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THE CURRENT STRATEGIES, RECENT PROGRESS AND REMAINING CHALLENGES FOR DEVELOPING MRNA VIRAL VACCINE

Strategi Saat Ini, Kemajuan Terkini dan Tantangan yang Masih Ada dalam Pengembangan Vaksin Virus MRNA

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ABSTRACT

The mRNA expression system has revolutionised biotechnology, notably in viral mRNA vaccine development, cancer immunotherapy, and gene therapy. However, recent safety concerns regarding the COVID-19 mRNA vaccine have emerged, particularly regarding its rare adverse effects and its possible connection to cancer. This review explains several approaches used in developing viral mRNA vaccines, the past obstacles solved in generating the current COVID-19 mRNA vaccine, and finally the current advancements and ongoing challenges in the viral mRNA vaccine field. We particularly focus on strategies and methods to improve the safety and translation efficiency of the mRNA vaccine, such as enhancing the vaccine's transfection specificity to targeted dendritic cells (DC) and using viral IRES or self-amplifying mRNA format to improve mRNA translation efficiency.

Keywords: *DC-specific-LNP-targeting, mRNA viral vaccine, safety, translation efficiency, viral IRES*

ABSTRAK

Sistem ekspresi mRNA telah merevolusi bioteknologi, terutama dalam pengembangan vaksin virus mRNA, imunoterapi kanker, dan terapi gen. Namun, kekhawatiran keamanan baru-baru ini mengenai vaksin mRNA COVID-19 telah muncul, terutama mengenai efek samping yang jarang terjadi dan kemungkinan kaitannya dengan kanker. Tinjauan ini menjelaskan beberapa pendekatan yang digunakan dalam pengembangan vaksin virus mRNA, hambatan yang pernah diatasi dalam menghasilkan vaksin mRNA COVID-19 saat ini, dan yang terakhir adalah kemajuan terkini dan tantangan yang sedang berlangsung di bidang vaksin virus mRNA. Kami secara khusus berfokus pada strategi dan metode untuk meningkatkan keamanan dan efisiensi translasi vaksin mRNA, seperti meningkatkan spesifisitas transfeksi vaksin ke sel dendritik (DC) yang ditargetkan dan penggunaan IRES virus atau format mRNA yang dapat menduplikasi dirinya sendiri untuk meningkatkan efisiensi translasi mRNA.

Kata kunci: *Efisiensi translasi, IRES virus, keamanan, penargetan-LNP-spesifik-DC vaksin virus mRNA*

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INTRODUCTION

Overview of mRNA Technology and Its Applications

Messenger RNA (mRNA) technology represents a powerful platform for the transient production of proteins, utilizing synthetic mRNA molecules to instruct cells to produce specific proteins. This versatile system has found widespread use in various fields, including basic research, regenerative medicine, therapeutic protein production, and vaccine development. Prophylactic applications, such as mRNA vaccines, have gained significant attention for their success in combating infectious diseases, notably the SARS-CoV-2 virus, which caused the COVID-19 pandemic (Fang et al. 2022). Therapeutic applications include cancer immunotherapy (Miao et al. 2021) and gene therapy (Liu et al. 2017; Popovitz et al. 2023). One of the key advantages of mRNA-based approaches is their ability to bypass DNA and directly program protein expression without the risk of genomic integration, offering a safer and more controlled alternative to traditional gene therapy methods (Rosa et al. 2021).

Viral mRNA Vaccine Mechanism of Action

In the context of vaccine development, mRNA technology has emerged as a groundbreaking tool. When mRNA is delivered into cells, it is translated into antigen proteins within the cytoplasm. A portion of these proteins remains intracellular, while the rest are secreted outside the cells. These secreted antigens are subsequently internalized by dendritic cells (DCs), which undergo maturation and migrate to nearby lymph nodes. There, they initiate T lymphocyte activation, sparking a robust adaptive immune response to target the pathogen (Fang et al. 2022).

Key Structural Elements of mRNA and Their Functional Roles

The typical mRNA template consists of several key components: a 5’ cap, a 5’ untranslated region (5’ UTR), a gene of interest (GOI), a 3’ UTR, and a poly(A) tail. Each of these elements plays a vital role in the translatability, stability, and longevity of the mRNA (Fang et al. 2022).

The 5’ cap structure, particularly the 5’ cap 1 structure as opposed to the 5’ cap 0 structure, functions as a molecular tag. This tag signals that the mRNA is endogenous, or cellular, rather than foreign or viral (Drazkowska et al. 2022). As a result, it reduces innate immune sensing and immunogenicity within the cell (Ramanathan et al. 2016). The 5' cap also aids in nuclear export (Hyde and Diamond 2015). Due to this molecular tag, mRNA stability in the cell’s cytoplasm is enhanced by preventing degradation via 5' exonucleases (Picard-Jean et al. 2018). Finally, the 5’ cap structure is essential for initiating translation through a cap-dependent pathway by binding to the eukaryotic initiation factor 4F (eIF4F) complex (Hyde and Diamond 2015).

The untranslated regions (UTRs) of an mRNA, located at the 5' and 3' ends, are non-coding sequences crucial for regulating mRNA stability, translation efficiency, and localization. The 5' UTR, positioned upstream of the start codon, helps translation initiation by controling how efficiently the ribosome initiates translation. It often contains elements like the Kozak sequence, which assists the ribosome in identifying the correct start codon in the main ORF from other existing AUG codons. In humans, the 5' UTR is typically longer than the 3' UTR, averaging about 218 nucleotides (Leppek et al. 2018). The length and structure of the 5' UTR can influence translation, harboring regulatory elements that respond to cellular conditions.

