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The Genetic Contribution to Vaccine Development: Insights from mRNA Technologies-An Updated Review.

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Abstract:

Background: Vaccination remains the most effective tool in preventing infectious diseases. mRNA vaccines represent a groundbreaking development in vaccine technology, offering several advantages over traditional vaccine types. These advantages include safety, scalability, and rapid production, addressing the key challenge of time delays in vaccine development during outbreaks. mRNA vaccines work by encoding the target antigen, stimulating both humoral and cellular immune responses without integrating into the host genome, providing a promising approach for future vaccine development.

Aim: This review aims to explore the genetic contributions to vaccine development, focusing on mRNA technologies and their evolution, particularly in the context of COVID-19. It examines the history of mRNA vaccine development, the types of RNA-based vaccines, their mechanisms, and the challenges in enhancing mRNA stability for better efficacy.

Methods: A thorough review of scientific literature was conducted to trace the development of mRNA vaccines, from early research in the 1980s to their application in modern viral pandemics. This work was carried out by a specialized team from the Jazan Health Cluster, Kingdom of Saudi Arabia, demonstrating a commitment to advancing the understanding of this critical technology. Key findings in the areas of mRNA stability, immune responses, and delivery methods were discussed, alongside significant advances in enhancing vaccine effectiveness.

Results: Significant advancements have been made in the stability of mRNA and the efficiency of its delivery, especially with the modification of nucleotides and improvements in mRNA capping techniques. mRNA vaccines, such as those for COVID-19, have proven to be highly effective, with rapid production timelines and high immunogenicity. However, challenges remain in addressing the instability of mRNA and the need for better delivery systems.

Conclusion: mRNA vaccines have revolutionized vaccine development by offering a rapid, scalable, and flexible platform. While challenges in stability and delivery remain, ongoing research into improving these areas will make mRNA vaccines a cornerstone of future vaccine strategies, potentially combating a wide range of infectious diseases.

Keywords: mRNA vaccines, vaccine development, genetic contribution, COVID-19, vaccine technology, stability, immune response, mRNA capping.

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Introduction:

The best defense against infectious diseases is still vaccination. mRNA-based vaccines, which have a number of important advantages over conventional vaccine types, provide a revolutionary method to vaccine development. First off, because mRNA vaccines only encode the target antigen, they are safe and do not carry the same danger of infection as attenuated or inactivated vaccinations. Additionally, less strain is placed on the immunological system. Second, the possibility of mRNA integration into the host genome is reduced since mRNA vaccines work by expressing genetic information only in the cytoplasm, avoiding the necessity for nuclear entrance. Furthermore, mRNA vaccines have the same ability to elicit humoral and cellular immune responses as DNA vaccines. Moreover, mRNA is a minimum genetic vector that stops antivector immune response development, enabling mRNA vaccines to be used again. Crucially, mRNA is quickly removed from the body, and by altering structural components and using different delivery techniques, its half-life can be controlled [1]. Manufacturing mRNA-based vaccines is not only quick and affordable, but also scalable and rather simple thanks to a well-established production infrastructure. The mRNA vaccine production platform makes it simple to swap out target genes without changing the manufacturing procedure. These benefits tackle a crucial problem in the prevention of viral diseases: the time lag between the start of an outbreak and the release of a vaccine.

History of mRNA Vaccines

Krieg, Melton, Maniatis, and Green used T7 RNA polymerase to create physiologically active mRNA, a technique that is still in use today, and published the first reports of biologically active mRNA creation in 1984. Notably, Krieg et al. showed in a 1984 study that when injected into frog egg cells, the mRNA may behave similarly to naturally occurring mRNA [2]. Melton discovered in 1987 that the mRNA had the ability to simultaneously stimulate and prevent the creation of proteins. At first, neither Melton nor Krieg saw synthetic mRNA as a viable platform for vaccine development, but rather as a research tool for examining gene function. Wolff et al. showed in 1990 that animals could express synthetic mRNA. They confirmed the presence of the associated protein products by injecting mice with mRNAs encoding luciferase, β-galactosidase, and chloramphenicol acetyl transferase. A 1992 follow-up study provided more evidence that introducing vasopressin-coding mRNA into rats' hypothalamus caused physiological alterations [3][4]. After these discoveries, the focus of the study switched to examining the potential of synthetic mRNAs as preventative and therapeutic agents. Nevertheless, a number of issues surfaced, such as mRNA's immunostimulatory characteristics, physical instability, and limitations with cellular absorption [5]. Due to its easier in vitro manufacturing and higher natural stability, DNA became the preferred option for many researchers. Notwithstanding its benefits, DNA has drawbacks, including decreased immunogenicity and the possibility of incorporation into the host genome. Consequently, mRNA vaccination research proceeded [6, 7].

Direct mRNA injection was shown to elicit an immunological response in mice in 2000 by Hoerr et al. [8]. Hoerr established CureVac, which carried out the first mRNA experiments on humans, in the same year. Steve Pascolo, the company's chief scientist, was the first to give an mRNA vaccination to himself [9]. Katalin Kariko made a significant contribution to the creation of mRNA vaccines. Kariko co-authored a report on the development of an mRNA-based HIV-1 vaccine with Drew Weissman in 1999. When synthesized mRNA was first introduced into mice, it caused significant inflammatory reactions [10]. Toll-like receptors, important immunological sensors that react to foreign RNA, were activated by synthesized mRNA, which was found to be the root cause [11]. Kariko and coworkers found in 2005 that replacing uridine with the uridine analog pseudouridine reduced inflammation by preventing the immune system from identifying mRNA as foreign [12]. The scientific community quickly recognized the medicinal potential of modified nucleotides, despite the fact that it was not well known at the time [13].

