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REVIEW ARTICLE

Downstream Critical Process Parameters for COVID-19 mRNA LNP Vaccine Production

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A B S T R A C T

The COVID-19 pandemic, triggered by SARS-CoV-2, has pushed mRNA vaccines to the forefront of global mitigation efforts, utilizing the virus's genetic sequence to stimulate an immune response without introducing the live pathogen. These vaccines undergo complex production processes, with downstream purification and formulation stages being critical for vaccine purity, potency, and safety. Essential structural components like the Open Reading Frame (ORF), untranslated regions (UTRs), cap structure, and poly(A) tail, along with lipid nanoparticle (LNP) delivery systems, are key to their functionality and efficacy. Different mRNA vaccine types, including conventional, self-amplifying, and trans-amplifying, use structural and delivery innovations to provoke a robust immune response. This review underlines the significance of thorough control in the manufacturing process, highlighting its impact on global health security and the advancement of vaccine technology. This review paper focuses on the downstream stages of mRNA vaccine production, comparing studies on CPP to emphasize the importance of stringent control measures for vaccine quality, safety, and efficiency.

K E Y W O R D S

mRNA vaccines, Vaccine manufacturing, Downstream processing, Critical process parameters, Lipid nanoparticles

H I G H L I G H T S

- ❖ mRNA vaccines use genetic sequences to elicit an immune response, showcasing their effectiveness in providing protection against COVID-19.
- ❖ The overview of different mRNA vaccine types, highlighting their specific approaches to targeting the virus.
- ❖ The significance of the vaccine's structural components and the lipid nanoparticle delivery system, which are vital for the vaccine's functionality and efficacy.
- ❖ The critical role of downstream processes in vaccine production, emphasizing the need for stringent control measures to ensure the vaccine's purity, potency, and safety.
- ❖ The importance of optimizing manufacturing processes to enhance vaccine quality and contribute to global health security and future vaccine development efforts.

INTRODUCTION

COVID-19, which was first identified in 2019 in Wuhan, China, is caused by the SARS-CoV-2 virus (UNICEF, 2020). The disease is transmitted through respiratory droplets and has a wide range of symptoms both mild and severe, including fever, coughing, sore throat, shortness of breath, congested nose, loss of smell, and more (Center for Disease Control and Prevention, 2022). The global spread of the virus has

necessitated concerted efforts worldwide to control its transmission and mitigate its impact. Among these efforts, vaccination, particularly through the innovative use of mRNA technology, has been the most prevalent. mRNA vaccines represent a novel approach to combating infectious diseases by using the genetic sequence of a virus's antigen to stimulate an immune response without introducing the live pathogen. This technology has been pivotal in the rapid development and deployment of vaccines against COVID-19, showcasing significant efficacy in inducing protective immunity.

The production of mRNA vaccines is a multistage process, divided into upstream and downstream phases. While upstream processes involve the synthesis of the mRNA template, the downstream processes focus on the purification and formulation of the vaccine, which are crucial for ensuring its purity, potency, and safety (Rosa et al., 2021). The downstream processes are particularly vital, as they directly impact the vaccine's effectiveness and safety by removing impurities, ensuring formulation stability, and achieving efficient encapsulation. In the context of medicinal development, the emphasis on control throughout the manufacturing process cannot be overstated. Stringent regulatory standards and quality control measures are implemented at every stage of the drug development process to ensure that every batch of medication meets the highest standards of quality, efficacy, and safety (Yu, 2008). These measures are critical for protecting public health and maintaining trust in medical interventions.

Given the critical role of mRNA vaccines in combatting the COVID-19 pandemic, this review paper aims to explore the downstream manufacturing stages of these vaccines in detail. It seeks to compare studies measuring the critical process parameters (CPP) associated with downstream processes, emphasizing the importance of stringent control measures. By analyzing how these controls are implemented and their impact on vaccine quality, safety, and production efficiency, the paper highlights the essential nature of precise control in pharmaceutical manufacturing. By understanding the CPP in the downstream processes, the pharmaceutical industry can ensure the highest standards of vaccine production, contributing significantly to global health security.

mRNA VACCINE FOR COVID-19

mRNA vaccine

Vaccination plays a crucial role in preventing infectious diseases and significantly contributes to the economy of the healthcare system, capable of driving down treatment costs (Gote et al., 2023). Inactivated pathogens, protein subunits, or viral vectors are utilized for the traditional vaccine; however, these vaccines require a complex manufacturing process (Al Fayez et al., 2023). As an alternative, messenger RNA or mRNA vaccines have emerged as an innovative technology in recent years. An mRNA vaccine is a class of vaccines that utilizes a copy of an mRNA molecule to create a specific protein that stimulates an adaptive immune response (Suzana et al., 2022). It offers advantages over traditional vaccines by providing low-cost manufacturing, high potency, safety, and efficacy (Pardi et al., 2018). In light of the recent SARS-CoV-2 outbreak, mRNA vaccine technology has risen as a prime candidate against COVID-19 (Chaudhary et al., 2021).

Components and structure

The structural composition of messenger RNA (mRNA) comprises distinct elements essential for its function. It consists of five critical parts, which include the protein-encoding Open Reading Frame (ORF), flanked by 5' and 3' untranslated regions (UTRs), a 7-methyl guanosine 5' cap structure, and a 3' poly(A) tail with varying length across different cell types (Kim et al., 2021). The ORF is a sequence of nucleotides with the potential for protein translation and is pivotal in mRNA vaccines. They specify the nucleotide sequence encoding the desired viral protein that mimics the part of the virus to produce an immune response (Qin et

al., 2022). Optimization of the ORF to enhance the translation process through codon optimization and the introduction of functional peptides can enhance mRNA vaccine safety, efficacy, and stability (Fang et al., 2022). Increasing the GC content and replacing the rare codons in ORFs contribute to these objectives, hence holding the potential to prevent endoribonuclease degradation (Kang et al., 2023).

The 5' and 3' UTRs, although not encoding proteins directly, play crucial roles in the regulation of mRNA translation and protein expression (Qin et al., 2022). The 5'-UTR initiates the translation process, while the 3'-UTR affects mRNA stability and half-life (Hinnebusch et al., 2016; Matoulkova et al., 2012). Regulatory elements within both UTRs govern mRNA stability, ribosome recognition, interaction with translational machinery components, and mRNA secondary structures. The modification of their length and structure will affect the translation efficiency and half-life (Kim et al., 2021). An mRNA with longer 3’ UTRs will have a shorter half-life. Meanwhile, mRNA with shorter 3’ UTRs will be translated less efficiently, as it has its optimal length requirements (von Niessen et al., 2019).