Similarly, the 3' UTR, found downstream of the stop codon, plays a key role in mRNA stability. It regulates how long the mRNA remains protected from degradation and can also bind regulatory proteins or microRNAs (miRNAs) that further control translation efficiency. Additionally, the 3' UTR is involved in mRNA localisation (Mayr 2017; Mayr 2019).

The GOI represents the coding sequence of the target protein. Codon optimisation may be necessary to enhance protein expression in transfected human cells, especially if the GOI encodes a non-human protein (Fang et al. 2022).

Finally, the 3' poly(A) tail structure plays a crucial role in translation and mRNA stability. This tail aids in the cap-dependent translation initiation pathway by binding to poly(A)-binding proteins (PABPs), which also interact with the eIF4F complex, creating a looped structure (Kühn and Wahle 2004; Kühn et al. 2009). The poly(A) tail prevents mRNA degradation via 3' exonucleases, thereby contributing to mRNA stability in the cytoplasm. In mammals, including humans, the poly(A) tail typically ranges between 150-250 nucleotides (Kühn et al. 2009; Fang et al. 2022). The length of the tail also influences translation efficiency; up to about 150 adenine nucleotides, the tail improves translation efficiency. A longer tail results in an extended mRNA half-life (Jalkanen et al. 2014; Fang et al. 2022; Biziaev et al. 2024).

Adaptive and Innate Antiviral Immune Response Induced by Viral mRNA Vaccine

As a viral mRNA vaccine works, after the generation of viral antigen proteins in the cytoplasm, a portion of the proteins remain in the cells while the rest are secreted. Local DCs ultimately internalise the secreted viral antigen proteins. These DCs mature while migrating to the nearest lymphatic nodes, activating the T helper cells (CD4+) and T cytotoxic cells (CD8+). The activated T cells proliferate and differentiate into effector and memory cells, with most effector cells exiting the lymph node to patrol the body through the bloodstream. The crucial role of the T helper cells is to activate B cells in the lymph node, leading to their proliferation and differentiation into plasma and memory B cells. This process results in the production of neutralising and non-neutralising antibodies by plasma cells, which prevent cellular infection by the virulent virus, and the targeting and killing of infected cells by effector cytotoxic T cells. This entire event represents the antigen-specific antiviral immune response carried out by the adaptive immune system (Fang et al. 2022). For this reason, one of the main targets of the viral mRNA vaccine is the DC, the most proficient antigen-presenting cell (APC) in the immune system.

Remaining viral antigen proteins in the cell may trigger the antiviral state response within transfected cells. These cells, in a remarkable display of self-defense, inhibit almost all protein production activities. This is a proactive measure, as the cells believe they are under viral attack and are trying to prevent viral replication within the infected cells and viral spreading to neighbouring cells. The cells employ IFIT1 and PKR proteins to halt translation initiation and elongation from mRNAs lacking the 5’cap 1 structures and contain double-stranded RNA (dsRNA) (Ramanathan et al. 2016; Matsumiya et al. 2023). They also use the OAS/RNAse L complex to identify and cleave dsRNA structures from foreign or viral RNAs (Schwartz and Conn 2019). As these three sensor proteins activate the type 1 IFN pathway, the cell eventually generates and secretes interferon-γ (IFN-γ). This secreted IFN-γ works in an autocrine and paracrine way, activating the interferon-stimulated genes (ISG). The ISG in the IFN-γ affected cells causes the cells to produce more IFN-γ, IFIT1, PKR, OAS/RNAse L and other antiviral effector proteins. The result is that these cells enter an antiviral state even before exposure to the actual virulent virus. If a virus does infect the body, these pre-activated cells inhibit viral replication, limiting its spread much faster (McNab et al. 2015). This event represents the non-specific antiviral response the innate immune system carries out.

It's worth noting that the viral mRNA vaccines developed by Pfizer/BioNTech and Moderna are designed with 5’ cap 1 structures and modified nucleotides, particularly the N1-methyl pseudouridine (m1Ψ). These modifications ensure that the mRNAs are shielded from the PKR and OAS/RNase L protein complex (Anderson et al. 2010; Anderson et al. 2011) and intracellular exonucleases, guaranteeing that the mRNAs will not be prematurely degraded and are thus appropriately translated (Corbett et al. 2020; Polack et al. 2020).

The Synthesis of mRNA Vaccines

To produce the viral mRNA vaccine, synthetic mRNAs are generated via *in vitro* transcription (IVT) using T7, T3, or SP6

phage RNA polymerase, followed by enzymatic or co-transcriptional capping and co-transcriptional polyadenylation. While co-transcriptional capping with 5'cap 1 analogues has been attempted to entirely replace enzymatic capping, as it requires both the Vaccinia capping enzyme and 2'-O-methyltransferase to generate 5'cap 1 structure, the latter remains more efficient and is therefore still widely used (Beverly et al. 2016; Vlatkovic et al. 2022).