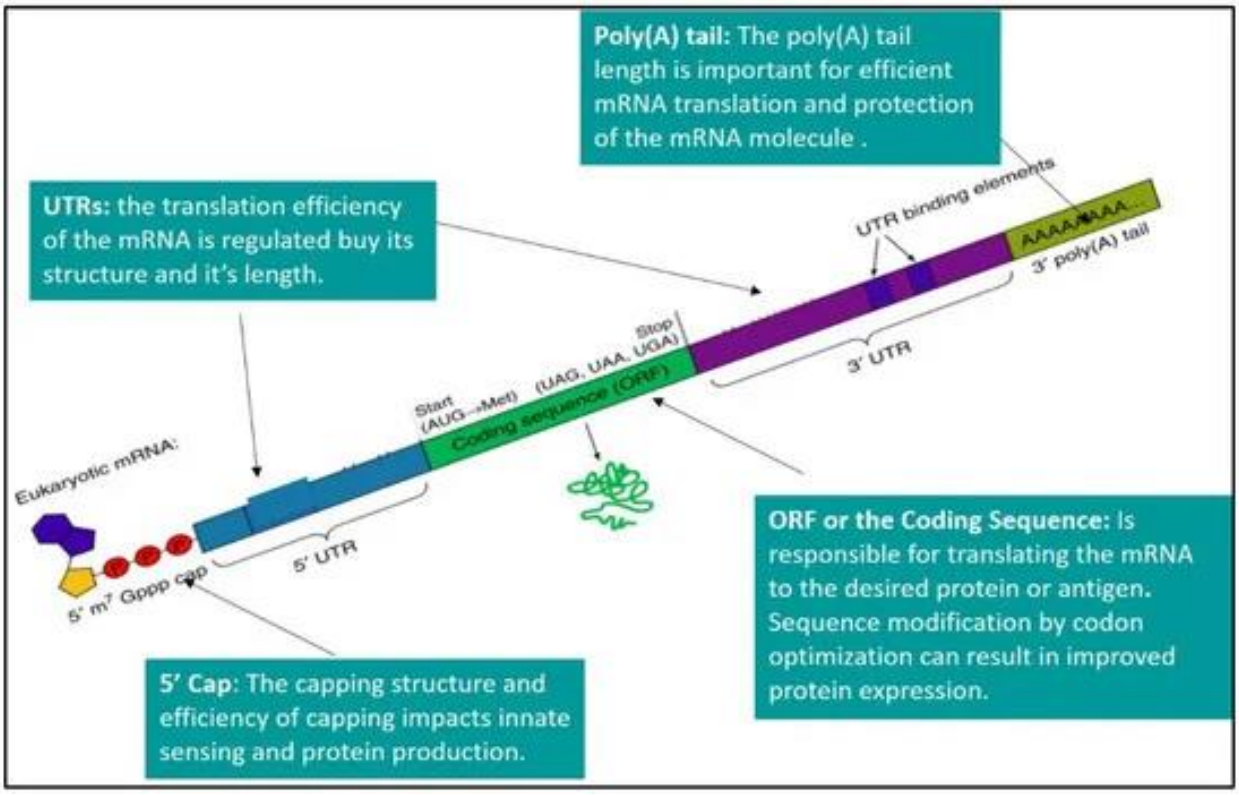

Figure 1: mRNA Components Structure.

By 2008, big pharmaceutical firms like Novartis and Shire had set up mRNA research departments, with Shire concentrating on treatments and Novartis on vaccines. Additionally, at this time, businesses like BioNTech and Moderna emerged and started creating mRNA-based technology. Targeting a variety of infectious disorders, such as those brought on by the coronavirus, respiratory syncytial virus, human metapneumovirus, cytomegalovirus, influenza virus, Epstein-Barr virus, HIV, Zika virus, and Nipah virus, Moderna had created nine mRNA vaccine candidates by 2019. Likewise, BioNTech had created a similar line of vaccines, although none of them had received a license at that point [14]. The early 2020 global COVID-19 pandemic greatly sped up the use of mRNA vaccinations in practice. For instance, Moderna Inc. and the National Institute of Allergy and Infectious Diseases (NIAID) worked together to develop the mRNA-1273 prototype vaccine against SARS-CoV-2 in a record 63 days, from choosing the viral nucleotide sequence to starting clinical trials. In a comparable period, BioNTech and Pfizer collaborated to create an mRNA-based COVID-19 vaccine [15]. Decades of study have significantly improved the stability of mRNA and the effectiveness of its distribution. The immunogenicity of mRNA vaccines can be significantly increased by combining different structural alterations and delivery systems, making this strategy a viable option for vaccine development in the future.

Types of RNA-Based Vaccines

RNA vaccines are now divided into two main categories: self-amplifying RNA vaccines and non-replicating mRNA vaccines. An mRNA molecule created by in vitro transcription using plasmid DNA encoding the target immunogene as a template makes up non-replicating mRNA vaccines. The mRNA molecule contains a poly-A tail at the 3' end, a 5' cap, and untranslated regions (UTRs) in addition to the coding sequence. These components are necessary for effective translation, defense against exonucleolytic destruction, and appropriate splicing of the transcript. Notably, non-replicating mRNA served as the basis for the first SARS-CoV-2 vaccinations that were authorized for use in humans [16, 17]. Nevertheless, issues with mRNA instability and inadequate delivery methods remain unresolved. Self-amplifying RNA (saRNA) vaccines are based on replicons, which include portions of the viral genome that aid in the replication of the target RNA in addition to the gene encoding the target immunogene. DNA or RNA viruses can penetrate

the nucleus and usually reproduce their genetic material in the cytoplasm. Some viruses replicate cytosolically without entering the nucleus [18]. In contrast to non-replicating mRNA, the encoded target immunogene self-amplifies, increasing the production of the immunogenic protein, but the resultant saRNA replicons are unable to generate infectious virus particles. At lower dosages, saRNA vaccines can attain comparable immunogenicity to non-replicating mRNA vaccines due to the prolonged immunogene synthesis. Single-stranded, positive-sense alphaviruses including the Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus are the source of the majority of saRNAs [20, 21]. Nonetheless, a number of issues still surround the use of viral vectors in vaccine production, such as the vector's immunogenicity, which may cause unintended immune reactions and conflict with booster shots in the future. Furthermore, replicating alphaviral vectors have the same risk of viral reactivation as live attenuated vaccines [22–24].

Enhancing mRNA Stability

Five essential components make up eukaryotic mRNA: a 5' cap (m7GpppN, where N can be any nucleotide), a 5' untranslated region (5'-UTR), the open reading frame (ORF), a 3' untranslated region (3'-UTR), and a 3' poly-A tail, which has a length that varies depending on the type of cell and contains 100–250 adenyl residues [25]. The hydroxyl group on the ribose sugar's 2'-carbon, which promotes hydrolytic destruction, is mostly responsible for mRNA's instability when compared to DNA. Additionally, foreign RNA can be recognized by the innate immune system, which will cause it to degrade. A number of processes that render foreign mRNA intrinsically unstable and less immunogenic are involved in the main pathway for mRNA breakdown in eukaryotic cells. In order to improve stability and immunogenicity, certain modifications are made to mRNA components during the vaccine development process. Important structural elements of in vitro transcribed (IVT) mRNA can change. It has been demonstrated that some chemical changes, such as the use of modified 5'-cap analogs like CleanCap® and Anti-Reverse Cap Analog (ARCA), improve the cap's attachment and, consequently, the effectiveness of translation in cells. These changes are discussed in more detail below [5].