The poly(A) tail is composed of 10 to 250 adenine ribonucleotides, and their length influences mRNA translation efficacy and protein expression (Jalkanen et al., 2014). It stabilizes mRNA, inhibits exonuclease-mediated degradation, binds to poly(A)-binding proteins (PABPs), and synergizes with the 5’ m7G cap to regulate translational efficiency (Fang et al., 2022). The poly(A) tail’s length is proportional to the translation efficiency, making it a pivotal determinant of mRNA molecule longevity (Kim et al., 2021).

The 5' cap is located at the mRNA's 5' terminus with varying methylation degrees. It contains a 7-methylguanosine that attaches the subsequent nucleotide via a 5’-5’ triphosphate bridge (Decroly et al., 2012; Qin et al., 2022). Its structure prevents mRNA degradation by exonucleases, ensuring stability and facilitating translation initiation (Warmiński et al., 2023). Post-transcriptional modifications such as 5'-capping are crucial for mRNA stability and efficient translation, emphasizing the importance of a functional 5' cap structure in robust mRNA translation (Kim et al., 2021).

Besides the mRNA itself, the vaccine contains a delivery system due to its large molecules (104-106 Da) and negative charge, making it unable to pass through the anionic lipid bilayer cell membranes (Wadhwa et al., 2020). Moreover, naked mRNA could be degraded and destroyed by nucleases. Therefore, lipid nanoparticles are used to encapsulate the mRNA to protect it and prevent the degradation of the nucleic acid core (Zhang et al., 2022). It represents the sole drug delivery system that has exhibited clinical efficacy and received approval for human use among various delivery approaches for mRNA vaccines (Gote et al., 2023). The lipid nanoparticles are usually composed of components that include ionizable cationic lipid, helper phospholipid, cholesterol, and PEGylated lipid (Mashima & Takada, 2022).

Types of mRNA vaccine

Currently, there exist three distinct types of mRNA vaccines that have been developed: conventional mRNA, self-amplifying mRNA, and trans-amplifying mRNA. Conventional mRNA is structurally akin to the endogenous mammalian mRNAs, which consist of a 5’ cap, 5’ UTR, coding region, 3’ UTR, and a polyadenylated tail (Schmidt & Schnierle, 2023). It is also called non-amplifying or non-replicating due to its structure, namely the UTRs and coding regions that can be transcribed into a single copy of the immunogenic protein (Al Fayez et al., 2023). Upon delivery to the cytosol, they undergo translation until degradation without additional replication (Zeng et al., 2020). This type has a typical size of 2-4 kb, and the number of transcribed mRNAs is directly proportional to the resulting immune response, potentially necessitating a high mRNA dose as well as repeated administration (Bloom et al., 2020; Schoenmaker et al., 2021).

Self-amplifying mRNA vaccines involve genetic modification by incorporating engineered replicons from self-replicating RNA viruses. It has conserved sequence elements (CSE) at the 5’ and 3’ ends that can regulate the synthesis of viral RNA besides its ability to attach to protein or viral proteins (Al Fayez et al.,

2023). Self-amplifying mRNAs are derived from single-stranded RNA viruses like alphaviruses and encode not only the proteins of interest but also replication machinery comprising viral non-structural proteins (nsPs) (Brito et al., 2015). With a typical size of 8–12 k nucleotides, larger than conventional mRNA vaccines, self-amplifying mRNAs replicate and express designated proteins upon cytosolic delivery in large amounts (Zeng et al., 2020).

Despite the similarity in having CSE at the 5' and 3' ends, trans-amplifying mRNA differs in that it lacks non-structural proteins 1-4 (nsP 1-4) sequence compared to self-amplifying mRNA (Al Fayez et al., 2023). The replicase and ORF encoding the immunogenic protein requires two RNA genes to be co-delivered to the target cells: one without nsP 1-4 and the other encoding nsP1-4 genes. However, both feature a cap structure, 5´ UTR, 3´ UTR, and a 3' poly(A) tail. The replicase translates into the RdRp complex, which is then utilized for amplification. Subsequently, mRNAs containing the ORF encoding the immunogenic protein are translated by ribosomes (Nafian et al., 2021).

mRNA vaccine mechanism of action

mRNA technology starts with the selection of target antigen and the mRNA sequence is synthesized optimally outside the human body. The synthesized mRNA vaccine must contain a 5'cap, 5'UTR, translated region, 3'UTR, and poly-A tail to be recognized by ribosomes (Liu et al., 2021). Once it is successfully done, it can proceed to administration via intramuscular. Through endocytosis, it is released in the cytoplasm for translation. The poly-A tail binds to PABP and eIFs bind to the 5'UTR cap to initiate the translation (Al Fayez et al., 2023). The process uses ribosomes to translate each codon of the mRNA in the translated region (consisting of three nucleotides) to amino acid and form the spike protein (Fabbri et al., 2021). Afterward, the spike protein is released extracellularly through the Golgi apparatus and undergoes breakdown into peptides. Formation of MHC class II is triggered by the spike protein, whereas through cross-presentation on dendritic cells, the spike protein is presented as MHC I. Hence, MHC I presents itself as an antigen to induce CD8+ T cells while MHC II induces CD4+ T cells (Liang et al., 2017). After induction, with the mediation of CD8+ T cells, an antigen-specific cytotoxic T-cell immune system will be formed. On the other hand, due to the CD4+ T cell activity, B cells become memory B cells (Suzana et al., 2022). The formation of those antibodies will damage the spike protein and the mRNA of the vaccine to form an adaptive immune system to fight off SARS-CoV-2.