PAST CHALLENGES SOLVED TO ACTUALISE VIRAL MRNA VACCINES

Before the successful deployment of Pfizer/BioNTech and Moderna's COVID-19 mRNA vaccines in late 2020, two major challenges limited mRNA technology. The first was the inherent instability of mRNA, making it prone to rapid degradation by ribonucleases (RNases) in biological fluids. Ribonucleases are enzymes that degrade RNA by cleaving its phosphodiester bonds. Depending on their cleavage points on RNA molecules, ribonucleases are categorised as endonucleases or exonucleases. Endonucleases cleave RNA internally, while exonucleases digest RNA from the ends. Extracellular ribonucleases—such as RNAse 1 and angiogenin found in blood—and intracellular ribonucleases—such as the XRN1 and DXO in the cytoplasm (Picard-Jean et al. 2018; Galloway and Cowling 2019; Drazkowska et al. 2022)—can easily mark and digest naked mRNAs before even being transfected to the target cell. Obviously, this is a significant concern for the stability and functionality of mRNA vaccines.

To protect mRNAs from extracellular ribonucleases, lipid nanoparticles (LNPs) were developed to encapsulate the mRNA, protecting it from degradation and facilitating cellular uptake via endocytosis and endosomal escape. To prevent mRNA degradation by intracellular ribonucleases, protective measures include adding 5' cap 1 structure to the mRNA to improve its stability. The 5' cap 1 structure, which features a methylated guanosine cap, effectively shields the mRNA from degradation by 5' exonucleases such as the XRN1 and DXO, in contrast to the 5' cap 0 structure, which lacks this modification and provides less protection (Hyde and Diamond 2015; Picard-Jean et al. 2018; Galloway and Cowling 2019; Drazkowska et al. 2022). Furthermore, appending poly(A) tails to the 3' end of the mRNA also protects it from exonuclease degradation (Kühn and Wahle 2004; Kühn et al. 2009; Fang et al. 2022).

The second major challenge was the high immunogenicity of mRNA. When produced without certain chemical modifications, mRNA molecules have the natural ability to trigger an immune response, leading to an unintended activation of the innate immune response. Reducing mRNA immunogenicity is vital to ensure the mRNA vaccine is efficiently translated into antigen proteins without causing excessive inflammation.

Generating mRNAs without those specific chemical modifications increases the likelihood of them being perceived as foreign mRNAs by the cells. Foreign mRNAs, such as viral RNAs, are typically detected by the cell's pattern recognition receptors (PRRs)—such as the TLR3, TLR7, and TLR8 (Karikó et al. 2004; Karikó et al. 2005)—and cytoplasmic RNA sensors—such as RIG-I and MDA5 (Tatematsu et al. 2018; Liu and Wang 2022). Several mRNA traits that might prompt detection include the absence of a 5'cap 1 structure, the presence of dsRNA structures, secondary RNA structures, unmodified nucleotides, and ample uridine sequence (Tatematsu et al. 2018). Since these features are often found in viral RNAs, detecting one, several, or all traits signals the cell of a potential viral invasion. Therefore, when a cell strongly detects the mRNA vaccine, it activates the innate antiviral response, triggering the IFIT, PKR, and OAS/RNAse L protein complex to degrade the mRNA vaccine prematurely and prevent antigen protein translation.

To address this challenge, the work of Katalin Karikó and Drew Weissman's team has been pivotal. They have demonstrated the importance of incorporating modified nucleotides, particularly the N1-methyl pseudouridine (m1Ψ) or pseudouridine (Ψ), in evading or significantly reducing immune detection by all PRRs and cytoplasmic RNA sensors (Karikó et al. 2008; Anderson et al. 2010; Anderson et al. 2011; Andries et al. 2015). This approach decreases the

mRNA's visibility to immune surveillance, effectively reducing its immunogenicity while maintaining sufficient self-adjuvant properties for effective vaccine performance. Additionally, this strategy enhances mRNA stability and translation efficiency, mimicking the properties of naturally occurring eukaryotic mRNAs.

To further enhance translation efficiency, Katalin Karikó and Drew Weissman discovered that purifying synthetic mRNA post-IVT using high-performance liquid chromatography (HPLC) effectively removes RNA contaminants generated during the IVT process by phage RNA polymerases. These contaminants include dsRNA, short RNAs from abortive transcription initiation, and random, short RNA products resulting from the RNA polymerase's RNA-dependent RNA polymerase activity. These impurities can trigger innate antiviral responses, leading to the unwanted production of interferons (IFNs) and inflammatory cytokines upon mRNA transfection. By eliminating these contaminants, the undesired immune response is reduced, allowing modified nucleotide mRNAs to achieve translation levels 10-1,000 times higher in primary cells, such as murine and human DCs and primary keratinocytes (Karikó et al. 2011).

VARIOUS STRATEGIES TO DEVELOP VIRAL MRNA VACCINES

There are many approaches to developing a viral mRNA vaccine. In terms of the use of modified nucleotides, while some opted to replace uridine with m1Ψ or Ψ—like Pfizer/BioNTech's BNT162b2 and Moderna's mRNA-1273—others chose not to. An example of the latter approach is CureVac's SARS-CoV-2 mRNA candidate vaccine, the CVnCoV. The BNT162b2 and mRNA-1273 vaccines showed the efficacy of 95.0% and 94.1%, respectively (Fang et al. 2022; Polack et al. 2020), while the CVnCoV demonstrated an efficacy of 48.2% against symptomatic COVID-19 in the entire study group, with a slightly higher efficacy of 52.5% observed among participants aged 18–60 years (Kremsner et al. 2022; Hein et al. 2022). When it came to preventing moderate to severe cases, the CvnCoV vaccine performed better, with an efficacy of about 70.7% overall and 77.2% for individuals aged 18-60 (Kremsner et al. 2022; Hein et al. 2022). Still, when comparing the efficacy of modified and unmodified mRNA vaccines, the use of modified nucleotides is preferred, as demostrated by the work of Karikó and Drew Weissman's team.