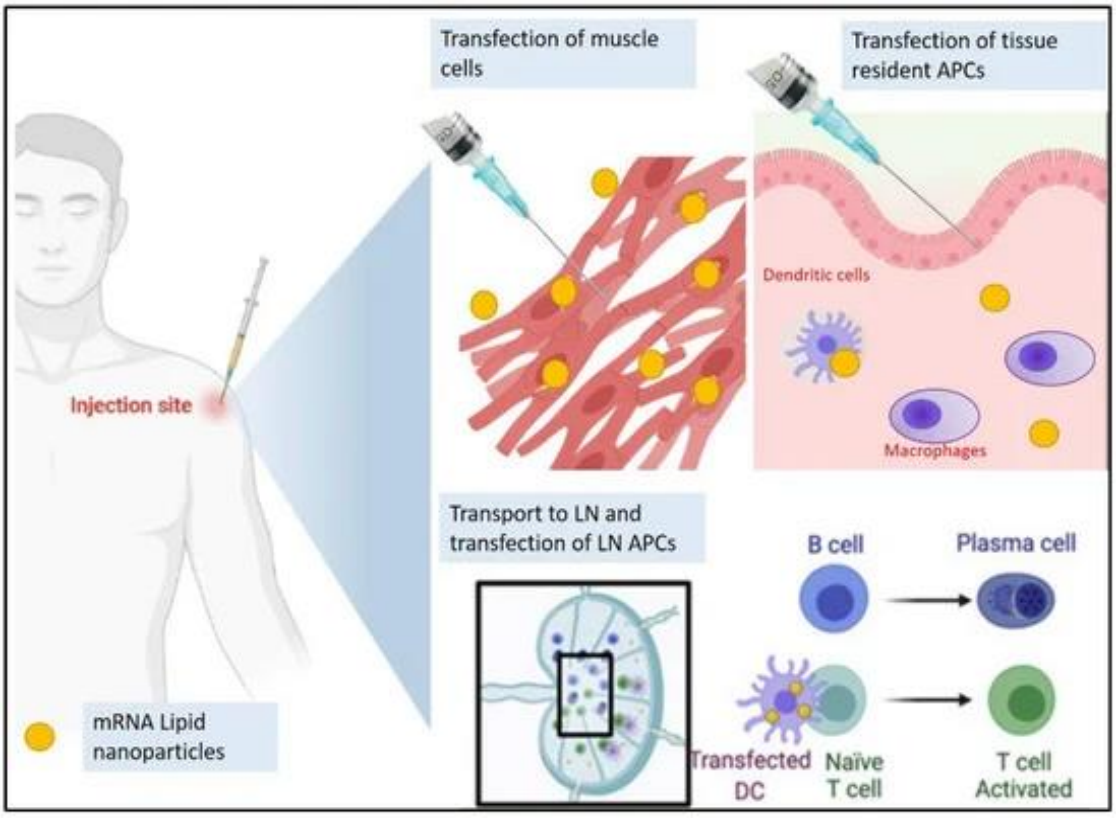

Figure 2: mRNA Lipid Delivery System for Vaccination.

Cap Structure

The m7GpppN motif, which includes the modified nucleoside N7-methylguanosine (m7G), is part of the 5'-cap structure of native eukaryotic messenger RNA (mRNA). It is connected to the first transcribed nucleotide by a 5'-5'-triphosphate bridge. In order to support mRNA stability, translation, splicing, polyadenylation, nuclear export, and defense against exonucleolytic destruction, the cap structure is essential. Three sequential enzymatic steps cause capping to occur within the nucleus during transcription of the first 20–30 nucleotides: RNA triphosphatase removes the phosphate group from the 5'-terminal nucleotide, guanyltransferase guanylates the terminal nucleotide, and guanyl-N7-methyltransferase N7-methylates the guanine to form N7-methylguanosine. By attracting crucial translation initiation proteins like eIF4E and facilitating the creation of the mRNA closed-loop structure, the cap structure plays a crucial function in translation initiation. Furthermore, mRNA nuclear export is facilitated by interactions between the cap and cap-binding proteins (CBPs) [26].

Two new 5'-cap structures, cap1 and cap2, as well as the enzymes that synthesize them, have been discovered recently. In these variations, the second or third ribonucleotide at the 2'-O-ribose site is methylated by the m7G-specific 2'-O-methyltransferase (2'-O-MTase), producing cap1 or cap2, respectively. According to research, the cap1 structure shields mRNA from cytosolic sensors like RIG-I and MDA5, which would otherwise activate the type I interferon signaling cascade and cause mRNA destruction. In comparison to cap0 mRNA, mRNA with the cap1 structure is more resistant to innate immune responses and has higher translation and protein production efficiency [27]. Notably, the lack of the cap that separates cellular mRNA from viral RNA causes the immune system to identify uncapped RNA as alien [5]. When incorrectly capped or uncapped RNA is recognized by pattern recognition receptors (PRRs) such RIG-I and IFIT, type I interferon production and RNA destruction result. Therefore, it is essential that synthetic RNA transcripts be capped in order to guarantee that synthetic RNA resembles the structure of eukaryotic mRNA. To prevent overstimulation of the innate immune system, it is essential for mRNA vaccines to achieve optimal capping efficiency, which includes filtering the finished product to remove uncapped or incorrectly capped RNA [28]. Capping can be done cotranscriptionally by adding cap analogs like ARCA and CleanCap® to the reaction mixture, or posttranscriptional by employing recombinant enzymes (such those from the smallpox virus).
Practically speaking, cotranscriptional capping minimizes the number of enzymes needed, cuts down on the steps in the mRNA manufacturing process, and lowers production costs [29]. Nevertheless, this approach has drawbacks, such as the potential for some mRNA molecules to become uncapped due to competition between cap analogs and guanosine triphosphate (GTP). An undesirable immunological reaction could be triggered by these uncapped RNAs. Triphosphates can be eliminated from the 5' end of uncapped mRNA using phosphatases to lessen this. The possible use of cap analogs in the wrong orientation, which would prohibit binding with CBPs and impede translation, presents another difficulty [30]. Chemical changes like methyl group additions at the 3'- or 2'-position of cap analogs can be made to address these problems and improve translation efficiency. This helps to prevent reverse cap orientation and improves the quality of produced mRNA. The Anti-Reverse Cap Analog (ARCA) is an example of a modified cap analog. It ensures correct cap attachment during RNA synthesis by having a methyl group at the 3'-OH of the m7G nucleotide [31]. Additionally, it has been demonstrated that other changes to the cap analogs, like the lengthened 5'-5'-phosphate bridges in ARCA, improve mRNA stability and translation efficiency. CleanCap®, a second-generation anti-reverse cap analog that can incorporate the cap1 structure into mRNA, is an example of how ARCA has been further refined. Compared to the lower yields seen with ARCA, CleanCap® increases capping efficiency and produces capped mRNA up to 95% of the time. BioNTech/Pfizer uses this second-generation cap analog to produce the BNT162b1 and BNT162b2 mRNA vaccines [33, 34].