Advantages of mRNA vaccine

DNA vaccination is a technique involving the delivery of plasmid DNA (pDNA) encoding a specific antigen, driven by a eukaryotic promoter, into host cells (Langer et al., 2012). The process leads to the encoded polypeptide(s)' intracellular synthesis that triggers an immune response (Hasson et al., 2015). DNA vaccines can closely mimic live infections and elicit both antibody and cell-mediated immune responses (Farris et al., 2016). This approach finds applicability across a spectrum of viral, bacterial, and parasitic diseases. Despite being easy to construct and well-tolerated in humans, DNA vaccines are yet to receive approval for human use and face critical disadvantages primarily in health and safety concerns (Mondal et al., 2021). The potential integration of exogenous DNA into the host genome poses risks of insertional mutagenesis, emphasizing safety issues associated with genomic incorporation (Wadhwa et al., 2020). Moreover, the inherently lower immunogenicity of DNA vaccines compared to other alternative platforms necessitates the use of adjuvants or repeated administrations to achieve robust and sustained immune responses (Ledesma-Feliciano et al., 2023).

The mRNA vaccines are superior compared to the conventional vaccines by offering several advantages. It has an easy, high-speed production where RNA synthesis can be carried out immediately upon the sequencing of the encoded immunogen on the same platform (Mirtaleb et al., 2023). The

manufacturing process is easily scalable and done in a cell-free manner with cost-effective production (Jackson et al., 2020). Moreover, the expressed target protein or antigen is translated from the mRNA by the host translation machinery rapidly after transfection. This process takes place in the cytoplasm, unlike DNA vaccines which take place in the nucleus (Park et al., 2021). Therefore, mRNA vaccines have a higher biosafety and eliminate the risk of genome interaction or integration that could lead to insertional mutagenesis. In addition, mRNA carries a short sequence that needs to be translated, which does not cause any host genome interaction. Hence, it is considered a safer vector than DNA (Wadhwa et al., 2020). This vaccine technology allows the encoding of multiple antigens. Thus, with a single formulation, it enables the target of multiple microbes or viral variants and enhances the immune response against resilient pathogens by stimulating both humoral and cellular immune responses (Schlake et al., 2012).

MANUFACTURING PROCESS OF mRNA VACCINE

Overview of the manufacturing process

The mRNA vaccine offers several advantages over traditional vaccines, including ease of development, scalability, and rapid manufacturing. Additionally, it is a more affordable option compared to other vaccines due to the streamlined production process. Unlike traditional vaccines, mRNA production does not require a costly qualified facility since it can be established in any space (Niazi, 2022). Moreover, the production of mRNA in cell-free systems eliminates the need for animal-derived materials, enhancing the safety of the manufacturing process. The manufacturing process for mRNA vaccines is sequence-independent and primarily depends on factors such as RNA length, nucleotide composition, capping chemistry, and product purification. mRNA vaccines for COVID-19 have also shown superiority due to the simple manufacturing and rapid response. The obtaining of RNA for large-scale production can be done in three ways, which are chemical synthesis using a solid-phase method, enzymatic synthesis by in vitro transcription, and purification from biological sources (Ryczek et al., 2022).

The solid phase for chemical synthesis was first developed by Bruce Merrifield. It is based on the cyclic elongation of the DNA/RNA chain on a solid support, such as a controlled pore glass or highly crosslinked polystyrene. It utilizes the reaction between an activated nucleoside phosphoramidite and a solid support-bound nucleoside to synthesize the desired RNA sequence from a 3' to 5' direction (Ryczek et al., 2022). The first step includes the removal of 4-4'-dimethyloxytrityl (DMTr), which protects the group at 5'-hydroxyl (OH) of tethered 3' nucleoside, with trichloroacetic acid or dichloroacetic acid. The nucleophile at 5'OH then attacks and removes the activated phosphoramidite to couple the two nucleosides with a phosphite triester bond. The final step is the oxidation of phosphite triester to phosphotriester with iodine or tet-butylhydroperoxide. After the sequence has been synthesized, RNA is deprotected and cleaved from solid support. However, it is only efficient for short oligomers up to 20 nt (Becette et al., 2019).

Alternatively, other methods can be done such as using plasmids that contain the genetic instructions for human cells to build coronavirus proteins and trigger immune responses to the virus. After the vials of DNA templates were harvested, the strands of mRNA were then transcribed, purified, and encapsulated into lipid nanoparticles. This was the method of manufacturing the Pfizer/BioNTech BNT162b2 vaccines. Alternatively, mRNA can be synthesized using the IVT reaction (Wei et al., 2023). The manufacturing process involves three main steps: upstream production, downstream purification, and formulation of the mRNA drug substance, including the use of LNP formulation and packaging (Gote et al., 2023). The upstream process involves enzymatic mRNA generation, while the downstream process includes the necessary operations to purify the mRNA product (Rosa et al., 2021).

Manufacturing process from upstream to downstream

The upstream production of the vaccine consists of the generation of mRNA transcript from a plasmid containing the gene of interest. This is called the *in vitro* transcription reaction (IVT). *In-vitro* transcribed mRNA is produced with the help of RNA Polymerase from bacteriophages and captures the native ability of these enzymes to synthesize long RNA transcript from DNA sources, whether it is from PCR-amplification templates or plasmids.

During the IVT enzymatic reaction, several components need to be included, such as RNA polymerases, nucleotide triphosphate substrate, polymerase cofactor $MgCl_2$, and a pH buffer containing polyamine and antioxidants (Rosa et al., 2021). There are different types of RNA polymerases used for the corresponding DNA template, such as T7 which is derived from the T7 bacteriophage, T3 which is derived from the T3 bacteriophage, and SP6 (Kang et al., 2023).

After the process of transcription, the 5' cap structure should be included, which is important for efficient translation initiation (Kang et al., 2023). The capping of mRNA can be in a one-step or two-step process. The one-step reaction involves the capping of the RNA concurrently during the IVT reactions by substituting part of the guanosine triphosphate (GTP) substrate for cap analog. However, the use of the cap analog can be more expensive. The two-step reaction involves a secondary enzymatic reaction which uses the ability of virus-derived capping enzymes such as the vaccinia capping enzyme and a methyl donor (from methyltransferase derived from the vaccinia virus) as a substrate. This method of capping proves 100% efficiency compared to using a cap analog which has 60%-80% capping efficiency. The two-step reaction provides a higher efficiency compared to the one-step reaction, but the production price can be a disadvantage when doing large-scale production (Rosa et al., 2021).