In terms of cell target, both the BNT162b2 and mRNA-1273 vaccines were initially designed to target primary cells near the injection site, like muscle cells, and particularly the most important APC: the local DCs (Fang et al. 2022). However, the LNPs delivering the mRNAs were not explicitly designed to facilitate endocytosis and endosomal escape selectively only to the muscle cells and DCs. Thus, other cells throughout the body can also internalise the vaccine. Should the SARS-CoV-2's S-protein only function as a surface protein, there might not be any problem. Unfortunately, this condition produced a potential safety issue to the vaccine. As opposed to this strategy, many scientists have been researching ways to deliver the mRNA cargo in a more discerning way, so that only the target cells would internalise and be affected by the vaccine. We will further discuss this below.

Another thing to consider is the format or platform of the mRNA, which also impacts mRNA stability and translation efficiency. Currently, there are 4 mRNA formats available: the conventional, non-replicating mRNA, the self-amplifying mRNA (saRNA) (Bloom et al. 2021), the trans-amplifying mRNA (taRNA), and the circular mRNA (circRNA) (Fang et al. 2022). The conventional mRNA has the typical mRNA components from the 5'cap structure to the 3' poly(A) tail. When scientists use modifidied nucleotides such as m1Ψ or Ψ, the mRNA is called nucleoside-modified mRNA (modRNA). When scientists optimise the mRNA sequences and the uridine contents to produce a stable and viable vaccine, the mRNA is called uridine-optimised mRNA (uRNA). Some scientists also use unmodified mRNAs (i.e. CureVac's CVnCoV). From Katie McCormik and colleagues' research, it was found that the protein expression of modified mRNAs, even in similar doses, far surpassed the protein expression of unmodified mRNAs. Although this research was conducted in the context of mesenchimal

stem cell (MSC) tissue engineering, the information obtained was noteworthy nonetheless (McCormick et al. 2024). The saRNA is an mRNA that has the ability to produce the GOI and replicate itself within the cell, supposedly leading to higher protein expression from smaller doses. The saRNA contains all the components of a typical mRNA from the 5'cap structure to the 3' poly(A) tail with the addition of the RNA-dependent RNA polymerase (RdRp) enzyme from alphaviruses before the GOI. The RdRp is the protein that enables RNA replication from an RNA template. As more mRNAs are produced within then cell, and each mRNA is translated, more protein antigen should be produced with lower initial saRNA vaccine dose. Note that since saRNA also produces viral proteins (RdRp), its immunogenicity is elevated, potentially increasing its immune response (Zhou et al. 2023). The taRNA is essentially a package of two conventional mRNAs; the first mRNA codes for the GOI while the second codes for the RdRp. The circRNA is an mRNA with a circular form, as opposed to all the aforementioned linier mRNA formats (Fang et al. 2022). The circRNA forms a closed loop connecting the mRNA's 5' and 3' ends. This form enhances its stability by resisting exonuclease degradation. For translation, this format utilises a cap-independent translation initiation mechanisms like viral IRES, m6A modifications or other elements like translational enhancer element (TEE) and cap-independent translation enhancers (CITE) described in reference (Deviatkin et al. 2023). This extends its cellular lifespan and increases protein output.

While researchers and pharmaceutical companies can combine any of these approaches to produce high-performing and effective mRNA viral vaccines, two crucial aspects of the vaccine are safety and translation efficiency.

ONGOING KEY CHALLENGES

MRNA Viral Vaccine Potential Safety Issues

The development of mRNA vaccines for SARS-CoV-2 marked a breakthrough in vaccine technology, providing a rapid and adaptable platform to combat the global pandemic, saving countless lives. Despite their best efforts to generate life-saving, effective, and safe mRNA viral vaccines, there have been some concerns regarding safety issues related to these vaccines. The first of these is their rare adverse events**.** The BNT162b2 and mRNA-1273 vaccines have been known to cause an extremely rare adverse event called myocarditis and/or pericarditis shortly after vaccination (Husby and Køber 2022).

The first myocarditis cases were reported in April 2021, primarily involving young men who developed myocarditis shortly after receiving the BNT162b2 vaccine (Husby and Køber 2022). Since then, observational studies from various regions, including Asia, Europe, and North America, have noted a short-term increase in myocarditis cases linked to mRNA vaccines (Husby and Køber 2022). Hui-Lee Wong and colleagues studied 15,148,369 individuals aged 18-64 in the U.S. who had received either Pfizer or Moderna vaccines between December 2020 and December 2021. Out of all those people, Wong and his team identified 411 cases of myocarditis or pericarditis in individuals aged 18-64 a week following any dose of the mRNA vaccines. The myocarditis or pericarditis case occurred the highest in individuals aged 18-25 (33-42%), mostly in men (58-78%), and mostly in people without prior COVID-19 diagnosis since 1 April 2020 (87-94%) (Wong et al. 2022).