Poly(A) Sequence at the 3'-End of mRNA: The Poly(A)-Tail

A crucial component of mRNA, the poly(A)-tail is important for mRNA stability and translation efficiency [35]. The most actively translated mRNAs in mammalian cells usually have poly(A)-tails with

100–250 adenosine residues [36]. Twenty adenosine residues are the bare minimum poly(A)-tail length required for exogenous mRNA translation [37]. By attaching itself to different polyadenyl-binding proteins (PABPs), the poly(A)-tail aids in translation. These PABPs then interact with the eukaryotic translation initiation factor 4G (eIF4G). In addition to promoting ribosome binding, this interaction aids in the development of the cap-eIF4E-eIF4G-PABP-poly(A) closed-loop structure, which shields mRNA from nuclease degradation [38]. There are two different ways to insert the poly(A)-tail into in vitro produced mRNA. One method uses poly(A)-polymerase to enzymatically add adenosine residues to the 3'-end. This process can add up to 200 adenosine residues, producing a heterogeneous product. As an alternative, a poly(A)-tail can be added by matrix synthesis, in which a DNA matrix with thymidine residues permits the addition of a poly(A)-tail with up to 120 adenosine residues because the poly(A)-sequence in the matrix is inherently unstable [39].

Noncoding Regions in mRNA

as RCCAUGG, where R stands for a purine, A, or G. The nucleotides at locations –3 and +4 in relation to the AUG start codon affect how well the start codon is recognized; ideally, the nucleotide at position +4 is G, while the nucleotide at position –3 is either A or G [5].

mRNA Coding Sequence Optimization

The open reading frame (ORF) of the mRNA's codon composition can have a major effect on both mRNA stability and translation efficiency. An ideal mRNA secondary structure can be formed, and translation elongation rates can be changed by altering the codon use inside the ORF [28]. Codon optimization techniques include using dicodons, which are pairs of codons that promote optimum translation, or replacing uncommon codons with more common substitutes for the same amino acid. Research indicates that in vitro produced mRNA can be protected from decapping enzymes by decreasing the frequency of UU and UA dinucleotides inside the ORF, improving mRNA stability and translation efficiency [41, 42]. Furthermore, it has been shown that optimizing translation can be achieved by using a codon that is similar to that of naturally occurring, highly expressed genes [5].

The overall amount of mRNA translation is increased by optimal codons close to the start codon, which tend to improve translation elongation. Rare codons, on the other hand, have the potential to impede elongation and result in ribosome accumulation on the mRNA molecule. After 5'-decapping, this ribosome stalling speeds up mRNA decay by facilitating the binding of DEAD-Box RNA helicases. As demonstrated by the decreased activity of codon-optimized firefly luciferase mRNA, a high translation elongation rate can interfere with the correct folding of the encoded protein, even though it typically increases mRNA efficiency [43]. Rare codons may be useful in these situations because they enable a slower translation rate, which is important for appropriate protein folding, particularly for target antigens that need precise folding for optimal function. As a result, the codon optimization approach ought to be customized to meet the unique needs of the target antigen. It could be most beneficial to optimize every codon for linear epitope-based mRNA vaccines. In order to promote appropriate protein folding and epitope conformation, it could be required to use techniques that reduce translation rates for more complicated antigens. In both situations, maximizing protein production requires minimizing the usage of uncommon codons [28]. As will be covered in the next section, codon optimization might also entail recoding terminal codons, such as substituting N1-methylpseudouridine or pseudouridine for U nucleotides. The main structure of the protein may be disrupted by noncanonical base pairing caused by these altered nucleotides, which can pair with any other nucleotide. For example, the tRNAGlu anticodon may recognize GAΨ, which codes for Asp, leading to a nonsynonymous replacement. Codons with terminal cytosine do not have this problem [44].

Modified Nucleosides in mRNA

It is crucial to understand that mRNA may be interpreted as alien by the body upon introduction, which could result in an unfavorable innate immune reaction. Inflammatory responses and mRNA degradation could result from this interaction. Through their interactions with cytoplasmic RNA sensors like Retinoic Acid Induced Protein I (RIG-I) and Pattern Recognition Receptors (PRRs) like Toll-like

receptors (TLRs), both DNA and RNA have been shown to activate the innate immune system in mammals. In particular, RNA chains rich in GU and AU sequences are known to activate both TLR7 and TLR8, whereas uridine residues are known to activate TLR7 [5]. There are 13 TLRs known to exist, and four of them—TLR3 for double-stranded RNA (dsRNA), TLR7 and TLR8 for U-rich single-stranded RNA, and TLR9 for the DNA CpG motif—are essential for recognizing nucleic acids. Research has demonstrated that dendritic cells (DCs) respond strongly to in vitro transcribed (IVT) mRNA preparations in terms of TNF-α [45].

It is thought that TLR-mediated dendritic cell activation is greatly decreased when modified nucleosides are added to in vitro transcription. It is still unknown how these altered nucleosides affect immunological responses that are not dependent on TLRs. By inhibiting the synthesis of dsRNA during transcription and reducing the activation of Pattern Recognition Receptors (PRRs) during mRNA administration into the body, modified nucleosides enhance the effectiveness of mRNA vaccines in two main ways [28]. Mammalian RNA has a number of modified nucleosides that can be used to reduce the undesirable immune response to the injected mRNA, including pseudouridine, N1-methylpseudouridine, 2-thiouridine, 5-methylcytidine, 6-methyladenosine, inosine, and 2'-O-methylated nucleosides [46]. Notably, the advantages of N1-methylpseudouridine are utilized by the mRNA vaccines BNT162b2 (BioNTech/Pfizer) and mRNA-1273 (Moderna) [16, 17]. CureVac uses a different strategy for developing mRNA vaccines that does not include modified nucleosides. Using its in-house mRNA synthesis technology, CureVac has created a SARS-CoV-2 vaccine that maximizes protein synthesis and attains a balanced immune activation. This strategy involves changing the poly(A)-tail, tweaking the untranslated regions (UTRs) by adding sequences from stable, highly expressed mRNAs, and improving the composition of the coding region. In particular, a "histone stem-loop" sequence was substituted for the traditional poly(A)-tail, UTRs from known stable genes were added, and the GC concentration in the coding region was increased. The resultant mRNA vaccines, CVnCoV and CV2CoV, are encased in lipid nanoparticles and encode the full-length S-protein with two proline mutations (S-2P) [47, 48]. Clinical studies, however, have shown that these vaccines' effectiveness was noticeably inferior to that of vaccines that used modified nucleosides [49].