The isolation and purification must be done once the mRNA is generated by IVT to achieve clinical purity standards, known as the process of downstream purification (Rosa et al., 2021). Impurifications that may derive from this method can include plasmids produced through fermentation, which may also contain immunogenic impurities such as endotoxin and proteins. Enzymes involved in IVT can introduce pollutants and exogenous factors, inducing proinflammatory cytokines and inflammation. Unpurified IVT mRNA might also contain unwanted RNA molecules, which include truncated or abnormal transcription, uncapped mRNA, and double-stranded RNA, all of which negatively impact the function of IVT mRNA. Efficient removal of these impurities is essential to improve the mRNA translation levels and prevent the activation of undesirable immune responses (Zhang et al., 2023). One of the most dangerous impurities is the dsRNA, as it is a potent pathogen-associated molecular pattern that is sensed by pattern recognition receptors in cellular compartments. The recognition of IVT mRNA contaminated with dsRNA can result in type I interferon production, which causes the inhibition of translation and degradation of the cellular mRNA and ribosomal RNA by the upregulated expression and activation of protein kinase R and 2'-5'-oligoadenylate synthetase (Pardi et al., 2018).

The process of purification depends on the production area. Lab-scale production is based on DNA removal by DNAse digestion followed by lithium chloride (LiCl) precipitation. This method can reduce the efficiency of the translation and modify the immunostimulatory profile. Using reversed-phase HPLC instead of LiCl can increase protein production up to 10-1000 fold (Gote et al., 2023). Chromatography is a widely used method for the purification process of various vaccines and biological products. This includes size exclusion chromatography, ion pair reverse phase chromatography, ion exchange chromatography, affinity-based separation, and tangential flow filtration (Rosa et al., 2021).

The fill-and-finish process of vaccine development involves a separate manufacturing facility that is capable of receiving the drug product to fill (squirt doses into vials) and finish (cap the vials with stoppers and then label and package) the vaccine for distribution. The plants required specializes assembly line capital

equipment in addition to variable inputs like glass vials and stoppers. Materials are also needed for shipping, including cold storage. The delivery is the final stage of delivery that contains the distribution of the vaccine, which incorporates skilled personnel (Bown & Bollyky, 2021). The COVID-19 vaccine necessitates a specialized end-to-end chain for its supply, spanning from manufacturing and transportation to warehouse and healthcare facilities. If issues concerning the storage and distribution of the vaccine are not addressed, it will hinder our society's progress toward achieving herd immunity. Enhancing storage conditions may involve optimizing the formulation by improving stability profiles through the introduction of dry powders, thereby enhancing shelf-life and allowing for storage in refrigerators rather than freezers (Fahrni et al., 2022).

Table 1. Different types of chromatography used for purification.

Purification process	**Process**	**Advantage**	**Disadvantage**
Size-exclusion Chromatography	It uses gravity-flow mode to separate molecules according to size		Not able to remove similar size impurities such as dsDNA
Ion pair reverse phase chromatography	Negatively charged sugar-phosphate backbone of oligonucleotides will pair with quaternary ammonium compounds present in the mobile phase to become lipophilic and interact with the stationary phase of the reversed-phase chromatography column. Elution is then performed with a gradient of adequate solvent	dsRNA impurities are removed while maintaining process high yield	Challenging, costly, use of acetonitrile is toxic
Ion exchange chromatography (IEC)	Explores charge differences between the target mRNA species and the different impurities	Scalable, cost-effective, allows separation of longer RNA transcript, and presents higher binding capacity	Performed under denaturing conditions which makes the process more complex as it requires a mobile phase heater and tight control of temperature
Affinity-based separation	Sequence binds to the poly-A tails present in the mRNA. Chromatographic beads with immobilized oligo dT	High purity products	Low binding capacities, less cost-effective
Tangential flow filtration (TFF)	Small impurities are trapped inside the beads while the product will flow through		

LNP delivery system

There are different vector systems for the delivery of genes, such as viral vectors and non-viral vectors. Viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, pox virus, and lentivirus. They have been modified by deleting the areas of the genomes to ensure the safety of replication. However, these vectors are immunogenic and can lead to the induction of inflammation. Non-viral vectors comprise physical and chemical systems. Physical methods include electroporation and chemical methods, including cationic delivery such as monovalent cationic lipids, polyvalent cationic lipids, and cationic polymers (Nouri et al., 2012).

The mRNA molecules are negatively charged and therefore should be formulated in a lipid-based drug delivery system for the prevention of mRNA degradation as well as improvement of transfection efficiency and half-life (Rosa et al., 2021). LNPs are the most common delivery vehicles used in mRNA delivery systems in preclinical and clinical studies. They present a micelle-like structure that encapsulates nucleic acid inside a non-aqueous core. The LNP system enables the uptake by host cells and the delivery of mRNA inside the cytosol (Schoenmaker et al., 2021).

Most of the COVID-19 mRNA vaccines are delivered by lipid nanoparticles (LNP). They encapsulate mRNA with a solid lipid structure composed of four components: Cationic or ionizable lipid for mRNA complexation, cholesterol for stability, helper phospholipid to aid formation and intracellular release, and PEGylated lipids to reduce non-specific interactions. These LNPs have many advantages such as efficient encapsulation and condensation of the mRNA, promotion of mRNA intracellular delivery to the cytosol by increasing the cellular uptake and triggering the endosomal escape, increase of the mRNA stability by protecting them from degradation in extracellular space, biocompatible composition which is safe for human use (Park et al., 2021).

The key design aspects of the drug delivery system for COVID-19 vaccines to avoid degradation and optimize stability is positive-charged lipids in nanoparticle formulation to associate with negative charges on the interface of the nucleic acid, a crucial aspect for choosing an appropriate cationic or ionizable lipid. However, these permanently positive-charged lipids are toxic, so they develop ionizable cationic lipids which can be protonated at acidic pH to become positively charged. Optimizations have also been done to coat nanoparticles with PEG (Labouta et al., 2022). LNP formulations need to overcome multiple extracellular and intracellular barriers, such as the protection of mRNA from nuclease degradation in physiological fluid, evasion of interception and clearance by renal glomerular filtration post systemic administration, and the need to reach target tissues, which requires evasion of the endosome to reach the cytoplasm where translation occurs (Hou et al., 2021).