In particular, they discovered that men aged 18–25 after their second dose of any COVID-19 mRNA vaccine were more prone to developing myocarditis and/or pericarditis a week post-vaccination. Note that out of the 411 cases from 15,148,369 mRNA vaccinated people (0.0027%), most of them were people without prior COVID-19 diagnosis. Fortunately, supporting the findings of previous studies, their research demonstrated that the association between mRNA vaccination and developing myocarditis or pericarditis was short-term (Wong et al. 2022). Symptoms of mRNA vaccine-induced myocarditis tend to be mild to moderate, including chest pain, fatigue, and shortness of breath. Although hospitalisation and monitoring is required, the majority of individuals recover within weeks. Symptoms typically resolve with supportive care. Most

patients recover fully without significant long-term effects (Ammirati and Cooper 2022).

In an effort to confirm the safety of SARS-CoV-2 mRNA vaccines, Hibino and colleagues observed whether the mRNA vaccine increased or decreased immune-related adverse events (irAEs) in lung cancer patients—immunocompromised individuals—undergoing immune therapies post-vaccination (Hibino et al. 2022). Of the 126 lung cancer patients involved in the study, 26 patients developed irAEs pre-vaccination, and only seven patients developed irAEs post-vaccination. No patients showed worsening of preexisting irAEs following vaccination. Interestingly, Spiliopoulou and colleagues in 2022 also conducted research to elucidate the safety and efficacy of the SARS-CoV-2 mRNA vaccine on cancer patients. Similarly, they reported that the mRNA vaccine did not exacerbate preexisting irAEs in the patients. However, they did note a significant increase in five autoantibodies post-vaccination, including the IgG autoantibody against α-cardiac myosin heavy chain 6 (MHC-α, MYH6), providing insights into myocarditis pathophysiology from vaccination and natural infection. Nevertheless, clinical findings did not align with the rise in these five autoantibodies, particularly IgG autoantibody MHY6 (Spiliopoulou et al. 2023).

An extremely intriguing finding was observed when Mikel Urroz Elizalde and colleagues' research compared the risk of developing myocarditis and pericarditis in unvaccinated COVID-19 patients and mRNA-vaccinated COVID-19 patients. Out of 157 patients involved in the study, 18 unvaccinated COVID-19 patients developed pericarditis or myocarditis, and 20 mRNA-vaccinated COVID-19 patients developed pericarditis or myocarditis. Of the 20 patients, 14 had pericarditis, and 6 had myocarditis. Of the 20, only two were women with the condition post-vaccination, while the rest were young men ranging from 13-40 years old. Although the study's result was similar to that of Hui Lee-Wong and colleagues, that the incidence of myocarditis and/or pericarditis post-mRNA vaccination was higher in young men, Elizalde's results seemed to show that the risk of developing a myocarditis and/or pericarditis post-mRNA vaccination or due to COVID-19 infection was roughly similar. Nevertheless, myocarditis cases resulting from COVID-19 infection were associated with a significantly older median age, extended hospital stays, higher severity, and increased mortality rates compared to post-vaccination (Elizalde et al. 2024).

These recent studies show that the question of "whether or not the COVID-19 mRNA vaccine causes myocarditis or pericarditis" has not yet reached a solid conclusion. We have many studies reporting that the use of the BNT162b2 and/or the mRNA-1273 may cause myocarditis and/or pericarditis soon after vaccination, but only at exceedingly low rates. Elizalde and colleagues' research also demonstrated that COVID-19 infection and mRNA vaccine could cause myocarditis or pericarditis with similar risks, despite the former causing higher severity and mortality rates. Hence, one should question whether the problem lies with the mRNA technology format—including each of the mRNA's components—, the GOI itself, or both. Suppose the issue lies with the mRNA format. In that case, this rare adverse event will and should be a recurring issue with other mRNA vaccines developed for other diseases, especially those developed from the same mRNA sequence template to the BNT162b2 and mRNA-1273. If the problem lies with the GOI, more research should be done to unearth other mysterious properties of the SARS-CoV-2's S protein.

In line with that, additional information regarding the traits of the S protein has emerged. An example is the SARS-CoV-2 S protein's ability to present estradiol-like effects when given to MCF-7 cancer cells. Oscar Solis and colleagues' research discovered that the S protein had a strong binding affinity to estrogen receptor α (ERα). Akin to estradiol (E2), adding the S protein to MCF-7 cells induced cancer cell proliferation and formation. They also discovered that the addition of S protein to MCF-7 cells and Calu-3 cells considerably increased ACE2 membrane protein expression (Solis et al. 2022).