Purification of IVT-mRNA from dsRNA Contamination

When synthetic mRNA is administered, the presence of double-stranded RNAs (dsRNAs) produced during in vitro transcription may play a major role in the activation of innate immune responses. In particular, T7 polymerase, which is frequently employed in mRNA synthesis, frequently produces dsRNA byproducts, which have the ability to activate cytosolic sensors like MDA5 and RIG-I. When the sense and antisense RNA transcripts hybridize, this dsRNA is created. At the 3' end of the DNA template, promoter-independent transcription starts, producing the antisense RNA [42]. Purification is crucial for improving vaccine efficacy, as evidenced by recent research showing that separating mRNA from dsRNA can boost protein production in human dendritic cells by more than 100 times [50]. Reversed-phase high-performance liquid chromatography (RP HPLC), a commonly used technique for this purpose, efficiently eliminates dsRNAs from the final mRNA product. This strategy reduces the possibility of the body's innate immune system activating and degrading mRNA, which eventually results in increased protein expression. But RP HPLC is a costly procedure that needs expensive supplies and equipment, and it has problems with waste management and scalability [51]. Up to 90% of dsRNA can be removed using an alternate purification technique that overcomes the drawbacks of RP HPLC. This technique provides an economical, quick, and scalable solution by depending on the selective binding of dsRNA to cellulose in a buffer containing ethanol. Fast protein liquid chromatography (FPLC) can be used to purify large amounts of mRNA using this method without generating hazardous waste. Interestingly, after being administered intravenously to mice, mRNAs extracted using cellulose-based and RP HPLC techniques show comparable translation levels in vivo [50].

In Vivo mRNA Delivery

mRNA must successfully penetrate target cells, avoid extracellular nuclease destruction, and stay intact in order to perform its therapeutic role. Many delivery methods, including viral and nonviral systems, have been put forth in response to the ineffective cellular uptake of unbound nucleic acids. There are two primary categories of nonviral mRNA delivery systems: (1) encapsulation of mRNA in liposomes or

polycationic polymers, and (2) mechanical delivery techniques such high-pressure injection, gene guns, electroporation, or ultrasound [52]. These techniques work both in vitro and in vivo.

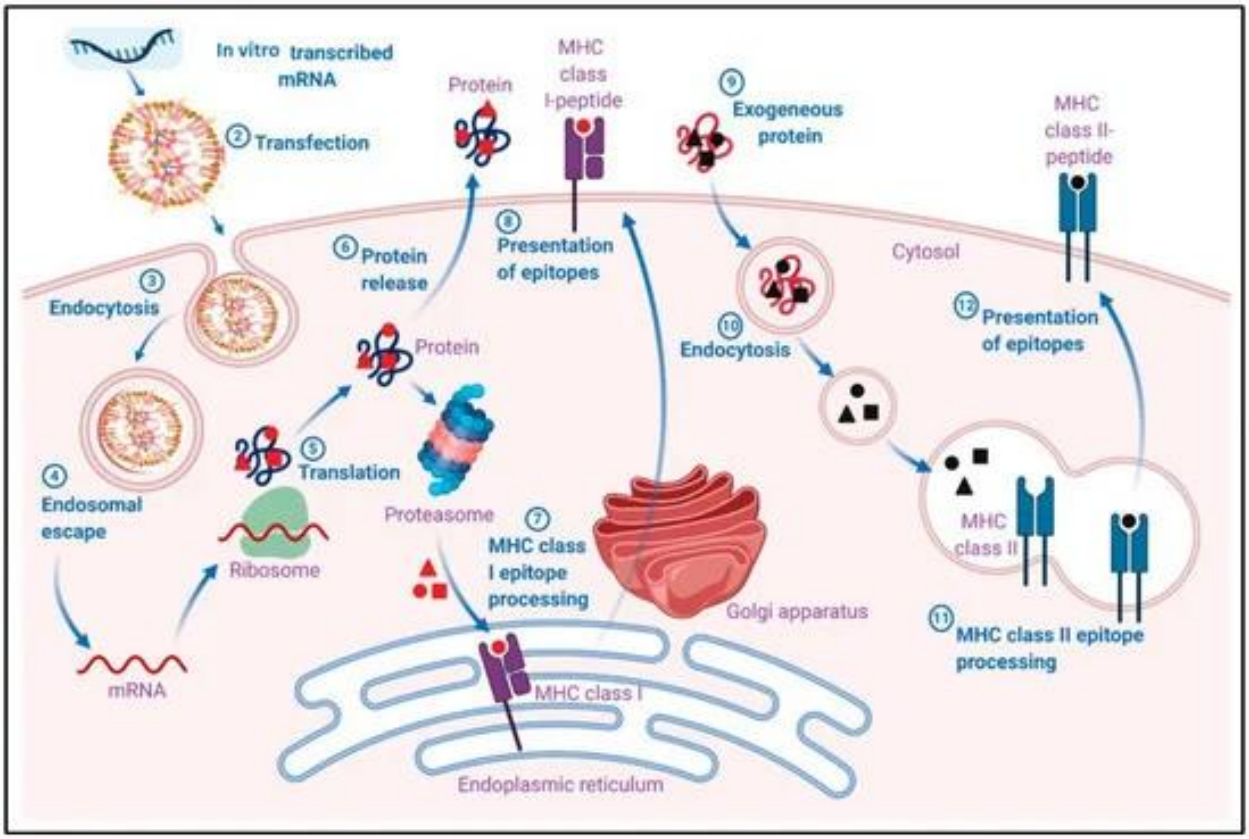

Figure 3: Mechanism of Adaptive Immunity for mRNA Vaccination.

Lipid Nanoparticles (LNPs)

Lipid nanoparticles (LNPs), one of the most commonly used systems for mRNA delivery, are generally composed of four major components (1) an ionizable cationic lipid, which promotes particle self-assembly and helps release endosomal mRNA; (2) lipid-conjugated polyethylene glycol (PEG), which increases stability; (3) cholesterol, which stabilizes the lipid bilayer; and (4) phospholipids, which preserve the bilayer structure [53, 54]. The method of delivery might affect the duration and effectiveness of mRNA translation in vivo; injections administered intradermally, intramuscularly, or subcutaneously result in extended protein production at the injection site [55]. To reach the ribosomes, the mRNA delivery mechanism needs to interact with the target cells, break through the cytoplasmic membrane, and release the mRNA into the cytoplasm. The delivery system's constituent parts can engage in electrostatic interactions with the cell surface, and the addition of ligands improves receptor-specific binding to the cell surface [56]. Endocytosis, a complex process that controls the intracellular localization of mRNA, is the main way that mRNA delivery systems enter cells. Endosomes, which eventually unite with lysosomes, contain mRNA upon entry. Optimizing the timing of mRNA release from endosomes is essential to preventing degradation and guaranteeing proper translation because the hydrolytic enzymes and acidic environment in lysosomes can break down the nucleic acid as well as the delivery system [5]. Although the processes that control the release of mRNA from lipid nanoparticles are still not fully understood, it is known that LNPs are internalized through both macropinocytosis and clathrin-dependent endocytosis [57].