LNP is composed of a cationic/ionizable lipid and helper lipids, such as phospholipids, cholesterol, and/or polyethylene glycol (PEG) lipids. Phospholipids and cholesterol contribute to the promotion of LNP stability and endosomal escape by modulating membrane integrity and rigidity. Cholesterol analogs with C-24 alkyl phytosterols in LNP enhance intracellular delivery of mRNA in vitro. PEG-lipid is used to control particle size and zeta potential; non-specific interactions are reduced to prevent aggregation in storage. The selection of an appropriate lipid composition or modifications of the structure is done to improve the biodegradability in vivo and to the biocompatibility to avoid the toxicity and side effects caused by ionizable lipids such as ester, amides, and mercaptans. The main advantages of LNPs include the tailored optimization of biophysical and biological characteristics to achieve the maximum efficiency of encapsulation and controlled release (Liu et al., 2022).

The flexibility of LNP-mRNA-based vaccine design and scalability of manufacturing in contrast to conventional technologies allows for a rapid response to other novel SARS-CoV-2 variants. Different approaches are in the pipeline, with the use of mRNA platforms nowadays aiming to have a variant-specific

response through modified, adjusted coding sequences for new strains or heterologous vaccine administration (Szabó et al., 2022).

mRNA-LNPs are prepared by the rapid mixing by microfluidic mixers using common methods such as ethanol dilution, in which ethanol solution is added to aqueous media, resulting in the formation of nanodroplets. This method showed significant nucleic acid encapsulation efficiency and the encapsulated mRNA molecule is protected from degradation by nuclease enzymes (Wilson & Geetha, 2022).

Different types of downstream processing of COVID-19 vaccines

New developments in the field of mRNA vaccination have been made possible by the use of lipid nanoparticles (LNPs), which offer protection and modified *in vivo* delivery of mRNA vaccines (Pilkington et al., 2021). LNPs have been proven to be highly adaptive to antigen presentation and improve immune stimulation to elicit powerful humoral and cellular immune responses (Liu et al., 2022).

In total, six studies on the downstream processes of COVID-19 vaccines were summarized in a review (Table 2). Based on the findings in Table 2, there are various downstream processes of COVID-19 vaccine production. These include tangential flow filtration (TFF), ultracentrifugation, hydrophobic interaction chromatography, size exclusion chromatography, flow-through chromatography, ion exchange chromatography, and affinity-based purification. These methods can purify the vaccine from any impurities with various grades of purity levels. However, most of the downstream process of COVID-19 vaccine production utilizes more than one method to remove impurities from its vaccine. Combining purification methods can provide more effective and yield more end products. However, the methods are not solely responsible for the purity level or yields.

CRITICAL PROCESS PARAMETERS IN DOWNSTREAM PROCESSING OF MRNA VACCINE

The primary objective of RNA-based vaccine manufacturing is to establish production methods that are able to consistently deliver high-quality end products. This includes creating accurate requirements for different critical process steps such as intermediates, drug ingredients, and drug products, and applying analytical methods that allow for robust product quantification and characterization (Rosa et al., 2021). In order to accomplish this, the production process is meticulously developed to reduce product variability while simultaneously improving process capabilities. This entails identifying key process parameters (CPPs) that are linked to critical quality characteristics (CQAs) (Daniel et al., 2022). The downstream process of mRNA vaccine contains the purification of the mRNA product that is combined with the LNP formulation and fill-to-finish procedures (Rosa et al., 2021). The downstream process may include the use of chromatography and filtration followed by formulation and bulk-fill processes.

Critical process parameters of chromatography in downstream processing of mRNA vaccine

The critical parameters in the chromatography process for mRNA vaccine manufacturing include characteristics such as sample load, RNA concentration, buffer type, pH, conductivity, flow rate, and column pressure (Daniel et al., 2022). The specific type of buffer used and the exact conditions used in chromatography have a significant influence on RNA binding and elution. Increasing the concentration of salt, for example, allows RNA to bind with positively charged support and accelerates elution. Increasing the ionic strength, on the other hand, leads to the retention and elution of the majority of RNA. The overall RNA recovery improves as the salt concentration rises. This dual binding pattern, found under both ionic and hydrophobic circumstances, may allow for the use of gentler buffers. This, in turn, helps both the entire process and the quality of the separated products (Carapito et al., 2023).

Table 2. Summary of different purification methods of COVID-19 vaccines.

Author (Year)	Title	Findings
Zhang et al. (2020)	A Thermostable mRNA Vaccine against COVID-19	Purification: Diafiltration with Tangential Flow Filtration (TFF) membrane 100 kDa MWCO, passed through a 0.22 mm filter
Lee et al. (2021)	Process development and scale-up optimization of the SARS-CoV-2 receptor binding domain-based vaccine candidate, RBD219-N1C1	Purification: Hydrophobic Interaction Chromatography (flow rate of 1 mL/min) and Size Exclusion Chromatography (10 mL/min flow rate) Yield: 50% Purity: 98.3%
Wang et al. (2020)	Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2	Purification: Utilizing sucrose gradient ultracentrifugation to achieve further purification and separation of desired viral particles from unwanted material
Lerer et al. (2021)	Highly Efficient Purification of Recombinant VSV-ΔG-Spike Vaccine against SARS-CoV-2 by Flow-Through Chromatography	Purification: Flow-through chromatography with the CC700 resin Yield: >85% Purity: 99%
Boggiano-Ayo et al. (2023)	Development of a scalable single process for producing SARS-CoV-2 RBD monomer and dimer vaccine antigens	Purification: Ion Exchange Chromatography and Size Exclusion Chromatography Purity: >98.5%
Makovitzki et al. (2021)	Evaluation of a downstream process for the recovery and concentration of a Cell-Culture-Derived rVSV-Spike COVID-19 vaccine candidate	Purification: Tangential flow filtration Yield: ~40% Purity: ~97%
Di et al. (2023)	Purification of SARS-CoV-2 RBD in Affinity Chromatography Using a Novel Nanobody Ligand	Purification: Affinity-based separation Purity: >90% Yield: ~50%

The pH level used in chromatography has a significant impact on the adsorption selectivity and capacity of the chromatographic support. Various pH settings may detect even minor changes in RNA adsorption processes, making them a valuable instrument for altering appropriate conditions to more efficiently isolate the target molecule. It is feasible to manage and improve selectivity and process yield by carefully selecting the ideal adsorption pH, thus adding to the efficiency and success of chromatographic separation in RNA-related applications (Cardoso et al., 2022).