Recent research has raised the possibility that SARS-CoV-2 may possess oncogenic potential. Certain viral proteins,

including Nsp3 and Nsp15, have been shown to promote the degradation of key tumor suppressor proteins, such as p53 and pRB, respectively (Gómez-Carballa et al., 2022; Costanzo et al., 2023). This degradation is crucial, as p53 and pRB are involved in controlling cell cycle regulation and preventing tumor growth. In addition, Zhang and El-Deiry's study revealed that the SARS-CoV-2 spike (S) protein may disrupt chemotherapy efficacy by reducing p53 activity in cancer cells. Specifically, in cancer cell lines like MCF-7, H460, and HCT116, the spike protein diminished the expression of p53 targets such as p21 and DR5, which are essential for DNA repair and apoptosis. This resulted in reduced DNA damage response markers, such as γ-H2AX, even under chemotherapy treatment, suggesting that the spike protein may contribute to chemotherapy resistance (Zhang & El-Deiry, 2024). Moreover, the ORF8 protein directly binds to major histocompatibility complex class I (MHC-I) molecules, leading to their downregulation. This could reduce the effectiveness of cytotoxic T cells in killing infected or cancerous cells (Costanzo et al., 2023). Such findings suggest that SARS-CoV-2 proteins may influence tumor progression, immune evasion, and even chemotherapy sensitivity. Further research by Gibo et al. (2024) observed a rise in cancer deaths among elderly individuals in Japan, potentially linked to the booster mRNA vaccines, especially in cancers sensitive to estrogen and estrogen receptor alpha (ERα). This highlights the need to better understand the interplay between the SARS-CoV-2 spike protein and cancer biology, particularly in hormone-sensitive cancers. These emerging findings point to a possible link between SARS-CoV-2 infection and oncogenic pathways, warranting further investigation to clarify the virus's role in tumorigenesis and cancer progression.

Additionally, George and Victor Tetz identified prion-like domains (PrLDs) in the receptor-binding domain (RBD) of the S1 region of the SARS-CoV-2 spike protein, which has been associated with sporadic Creutzfeldt-Jakob Disease post-vaccination (Tetz and Tetz 2022; Kuvandık et al. 2022; Doğru and Kehaya 2022). While we cannot definitively rule out the possibility that the mRNA format itself may contribute to safety concerns, evidence suggests that the spike protein, as the GOI, could also play a role in these issues. As the S protein has demonstrated a strong affinity for estrogen receptor alpha (ERα) and contains PrLDs, this raises the need for pre-testing the binding properties of candidate GOIs with extensive protein arrays before clinical use. In viral mRNA vaccines, the GOI typically encodes surface proteins that are responsible for receptor recognition and viral fusion. If a viral protein is found to have an unintended strong affinity toward non-target receptors, this information could guide modifications through protein engineering. For instance, in the case of the SARS-CoV-2 S protein, the vaccine might be safer if the PrLDs are removed or reduced within the GOI sequence. The goal of this approach would be to retain the protective immunogenic effects of the vaccine while mitigating potential risks, such as oncogenic or prion-like activities.

That said, we must emphasize that both the GOI and the mRNA format could contribute to mRNA safety concerns, and further research is necessary to better understand their respective roles. Only with more comprehensive studies can we draw definitive conclusions and address these safety considerations effectively.

A second safe concern regarding mRNA vaccines is the non-specific targeting of LNPs, which may lead to uptake by various cell types. In studies on BALB/c mice, the mRNA-LNP complex was found to predominantly accumulate in organs such as the ovaries, liver, spleen, and adrenal glands (Pateev et al. 2023). However, it is essential to note that only limited data exists on the biodistribution of mRNA vaccines in humans, and findings from animal models cannot be directly extrapolated to humans without further investigation. Should similar patterns of LNP-mRNA distribution occur in humans, there may be implications for where the S protein is expressed in abundance, potentially linking to outcomes such as those observed in cancer patients in the study by Gibo and colleagues. More research is needed to determine whether this distribution occurs in humans and what its implications may be.

Increasing the specificity of the drug-delivery mechanism is required to solve the non-specific targeting of the LNP in existing mRNA vaccines. Evidently, BioNTech's approach to advancing their mRNA vaccine technology, be it for viral vaccine or cancer vaccine, is to specifically target the mRNA uptake into DCs (Kranz et al. 2016)**.** We agree with this approach to increase the mRNA vaccine's specificity for two reasons. Firstly, it directly focuses the mRNA uptake to the strongest APC: the DCs. Secondly, improving the specificity of the mRNA vaccine limits the number of impacted cells, even if unwanted inflammation occurs.

Specifically targeting the APCs, particularly the DCs, can be achieved by incorporating specific ligand proteins, such as DC-SIGN and CLRs (C-type Lectin Receptor), into the LNP structure, allowing only cells with corresponding receptors to internalise the mRNA-LNP complex (Clemente et al. 2023). Alternatively, manipulating nanoparticles' charge and particle size—such as those ranging from 200-500 nm with a strong negative charge—causes the vaccine to primarily accumulate in the spleen, targeting the splenic DCs (Sasaki et al. 2022). Further developments in biocompatible and less toxic cationic lipids and ionisable cationic lipids will also enhance mRNA vaccine safety.

Increasing The Translation Efficiency of Viral MRNA Vaccine

In addition to safety concerns, another critical challenge for mRNA vaccines is improving its translation efficiency. Translation efficiency is crucial because it dictates the rate and success of mRNA-to-protein conversion in cells. Improving this aspect can significantly boost protein production, enhance vaccine efficacy, lower dosage requirements, improve safety, minimise side effects, extend the duration of immune protection, and enhance cost-effectiveness (Rosa et al., 2021; Fang et al., 2022).