Current mRNA Vaccines and Lipid Nanoparticle Composition

Lipid nanoparticles are used to transport mRNA encoding the SARS-CoV-2 spike protein in the licensed mRNA vaccines for COVID-19, such as mRNA-1273 (Moderna) and BNT162b2 (BioNTech/Pfizer) [58]. ARCoV (Walvax) and CVnCoV (CureVac), two clinical trial candidates, use various LNP formulations

for mRNA delivery [59, 60]. Despite its efficiency, there are a number of difficulties when using lipid nanoparticles to transfer mRNA. When negatively charged proteins and nucleic acids interact with positively charged lipid nanoparticles, the plasma membrane may become unstable and vaccinated individuals may experience unfavorable side effects [61]. Furthermore, antibodies against PEG can cause the quick clearance of PEGylated nanoparticles, changing the bioavailability and biodistribution of the encapsulated mRNA. Additionally, some lipid components, like PEGylated lipids, may activate the complement system. Furthermore, proinflammatory cytokines and reactive oxygen species may be released in response to cationic and ionizable lipids; however, the exact processes behind this immunogenicity are unclear. The usage of lipid nanoparticles has been linked to liver and lung damage in animal models, possibly as a result of their cytotoxic qualities and the production of proinflammatory responses [64]. This raises concerns about the cytotoxicity of lipid materials. These difficulties have led to the creation of components for modified lipid nanoparticles, which enhance performance but also raise complexity and production costs [65]. Large-scale vaccination campaigns face logistical issues because to the sensitivity of lipid nanoparticles to freezing and thawing, which requires storage and transportation at extremely low temperatures (–80°C) [66,67].

Polycationic Polymers in Nucleic Acid Delivery

Despite being less common than lipids in the delivery of nucleic acids, polymeric materials have a number of drawbacks, including the inability to biodegrade high molecular weight polymers. These issues are being addressed by continuous study, though. To lessen the toxicity of high-molecular-weight poly(ethylenimine) (PEI), for example, fatty acid chain-modified low-molecular-weight PEI has been used for mRNA delivery. Furthermore, erythropoietin mRNA has been successfully delivered in murine models by poly(glycoamidoamine) polymers, such as TarN3C10, which has a tartrate backbone composed of esters and tartaric acid salts [68].
It has also been demonstrated that mRNA can be efficiently delivered into cells by other polymer types, including poly(methacrylates) with amine-bearing side chains, polyaspartamides with oligoaminoethylene side chains, and polyacrylic acids amidized with tetramine and alternating ethylpropylethyl spacers. An important development is the creation of self-degradable esters, often known as Charge-Altering Releasable Transporters (CARTs). After being rearranged and degraded at physiological pH (7.4), these polymers can release mRNA. Moreover, it has been shown that biodegradable aminopolyesters (APEs) can carry mRNA to specific tissues with selectivity [69]. Another topic of great interest in this area is chitosan, a biodegradable and biocompatible polymer made from chitin by removing its acetate group. Because chitosan has chemical functional groups that may be changed, it can be used in a wide range of ways. Chitosan and its derivative-based nanoparticles typically have mucoadhesive qualities and a positive surface charge, which allow for efficient attachment to mucosal membranes and aid in drug release [70]. The potential of dendrimers, such as poly(amidoamine) (PAMAM) and polypropyleneimine derivatives, in the delivery of nucleic acids has been studied. For instance, fatty acid chain-modified PAMAM dendrimers were created to deliver small interfering RNAs. These dendrimers were then utilized to create a self-replicating mRNA vaccine platform that expressed antigens from the Zika virus, H1N1 influenza, Ebola virus, and Toxoplasma gondii in intramuscular applications [71]. Benefits of these dendrimers include improved pharmacokinetics for drug delivery, stability, targeting, and water solubility. They are a viable basis for next-generation drug delivery systems because of their versatility [72]. But there are also issues, like the steric inhibition of enzymatic biodegradation because dendrimer units are repetitive, which can cause toxicity from the buildup of these materials in tissues [73].

In addition to these well-known agents, novel mRNA delivery materials are being investigated to improve vaccine efficacy, safety, and portability. The State Research Center of Virology and Biotechnology's "Vector" polyglucin-spermidine (PGS) conjugate is noteworthy because it acts as a vehicle for the mRNA-RBD vaccine, which encodes the receptor-binding domain (RBD) of the SARS-CoV-2 S-protein [74]. This conjugate, which consists of spermidine and polyglucin, allows nucleic acids to be lyophilized and subsequently kept at positive temperatures for extended periods of time. According to studies, PGS-encapsulated DNA vaccines that are kept at 4°C retain their effectiveness for at least two years. Preclinical

research and phase I clinical trials have shown the safety of PGS conjugates, which are already included in HIV-1 DNA vaccines [75,76]. The biodegradability and safety of PGS components for human usage are two of their main benefits. With a molecular weight of 40,000, polyglucin is a glucose polymer that is non-toxic to people and acts as a certified plasma substitute with hemodynamic activity, helping to restore the amount of blood in circulation. Additionally, it has been demonstrated to shield yeast dsRNA from serum nuclease destruction [77]. All living things naturally contain spermidine, a polyamine that is essential for maintaining cellular homeostasis, stabilizing DNA and RNA, promoting cell division, and controlling translation [78,79]. For the manufacturing and delivery of mRNA vaccines, the cheap cost and the capacity to lyophilize and store the PGS conjugate at 4°C provide important technological benefits. Because the mRNA-PGS complex is similar in size to virus particles (100–200 nm), it is thought to enter antigen-presenting cells by endocytosis. Furthermore, PGS's protective packaging strengthens the immune response by shielding the mRNA from nuclease destruction. Research shows that the polyglucin-spermidine polycationic conjugate has potential as a secure and efficient mRNA vaccine delivery method, especially for SARS-CoV-2 vaccines [80].

Physical Delivery Methods for Nucleic Acid Transfection

Various physical techniques are employed to enhance the efficiency of direct nucleic acid transfection, both in vivo and in vitro [52, 81]. Methods such as electroporation, gene guns, ultrasound, and high-pressure injection have been utilized for the direct delivery of nucleic acids into cells. Among these, electroporation stands out as one of the most effective approaches for mRNA delivery. Since mRNA does not require nuclear localization, electroporation utilizes mild electrical pulses that help minimize cellular toxicity while ensuring efficient delivery. Additionally, electroporation enables the direct introduction of mRNA into the cytosol, which may reduce the risk of triggering an unwanted immune response [82]. In addition to the well-established techniques, alternative delivery methods are actively under investigation. Despite the successes achieved with individual systems, there is a growing consensus that a combination of different mRNA delivery approaches may yield the most optimal results.