Pressure plays a role in chromatographic adsorption equilibrium and has an influence on many aspects of the process. It may lead to changes in the molecular molar volumes of analytes, stationary phases, and adsorbates, affecting the chromatographic system's interaction dynamics. Notably, research has shown that increasing pressure while maintaining a fixed flow rate and temperature can lead to an increase in the chromatographic retention factor (Makarov et al., 2014). However, contrast arises from a different study revealing that pressure had no significant effect on analyte retention time (Fallas et al., 2013).

The flow rate of the mobile phase is a critical parameter in chromatography, and it has a notable impact on the separation of RNA. Specifically, it affects the characteristics of the peaks observed in

chromatograms. A reduction in peak heights, as observed in some cases, can impose limitations on the method's utility, particularly when accurate quantification of degradation products similar in size to the primary mRNA peak is required (Currie et al., 2021). Furthermore, the sample load, which includes both volume and mass, is a significant factor that affects chromatographic resolution and sensitivity. This impact corresponds to the principles that influence other chromatographic processes. Sample load must be carefully examined in order to obtain the appropriate chromatographic performance. Overloading a column with an excessive sample load may result in a decrease in analyte resolution. As a result, striking a balance in sample load is critical for ensuring successful separation and the quality of the chromatographic results (Hong et al., 2012).

In chromatography, the relationship between retention period and peak height in a column is linear. The mobile phase flow rate significantly influences the separation of RNA and its peak characteristics. Reduced peak heights may hinder the accurate quantification of degradation products similar in size to the mRNA main peak, particularly when the injection volume is less than 10% of the mobile phase volume. Additionally, peak width increases with injection volume, demonstrating a linear connection below a certain volume threshold. However, increasing the injection volume results in a reduction in the resolution between the separated substances (Ren et al., 2013).

In addition to the critical process parameters mentioned, the column temperature and column hardware were discovered to be a critical factor in mRNA recovery and carry-over. The self-structure of mRNAs is altered by temperature. It is possible that as column temperature approaches the melting temperatures of some mRNA self-folded structures, chromatographic recovery decreases. Nucleic acids often face poor recovery in LC analysis due to strong adsorption on the stationary phase and interactions with column hardware surfaces. A new hydrophilic modified hybrid surface (h-HST) was developed for metal-based column hardware, reducing non-specific adsorption and secondary interactions (Fekete et al., 2022; Madsen et al., 2022).

Critical process parameters of filtration in downstream processing of mRNA vaccine

The filtering process in the manufacture of mRNA vaccines comprises numerous essential process parameters that are critical to their success. These factors include the feed flow rate, transmembrane pressure (TMP), the type of membrane utilized, RNA concentration, and the buffer composition. The right selection of these elements is critical in order to accommodate the specific properties of RNA molecules while avoiding undesired consequences such as phosphodiester ion generation, RNA precipitation, and denaturation.

The occurrence of gel formation and membrane fouling is one crucial observation, emphasizing the significance of employing membranes and purification buffers designed particularly for RNA molecules. This customization aids in avoiding risks associated with unsatisfactory filtering outputs (Daniel et al., 2022). In addition, a reasonably high flow rate avoids concentration polarization and membrane fouling. However, the high speed causes relatively minor concentration changes in the stream after a single pass, requiring recirculation. This elevates the energy consumption and may result in an undesirable temperature rise. The high flow rate additionally increases shear stress on the molecules, which can lead to the denaturation of sensitive biomolecules and challenges with foaming (Madsen et al., 2022).

The effect of increasing TMP (Transmembrane pressure) on filter capacity is notable in filtration. Higher TMP levels have been observed to result in greater filter capacity. This behavior differs from the pressure-independent predictions for an incompressible medium and the normal resistance increase associated with compressible fouling deposits. As TMP increases, the effective resistance of the fouled filter decreases. This fascinating discovery sheds light on the complex dynamics at work in the filtering process

for mRNA vaccine manufacturing (Messerian et al., 2022).

Tangential flow filtration is greatly impacted by a phenomenon known as concentration polarization, which results in decreased permeate flux and the presence of low concentrations in the retentate. The interaction of environmental and hydrodynamic forces is critical in managing this process. To optimize the filtering process, the membrane concentration should not approach the gel concentration, which might result in the creation of a gel-like layer on the membrane's surface, resulting in decreased flow. It is crucial that the target molecule does not reach this vital stage in the process. (Huter & Strube, 2019).

Several factors have a significant impact on the rate at which permeate flow declines. It was discovered that increasing feed concentration and membrane pore size, together with a drop in tangential flow rate, resulted in a faster decline in permeate flux. When tangential flow was absent, reversible fouling was the predominant cause of permeate flux reduction. Tangential flow, on the other hand, caused slightly greater levels of irreversible fouling. This difference was due to the greater permeation drag associated with tangential flow as compared to non-tangential flow conditions (Choi et al., 2005).

Critical process parameters of formulation and bulk fill in downstream processing of mRNA vaccine

The addition of excipients in the formulation and bulk fill process in the downstream processing of mRNA vaccine is crucial in maintaining the stability of the vaccine, wherein it affects the shelf-life of the vaccine. Several critical process parameters have been determined, such as the type of buffer used. It was found that TRIS buffer was more preferred to sodium phosphate buffer due to its property as a hydroxyl free-radical scavenger that has a more stabilizing effect and did not result in a pH shift (Ramachandran et al., 2022). Other critical process parameters were lyoprotectants concentration, pH, and temperature. It had been shown that the addition of trehalose or sucrose as lyoprotectants at a concentration of 20% (w/v) stabilized LNP throughout the freeze-thaw cycle. In addition, the formulation stored at room temperature was shown to experience a decrease in efficacy through degradation whereas, over 156 days, the LNP formulation stored in the freezer was able to maintain its efficacy. However, according to this study, the differences in the pH did not affect the stability of the LNP (Ball et al., 2016). In contrast, Schoenmaker et al. (2021) stated that the pH of the environment affects the stability of the mRNA LNP vaccine through the hydrolysis rate. A pH between 7 and 8 was appropriate since mRNA is stable in a weakly basic environment. Another method to maintain the stability of the mRNA LNP vaccine is through lyophilization.