Several strategies can enhance the translation efficiency of mRNA vaccines. One effective approach is optimising the 5' UTR, which is critical for initiating translation. For example, the BNT162b2 vaccine uses an optimised 5' UTR from the human α-globin gene (Xia, 2021; Fang et al., 2022). Another strategy involves incorporating the 5' UTR from RNA viruses that contain at least one internal ribosome entry site (IRES) domains. The IRES domain is a part of the RNA virus' 5'UTR that enables translation initiation without the need for a 5' cap structure by directly recruiting eukaryotic translation initiation factors (eIFs), ribosomal subunits, and IRES trans-acting factors (ITAFs) (Martinez-Salas et al., 2018; Mailliot & Martin, 2018).

This mechanism is facilitated by the complex secondary and/or tertiary structures of the viral RNA's IRES domain, which contain specific motifs that attract these proteins. Interestingly, aside from the motifs, the IRES domain of the RNA viruses does not truly have a conserved RNA sequence. Instead, what is conserved is the shape of the secondary and/or tertiary RNA structures, which aids in the recruitment and docking of the eIFs, ribosomal units, and ITAFs for a successful translation initiation process. (Lozano & Martínez-Salas, 2015; Martinez-Salas et al., 2018; Mailliot & Martin, 2018).

The structural similarities of viral IRES elements serve as the basis for classifying them into four distinct types: Type I (poliovirus), Type II (encephalomyocarditis virus and foot-and-mouth disease virus), Type III (hepatitis C virus), and Type IV (cricket paralysis virus). For further details on viral IRES classifications dan domain functions, please refer to the following sources: Lozano & Martínez-Salas, 2015; Martinez-Salas et al., 2018; Mailliot & Martin, 2018. Figures 1 and 2 illustrate the complexity of two RNA viruses containing an IRES domain. Note that not all RNA viruses have an IRES domain and some even have more than one.

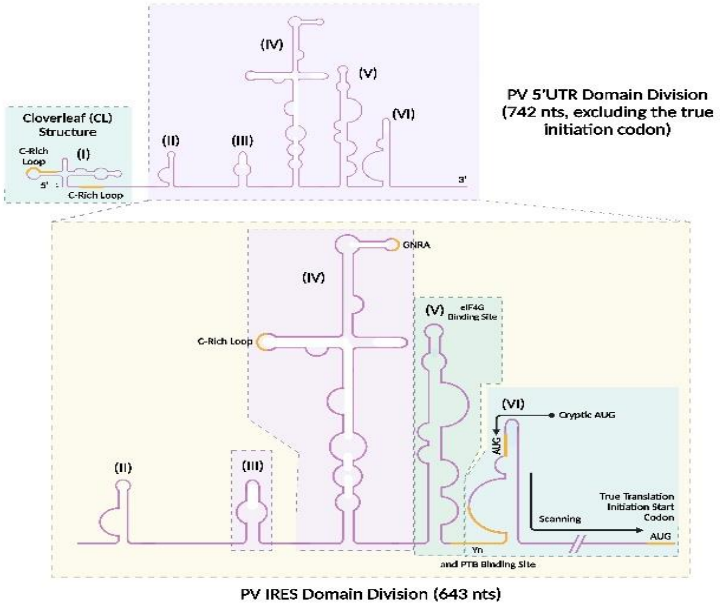

Figure 1. Poliovirus (PV) 5'UTR and IRES Domain and Their Divisions (Created with BioRender.com)

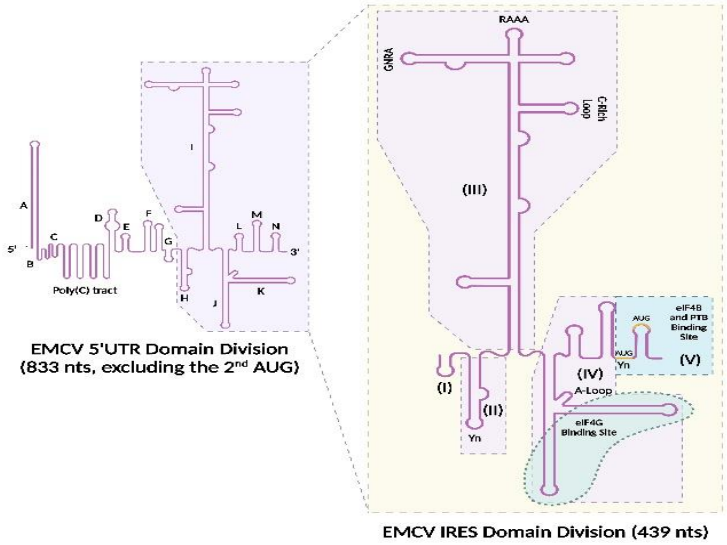

Figure 2. Encephalomyocarditis virus (EMCV) 5'UTR and IRES Domain and Their Divisions (Created with BioRender.com)

In addition to aiding translation initiation, certain ITAF proteins help maintain the integrity of the IRES structure and protect viral RNA from 5' exonuclease degradation. For example, in poliovirus (PV), the poly(rC)-binding protein (PCBP) binds to the C-rich loop and C-rich region of the cloverleaf structure (Figure 1), preventing mRNA degradation (Lloyd, 2015). Another ITAF, the polypyrimidine tract-binding protein (PTB, also known as hnRNP I), binds to domains V and VI (Yn region) of PV's IRES, helping preserve the IRES structure's shape. Similarly, these ITAF proteins also bind to the C-rich loop and polypyrimidine (Yn) region in the IRES of EMCV with relatable functions (Figure 2) (Lozano & Martínez-Salas, 2015; Martinez-Salas et al., 2018; Mailliot & Martin, 2018).