PEG-Lipid Component in Lipid Nanoparticles (LNPs)

A hydrophilic polymer, polyethylene glycol (PEG) finds extensive application in the food, pharmaceutical, and cosmetic sectors. PEG is commonly attached to an anchoring lipid in the formulation of lipid nanoparticles (LNPs) and is essential for improving the stability and effectiveness of these nanoparticles. PEGylation increases the colloidal stability of LNPs in biological fluids and reduces the uptake of nanoparticles by filtering organs. As a result, PEG greatly improves LNP circulation, half-life, and in vivo dispersion. PEG-lipids usually make up only 1.5% of the lipid components of LNPs, but they have a significant effect on important factors like stability, encapsulation efficiency, aggregation reduction, polydispersity, and particle size. The overall delivery efficiency can be influenced by adjusting the anchor lipid's carbon chain length and PEG's molecular weight to maximize immune cell absorption and circulation duration [70]. Additionally, the PEG-lipid coat functions as a steric hydrophilic barrier, preventing LNPs from aggregating and self-assembling while being stored, which is essential for preserving particle stability and size. The size of the LNP and the amount of PEG in the formulation are inversely correlated; smaller LNPs are generally produced by higher PEG content [71]. The carbon chain length of the anchor lipid normally spans 13 to 18 carbons, whereas the molecular weight of PEG typically falls between 350 and 3000 Da. According to studies, longer lipid chains and a higher PEG molecular weight both lengthen the nanoparticles' half-life and decrease the immune cells' ability to absorb them. However, PEG may shorten circulation time as it separates from the LNP surface, making it easier to transport mRNA payload into target cells—a phenomenon known as the "PEG-Dilemma." Notably, in vivo transfection effectiveness seems to be unaffected by the carbon chain length of the anchor lipid when the molar percentage of PEG-lipid is kept at roughly 1.5%. Furthermore, PEG-lipids have the ability to conjugate certain ligands to LNPs, enabling targeted drug delivery [83,84].

Helper Lipids in LNP Formulation

The stability of LNPs during storage and in vivo circulation depends on helper lipids. Typically, these lipids are non-cationic glycerolipids. The most often employed helper lipids in LNP formulations are phospholipids, especially cholesterol, and sterols. Because it stabilizes the lipid bilayer, cholesterol, a naturally occurring component of cell membranes, is frequently added to LNPs. It may exist in the lipid bilayer, on the surface, or coupled with the ionized lipid in the core. By bridging the spaces between lipids and controlling membrane fluidity, density, and absorption, cholesterol contributes to the stability of LNP. Cholesterol stops leakage by regulating the membrane's stiffness and integrity through a "condensing effect." The effectiveness of LNP distribution is greatly influenced by the hydrophobic tail, the flexibility of the sterol ring, and the polarity of the hydroxy groups in cholesterol [85]. Cholesterol plays a crucial role during cellular uptake by fusing with the endosomal membrane and helps to prolong the circulatory half-life of LNPs by decreasing protein binding on the surface. In order to release the mRNA payload into the cytosol, the lipid must undergo this fusion in order to change from a lamellar phase to a hexagonal phase [86]. When added to LNP formulations, phospholipids improve transfection potency, cellular delivery, and encapsulation efficiency. Phospholipids usually make up a smaller percentage of LNPs than cholesterol, but when combined with cholesterol, they increase the total effectiveness of delivery. Better delivery performance is linked to higher phospholipid molar percentages. The LNP is assembled in part by phospholipids, particularly those in their zwitterionic form, which stabilize electrostatic connections between cationic lipids, mRNA cargo, and surrounding water molecules. Although their exact function in mRNA transport through LNPs is still unclear, phospholipids are thought to be essential for improving particle stability and delivery effectiveness in vivo. To determine their exact role in these processes, more research is required. Ionizable lipids, cholesterol, helper lipids, and PEGylated lipids are among the components of LNPs.

Physicochemical Properties Affecting mRNA-LNPs

Lipid nanoparticles (LNPs) exhibit various physicochemical properties that significantly impact their performance, particularly when used to deliver mRNA. While many of these properties contribute positively to LNP functionality, some can lead to unwanted toxicities. Understanding these characteristics is essential for optimizing the formulation of mRNA-loaded LNPs and improving their efficacy and safety.

1. **Size and Surface Area:** The size and surface area of LNPs are critical factors influencing their interaction with biological systems, including distribution, elimination, internalization, degradation, and response. A reduction in particle size increases the surface area, making LNPs more reactive in the biological environment. For essential biological activities such as endocytosis and cellular uptake, particle size plays a pivotal role. Specifically, mRNA-LNPs with a particle size of approximately 50 nm are considered optimal for efficient delivery, including in the context of vaccines. Any size-dependent toxicity is typically linked to the ability of LNPs to enter the biological system, potentially modifying macromolecules and disrupting essential biological functions.
2. **Charge:** The surface charge of LNPs is crucial in determining their biodistribution, cellular uptake, and efficacy. The charge facilitates the interaction between the LNP and the biological membranes, particularly for mRNA delivery. Cationic lipids, which are positively charged, can form electrostatic interactions with negatively charged mRNA, leading to efficient encapsulation. Additionally, cationic liposomes interact with the anionic cell surface and endosomal membranes to release mRNA. The pKa (the ability to attain a positive charge) of cationic lipids is an important parameter affecting mRNA delivery, though the exact ideal pKa range for optimal gene delivery remains under investigation. Some studies suggest that the pKa range of 6.2 to 6.6 is ideal for intravenous (IV) delivery of LNPs. Charge modulation has been explored to mitigate toxic effects and improve the delivery of mRNA from LNPs.
3. **Shape and Structure:** The shape and internal structure of LNPs directly influence cellular uptake and their interaction with the biological environment. Research has shown that spherical nanoparticles are generally

more easily endocytosed compared to non-spherical particles, which tend to flow more effectively through capillaries. However, the exact mechanisms by which shape and structure affect in vivo behavior remain unclear. Given the technical challenges associated with shape and structure, further research is needed to understand how these properties influence membrane deformation and therapeutic efficiency.

4. **Surface Composition:** The surface composition of LNPs significantly affects their delivery efficiency and biodistribution. Surface modifications, such as the incorporation of PEG-lipids through PEGylation, are commonly used to improve the circulation half-life and modulate nanocarrier trafficking. PEGylation can enhance biodistribution and extend circulation time by preventing immune recognition. However, it can also limit the uptake of LNPs due to steric hindrance, reducing interactions with the plasma membrane. Over time, PEG-lipids may detach from the LNP surface in the serum, alleviating this steric hindrance and favoring endosomal uptake, which is crucial for the effective delivery of mRNA cargo into cells [87].

These physicochemical properties, such as size, charge, shape, and surface composition, are essential considerations in designing and optimizing mRNA-LNP formulations for effective therapeutic applications.

mRNA Vaccines Manufacturing:

mRNA vaccines present numerous advantages over traditional vaccines, particularly in their development simplicity, scalability, and rapid production. Like other vaccine formulations, mRNA vaccines undergo three core stages in their manufacturing process: upstream production, downstream purification, and the final formulation of the mRNA drug substance. This section provides a detailed discussion of these phases, along with recent innovations aimed at optimizing the production of mRNA vaccines.