Several studies have discussed the use of lyophilization techniques in increasing the stability of the mRNA LNP vaccine. There are three main steps involved in the lyophilization process, which are freezing, primary drying, and secondary drying. In the freezing step, the critical process parameters defined were crystallization, nucleation temperature, glass transition temperature, and collapse temperature. These parameters have been shown to affect the crystal size, inter/intra batch vial heterogeneity, ice crystal size distribution, and product porosity. During the primary drying process, heat transfer coefficient, vapor flow resistance, cycle time, product temperature, and sublimation rate were determined as the critical process parameters (Blue et al., 2015; Ghaemmaghamian et al., 2022). These parameters were shown to affect the stability, moisture content, appearance, and reconstitution time. The two main critical process parameters in the second drying process were drying time and product temperature. Both of these parameters influenced the residual moisture content (Ghaemmaghamian et al., 2022).

Latest challenges in the downstream processing of COVID-19 mRNA vaccine production

One factor that must be maintained in mRNA vaccines is the stability of the mRNA itself. Just like DNA, mRNA also undergoes modification when exposed to environmental changes. This modification will affect mRNA stability which eventually leads to a decrease in the end product quality. The stability of mRNA

is defined by its sequence of nucleotides, which influences both secondary and tertiary mRNA structures (Boo & Kim, 2020). To guarantee that mRNA utilized in vaccines remains intact throughout storage and delivery, it must be stable. Vaccines will not be able to work properly if the mRNA contained in the product degrades over time (Crommelin et al., 2021). As a consequence, no immune responses will be activated and no antibodies will be produced towards the pathogen, which is the case with the COVID-19 virus. Moreover, mRNA degradation will decrease the concentration of mRNA in the vaccine. Due to these reasons, ensuring vaccine stability is essential by examining and monitoring the vaccine's stability throughout its whole life cycle (Cheng et al., 2023).

Aside from their quality and efficacy, mRNAs' stability is also responsible for their shelf-life. When mRNA vaccines are able to be stored for a longer period of time, it can be said that it has good stability (Uddin & Roni, 2021). Moreover, the availability of the vaccine for a long period will prevent vaccine shortages during an outbreak or in areas with limited health facilities access. A stable mRNA vaccine also allows production in big batches and can be stored for a long time, leading to cost efficiency (Cheng et al., 2023). Moreover, it would also elevate the trustworthiness of the public as the vaccines administered to them have a reputation of having good stability and effectiveness.

The importance of vaccine stability has been discussed, but the factors that can influence vaccine stability have not been covered in this review. mRNA is very prone to changes resulting from non-optimal environments, such as high temperature which can lead to mRNA degradation. In a study done by Hollams et al. (2002), it was found that mRNA half-life is decreased as a result of heat shock exposure. The translation of mRNA plays an important role in affecting its degradation Thus, it can be suggested that both of the processes establish the mRNA stability temperature dependence (Jaquet et al., 2022). In addition, mRNA codon arrangement affects the efficiency of translation as well as the degradation rate of mRNA. Hence, maintaining an optimal temperature for the mRNA vaccine is essential. This can be a challenge in the manufacturing process of the COVID-19 mRNA vaccine because it complicates the process of distribution as well as logistics, especially in facilities or areas without the availability of adequate storage infrastructure to keep the low temperature needed for maintaining mRNA stability. For example, the Pfizer COVID-19 vaccine requires a temperature of -80℃ to -60℃, while the Moderna vaccine obliges a temperature range of -25℃ to -15℃ (Meo et al., 2021). Aside from that, chromatography also requires a certain column temperature during the downstream process. For these reasons, researchers are needed to find a solution to tackle this problem.

Another factor contributing to mRNA instability is direct light exposure to the vaccine. Direct exposure to light will result in photodegradation, a condition where compounds deteriorate as they are exposed to light (Yousif & Haddad, 2013). Strack (2020) explained that blue light causes mRNA to form large clusters upon exposure. The high photochemical energy emitted by blue light eventually generates free radicals that contribute to the destruction of mRNA. In addition, it stimulates the activation of PspCas13b expression, the proteins responsible for mRNA knockdown, that downregulates the mRNA (Blomeier et al., 2021). Aside from blue light, ultraviolet light may contribute to mRNAs losing their functionalities through inducing a cross-linking process. Cross-linking is defined as a covalent bond formation process that connects adjacent nucleotides of mRNA together or with other components (Harris & Christian, 2011). The resulting effects may vary depending on the location of the linking as well as the type of component. It can be beneficial in some applications, such as in a study by van Damme et al. (2022) that utilized RNA cross-linking to measure RNA function through RNA 3D structure. Through a method called Spatial 2'-Hydroxyl Acylation Reversible Crosslinking (SHARC), an accurate and precise transcriptome-wide 3D structure contact maps that show different RNA structures as well as interactions can be obtained. As opposed to this, the downside of cross-linking to mRNA stability is it could decrease the function of mRNA, as it may alter the structure that

affects its translation process (Huch & Nissan, 2014). Abnormal polypeptide accumulation due to early termination and misincorporation of amino acids will give rise to a decline in the translation process (Tanaka & Chock, 2021). In addition, other effects of mRNA cross-linking include mRNA binding protein dysregulation and decreasing protein balance because of aggregation of protein. Hence, providing a solution to this matter is necessary to maintain the stability of the mRNA vaccine.

The presence of RNAse enzymes in all equipment used during the upstream and downstream processes contributes to the degradation of mRNA in the vaccine. RNAse is an enzyme involved in the process of RNA hydrolysis (Messmore et al., 1995). On the other hand, this enzyme is essential for the elimination of defective RNAs that are unnecessary anymore to the cell during the maturation phase. However, the existence of RNAse in unfitting conditions will eventually be detrimental to the RNA component (Vincent & Deutscher, 2009). Therefore, ensuring the absence of RNAse in the whole process of mRNA vaccine is critical to ensure its efficacy, safety, and quality.