Furthermore, some viral RNAs have 5' UTRs containing complex secondary or tertiary structures strong enough to protect it from 5' exonuclease degradation, even in the absence of ITAF proteins (Akiyama et al., 2016; Schult et al., 2018). Studies have shown that cap-independent viral 5' UTR-IRES mRNAs, particularly those from encephalomyocarditis virus (EMCV) and cricket paralysis virus (CrPV), can sometimes outperform traditional capped mRNAs

in terms of translation efficiency when transfected into compatible cells, such as HEK 293T, A204, and DCs (Tan & Wan, 2008; Ko et al., 2019). However, these results can vary depending on the specific cell type and the viral IRES used.

Another promising way to enhance the translation efficiency of mRNA vaccines is to choose different mRNA platforms. In theory, both saRNA and circRNA formats should offer higher translation efficiency than conventional mRNA. Since conventional mRNA uses modified nucleotides to reduce mRNA immunogenicity, similar modifications should be applied when using saRNA or circRNA formats. Because the modified nucleotides reduce the mRNA's detection by PRRs and cytoplasmic RNA sensors, the chances of successful mRNA translation are enhanced, thus improving the mRNA translation efficiency. Unfortunately, attempts to incorporate modified nucleotides like m1Ψ or Ψ into saRNA have been unsuccessful, suggesting that modNTPs are incompatible with saRNA, which hinders further development (McGee et al., 2024).

Fortunately, Joshua E. McGee and colleagues' research have identified three modified nucleotides with high compatibility for saRNA: 5-Hydroxymethylcytidine (5OHmC), 5-Methylcytidine (5mC), and 5-Methyluridine (m5U). ModsaRNAs with full substitution using these modified nucleotides showed significantly higher transfection efficiency compared to the conventional m1Ψ modRNA. In transfection efficiency, 5OHmC, 5mC, and m5U modsaRNAs demonstrated 14-fold, 10-fold, and 8-fold improvements, respectively, compared to m1Ψ modRNA. In contrast, saRNAs modified entirely with Ψ exhibited even lower transfection efficiency than m1Ψ-modified saRNA (McGee et al., 2024).

To further explore the impact of these modifications on translation efficiency, McGee and colleagues employed a luciferase-based assay to compare 5mC-modsaRNA, unmodified saRNA, and conventional m1Ψ-modmRNA. Results indicated that 5mC-modsaRNA showed a 4.9-fold higher translation efficiency than unmodified saRNA and 68-fold higher than m1Ψ-modmRNA in HEK293T cells. This improvement led to the development of a modsaRNA SARS-CoV-2 S protein vaccine, using the 5mC modification with a dose of 10 ng per mouse. When compared to a conventional modRNA SARS-CoV-2 S protein vaccine (which used m1Ψ with a dose of 1 µg per mouse), the modsaRNA vaccine provided comparable protection against lethal SARS-CoV-2 challenges in C57BL/6 mice, despite the significantly lower dosage (McGee et al., 2024).

Interestingly, in mesenchymal stem cell (MSC) transfection studies related to tissue engineering, modRNA was found to be 5.6 times more efficient than unmodified saRNA. One potential explanation is the use of vectors that are optimised for non-replicating mRNA but not for saRNA translation (McCormick et al., 2024). This highlights the need for further research to develop vectors specifically tailored for saRNA. Additionally, the lack of modified nucleosides in the saRNA transcription could have contributed to the disparity, suggesting that applying modified nucleosides to saRNA may enhance its translation efficiency in future studies.

CONCLUSION

This review highlights the significant potential of developing a highly effective and safe mRNA viral vaccine. Despite emerging safety concerns with existing SARS-CoV-2 mRNA vaccines, there is room for refining the mRNA vaccine technology before future pandemics, particularly in the safety and translation efficiency aspect. To refine the technology, three key steps are proposed for future mRNA vaccines.

First, it is essential to investigate whether vaccine-induced myocarditis and/or pericarditis are caused by the mRNA platform itself, the specific GOI used in the vaccine, or both. To enhance safety from the GOI's side, we suggest pre-testing the binding properties of candidate GOIs using comprehensive protein arrays before they are used for clinical purposes. This approach aims to preserve the vaccine's protective immune response while reducing potential risks, such as oncogenic or prion-like effects and rare adverse events like vaccine-induced myocarditis or pericarditis.

Second, improving the targeting of LNPs to APCs, especially the DCs, can enhance vaccine safety. This can be done by adding specific proteins, like DC-SIGN and CLRs, to the LNPs and adjusting their charge and size. For example, maintaining a strong negative charge and a particle size between 200-500 nm helps the vaccine accumulate in the spleen, where it can effectively target splenic DCs.

Third, boosting translation efficiency can improve vaccine safety and effectiveness. This could allow for lower doses while still offering strong protection and reducing side effects. One approach is replacing the 5'UTR with viral IRES, which have been shown to outperform traditional capped mRNA in certain cell types, including in DCs. Additionally, alternative mRNA platforms, such as saRNA and circRNA, offer potential improvements. Recent studies have identified compatible modified nucleotides for the saRNA platform, potentially enabling similar protection at significantly lower doses.

In summary, this review calls for further research to refine and perfect the mRNA viral vaccine technology, enhancing safety and effectiveness for a safer tomorrow.

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