Upstream Production:

In the process of making mRNA vaccines, upstream production entails creating the mRNA transcript from a plasmid containing the desired gene. The in vitro transcription (IVT) response is the name given to this process. RNA polymerases, such T7, SP6, or T3, are used in the IVT enzymatic reaction to catalyze the synthesis of target mRNA from a linearized DNA template. This template is created either by amplifying the desired gene using the polymerase chain reaction (PCR) or by enzymatically cleaving the plasmid with restriction endonucleases. RNA polymerase, which transforms DNA into RNA, inorganic pyrophosphatase (IPP), which increases IVT production, and guanylyl transferase, which appends GMP nucleoside to the 5′ end of the mRNA, are the main enzymes involved in IVT. (iv) DNase I, which eliminates contaminating genomic DNA and breaks down DNA templates; (v) Cap 2′-O-Methyltransferase (SAM), which methylates the 5′ cap's 2′ position; and (vi) poly(A) tail polymerase, which inserts the poly(A) tail. SAM and guanylyl transferase, two enzymes involved in the capping process, enzymatically form a 5′ cap on the mRNA, and poly(A) tail polymerase adds the poly(A) tail. Co-transcriptional capping is an alternate technique for 5′ capping in which CleanCap® Reagent AG is used to add the 5′ cap to the mRNA non-enzymatically after it has been prepared beforehand [88].

Downstream Purification

The mRNA is extracted and purified via a number of downstream processing steps after being produced during the IVT reaction in upstream manufacture. Remaining nucleoside triphosphates (NTPs), enzymes, misformed mRNAs, and plasmid DNA templates are among the contaminants present in the IVT reaction mixture. Lithium chloride (LiCl) precipitation is usually used to separate the mRNA after DNase enzyme digestion is used to remove DNA in laboratory-scale purification [89]. Nevertheless, truncated fragments and double-stranded RNA (dsRNA) are examples of aberrant mRNA species that are not entirely eliminated by lab-scale techniques. For the final mRNA product to maintain its performance and safety , these contaminants must be effectively removed. Reduced translation efficiency and possibly undesirable immunostimulatory effects can arise from inadequate purification.

Reverse-phase high-performance liquid chromatography (HPLC) purified mRNA, for instance, demonstrated a 10–1000-fold improvement in transfection effectiveness and protein production prior to transport to dendritic cells [90]. Size exclusion chromatography (SEC) was the first approach used for large-

scale nucleic acid purification of RNA oligonucleotides in 2004 [91,92]. Chromatography is a purification technology that is widely used in the biopharmaceutical business. Although SEC has several advantages, such as high purity, scalability, and selectivity, it cannot effectively remove contaminants of the same size, such dsDNA. Since Ion-pair reverse-phase chromatography (IEC) separates mRNA from IVT reaction contaminants according to their charge differences, it has been shown to be more successful for mRNA purification. Although the method necessitates denaturing conditions, which makes it temperature-sensitive, IEC has benefits such increased binding capacity, scalability, and cost-effectiveness [93, 94, 95] Another purification technique is affinity-based chromatography, which uses dT-Oligo dT beads to collect the poly(A) tail of mRNA [96]. Furthermore, methods such as core bead filtration and tangential flow filtration (TFF) are used to eliminate minor contaminants [97]. For final polishing, mRNA vaccines can be purified using hydrophobic interaction chromatography (HIC) with connective interaction medium monolith (CIM) columns containing OH or SO3 ligands [97].

Formulation

Due to their intrinsic negative charge, mRNA molecules must be formulated within lipid-based delivery systems in order to enhance transfection efficiency and half-life and prevent destruction. The most dependable and FDA-approved non-viral delivery system for mRNA vaccines is lipid nanoparticles (LNPs). Lipids dissolved in an organic phase are combined with mRNA in an aqueous phase to generate LNPs. Ionizable lipids, cholesterol, helper lipids, and PEG-lipids are the main lipids utilized in the organic phase. Usually, the mRNA is dissolved in a pH 4 citrate or acetate buffer. The hydrophobic lipids help the LNPs self-assemble, encasing the mRNA inside the nanoparticle core, while the ionizable lipid becomes protonated upon mixing, forming electrostatic contacts with the anionic mRNA. After this procedure, called microprecipitation, the pH is adjusted to physiological levels and the organic solvent, usually ethanol, is removed using dialysis. To create tiny LNPs with low polydispersity and excellent mRNA encapsulation efficiency, microfluidic mixers are frequently used. These mixers produce homogenous nanoparticles by quickly combining the organic and aqueous phases in a matter of microseconds. The staggered herringbone micromixer (SHM) cartridge architecture of Precision NanoSystems' NanoAssemblr® platform is widely used for both lab-scale and GMP manufacture of LNPs. The aqueous and organic phases' flow rate and volume ratio may be precisely controlled with this method, resulting in LNPs with the appropriate size and distribution. However, solvent incompatibility limits the use of SHMs for GMP manufacturing since extended exposure to ethanol can cause internal system components to distort. Because T-mixers can manage higher flow rates and volumes while still being compatible with organic solvents like ethanol, they are being utilized more and more for LNP scale-up in order to resolve this issue [98,99, 100].

Conclusion:

mRNA-based vaccines have reshaped the landscape of vaccine development, particularly in the face of the global COVID-19 pandemic. These vaccines offer numerous advantages over traditional approaches, such as their ability to be rapidly developed, produced at scale, and adapted for new pathogens. Unlike conventional vaccines, mRNA vaccines do not require the use of live virus, reducing safety concerns associated with potential infection. Moreover, mRNA vaccines stimulate both cellular and humoral immune responses, contributing to a more robust and lasting immune protection. The history of mRNA vaccine development dates back to the 1980s, with early research focusing on understanding mRNA's potential in gene expression. Despite initial setbacks due to the instability of mRNA and immune system responses to foreign RNA, continued advancements in mRNA technology have led to significant improvements in vaccine stability and efficacy. Key developments, such as the use of pseudouridine to reduce inflammatory responses and the optimization of mRNA capping structures, have paved the way for the successful deployment of mRNA vaccines, especially for COVID-19. The rapid development of mRNA vaccines during the COVID-19 pandemic highlighted the potential of this technology, with vaccine candidates being developed in record time, thus proving the viability of mRNA as a platform for future vaccine strategies. However, challenges remain in further improving the stability of mRNA and the delivery methods used to administer these vaccines. The instability of mRNA and its recognition by the immune system continue to

pose obstacles. As such, the refinement of delivery systems, such as lipid nanoparticles, and the development of more stable mRNA formulations are crucial for the success of mRNA vaccines in broader applications, including other viral diseases such as Zika, influenza, and HIV. The future of mRNA vaccines is promising. With ongoing research into enhancing their stability and immune response, mRNA vaccines are expected to become a cornerstone in global vaccination strategies. The flexibility of the platform allows for the rapid adaptation to emerging infectious diseases, potentially transforming the way we respond to future pandemics. The continued development and optimization of mRNA vaccines will not only address current challenges but also shape the future of vaccine technology and public health worldwide.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. It was reviewed and approved by The Jazan Ethics Committee

Availability of data and material: All the necessary information is provided within the manuscript. Any other data that support the findings of this study are available from the corresponding author upon request.

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