In COVID-19 mRNA vaccine conservation, the biggest challenge faced is the requirement of very low temperatures for storing the vaccine appropriately. According to Jaquet et al. (2022), temperature caused the turnover of mRNA to be gradual and coherent. Additionally, the mRNA half-life decreases as the temperature increases. Whereas at 20 ℃ its half-life is 3.4 minutes, at 30℃ the half-life decreases to 2.0 minutes. Thus, the need for ultra-cold storage is required in the storage as well as distribution of COVID-19 mRNA vaccine. Brasil (2001) stated that the purpose of having a cold chain is to preserve the proper conditions for the vaccine during storage, transport, distribution, and administration to the patient so that the original vaccine characteristics are maintained and changes are prevented. In the distribution of Pfizer COVID-19 vaccine, additional packaging using dry ice is utilized for extra precaution and to maintain the quality of the vaccine in which they invested approximately $2 billion in creating their network of cold-chain (Uddin & Roni, 2021; Ahnert et al., 2021). Due to this reason, COVID-19 mRNA vaccine distribution is a challenge, especially in the area without adequate facilities. Furthermore, WHO (2023) also stated that mRNA COVID-19 vaccine advantages were not spread uniformly across the globe, especially in low-to-middle income countries due to a lack of resources for research and development, high-cost equipment needed for making the vaccine, and intellectual property obstacles.

Aside from the extreme temperatures required for mRNA vaccine storage and distribution, the scaling up process of the vaccine is also a challenge, as it involves multiple-step processes that may be complex to scale up. Furthermore, the scaling-up process of the COVID-19 mRNA vaccine requires a high amount of capital being invested. Kis et al. (2020) stated that there are two significant components of cost for most RNA vaccines for COVID-19 in November 2020, which are the cost of materials as well as consumables. The consumables cost is the net result of using one-time-use products during the production process. Additionally, maintaining batch-to-batch quality consistency during the scaling-up process is another crucial factor. Errors or deviations coming from the production process will affect the end product safety, efficacy, and quality, which will eventually affect the patient upon administration of the vaccine. Thus, during the process of scaling up, quality control plays an important role. Nonetheless, Louden (2022) explained that RNA vaccines are an excellent technology that can be applied for rapid scale-up compared to conventional vaccine production methods because RNA vaccine production does not involve the use of cell culture, making it simpler and more consistent in terms of yield. Moreover, mRNA molecule production only requires 2-6 hours and the whole production can be done within days, unlike the vaccines that utilize cell culture.

The emerging new COVID-19 strains further complicate the downstream process of vaccine production. COVID-19 viruses can eventually adapt to vaccines as they circulate more in the environment and give rise to new variants. A variant is a condition in which the virus has at least one new characteristic

differing from the initial virus (WHO, 2023). As of now, there are several outstanding variants of COVID-19 including the Alpha, Beta, Gamma, and Omicron variants. Their differences are caused by mutations that can take place in their genome that codes for spike protein, molecules required for allowing viral entry into host cells (Taha et al., 2023). The mutation occurs as a result of adaptation to the changes in the surrounding environment. In addition, they can also differ in the symptoms resulting, transmission rate, and detection place (Putri, 2022). These changes will cause the entire production of COVID-19 mRNA vaccines to adapt in order to produce variant-specific vaccines while preserving the quality since different variants of COVID-19 virus will react differently toward the vaccine. Some variants may be resistant to the current vaccine, thus requiring a new vaccine that would work against said variant of the virus.

Optimizing pH is another crucial factor that needs to be considered to ensure the stability of the mRNA vaccine. This is because pH will determine both the rate of mRNA hydrolysis and the stability of lipid nanoparticles (Schoenmaker et al., 2021). The environment with slightly basic conditions is where mRNA is the most stable. The process of mRNA hydrolysis in acidic and basic conditions is different. However, one study showed that mRNA hydrolysis proceeds through a phosphodiesterase backbone attack by the hydroxyl group (Wayment-Steele et al., 2020). At a pH below 5, the rate of phosphate migration, a process that leads to oligonucleotide cleavage, appears faster than hydrolysis. On the other hand, a pH above 8 shows hydrolysis occurring (Jarvinen et al., 1991). This information can be useful for choosing the correct buffer for the formulation or during the manufacturing process. Additionally, it would also be helpful for helping chromatographic pH optimization.

CONCLUSION

The emergence of mRNA-based vaccine technology represents a paradigm shift in the approach to vaccine development and manufacturing, as exemplified by COVID-19 vaccine development. This paper discusses the comprehensive advancements, critical process parameters, and innovative strategies that underpin the mRNA vaccine manufacturing process. mRNA vaccines offer advantages over traditional vaccine platforms due to their mechanism of utilizing the body's cellular machinery to produce viral protein. Therefore, mRNA vaccines are eliciting a potent immune response without the risk of infection on top of lowering the development time and cost.

Critical to the success of mRNA vaccine production are the upstream and downstream processes, which are meticulously designed to ensure the quality, safety, and efficacy of the final product. The upstream process involves the synthesis and in vitro transcription of the mRNA, which is then encapsulated within lipid nanoparticles (LNPs) to enhance stability and facilitate cellular uptake. This encapsulation addresses one of the primary challenges associated with mRNA vaccines: The inherent instability of mRNA and its susceptibility to degradation. The downstream process consists of a series of purification and formulation steps critical for removing impurities and achieving the required purity and concentration of the vaccine. Techniques such as chromatography and filtration play pivotal roles in this phase, with specific parameters including sample load, RNA concentration, buffer type, and pH being meticulously optimized to enhance product recovery and purity. Additionally, the formulation process involves the careful selection of excipients and conditions to ensure the stability and longevity of the vaccine, with strategies such as lyophilization becoming promising avenues for improving storage conditions and extending shelf life.

The manufacturing process of mRNA vaccines also highlights the importance of scalability and adaptability. The cell-free nature of mRNA synthesis, combined with the rapid prototyping capabilities afforded by the technology, allows for swift responses to emerging infectious diseases. However, challenges such as cold chain logistics underscore the need for ongoing innovation in vaccine formulation and delivery to enhance accessibility and distribution on a global scale.

In conclusion, mRNA vaccine technology for COVID-19 has not only played a critical role in addressing the current pandemic but also laid the groundwork for future vaccine development. Through a detailed understanding of the critical process parameters and a commitment to technological innovation, the field is poised to revolutionize the landscape of vaccine production, offering a robust platform for rapid and effective responses to a wide range of infectious diseases. This comprehensive approach, from the molecular design of the mRNA to the final formulation and delivery, represents a pivotal advancement in our collective ability to protect global health.

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