneously, intradermally, and directly to the respiratory system through inhalation (Pardi et al. 2018).

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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