The Impact of Nucleotide Modifications on the Immune Responses of mRNA Vaccines

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Abstract. Messenger RNA (mRNA) vaccines play an important role in preventing infections and treating cancer. They offer notable advantages, including rapid design, flexible antigen encoding, and the feasibility of large-scale production. Despite these benefits, mRNA vaccines face some challenges, such as unintended immune activation, poor stability, and limited protein yield. Chemical modifications can help overcome these problems. Examples include N1-methylpseudouridine, 2'-O-methylation, and 5-methylcytidine. These changes improve stability and reduce immunogenicity. In addition, adjusting the 5' cap, controlling poly(A) tail length, and modifying untranslated regions (UTRs) can increase protein production and extend mRNA lifespan in the body. Together, these improvements increase antigen production, also strengthen immune responses against viruses, tumors, and chronic infections such as hepatitis B. Some challenges remain, like we need to know how modifications affect translation accuracy, how cells need different poly(A) lengths and UTRs, and how to combine cap changes with large-scale production. Addressing these problems is important to achieve safer and more effective mRNA therapies. This review summarizes current knowledge on nucleotide changes and design rules. It explains how they influence stability, translation efficiency, and immune activation, and offers advice on making the next generation of mRNA vaccines safer and more effective in clinical use.

Keywords: mRNA vaccine, nucleotide modification, structural optimization, stability, translation efficiency.

1. Introduction

Messenger RNA (mRNA) vaccines are a new type of vaccine. They are flexible in design, quick to produce, and can encode complex antigens. These features make them promising for preventing infections and cancer therapy [1]. In recent years, several mRNA vaccines reached clinical trials and showed good results against rabies, Zika, and influenza. The success of COVID-19 vaccines confirmed that the mRNA platform works and boosted industry development [2]. Studies show that unmodified mRNA vaccines trigger type I interferon (IFN-I) and downstream signaling. This helps produce strong antitumor T-cell responses and control tumor growth [3]. However, unmodified mRNA has challenges. Its natural immune activity may over-activate innate immune pathways. This leads to too many inflammatory cytokines and reduces mRNA translation, which limits the production of therapeutic proteins. Although this immune activation can act as a vaccine adjuvant, the translation suppression and mRNA degradation limit its therapeutic use [4]. In contrast, nucleoside modifications provide clear benefits. They can stop in vitro transcribed (IVT) mRNA from being detected by the innate immune system. This reduces unwanted immune responses and increases antigen production [5]. As a result, chemical modification of mRNA has gained more research attention recently because of its wide application potential [6].

Multiple factors influence the expression and stability of mRNA vaccines, among which structural features of mRNA, including the 5' cap (CAP), poly(A) tail, and untranslated regions (UTRs), play decisive roles in determining stability and translational efficiency. Through chemical modification and structural optimization, mRNA stability and protein expression efficiency can be further improved [7]. The 5' cap is essential for protein synthesis and the recruitment of translation initiation factors; the length of the poly(A) tail is closely related to mRNA degradation; and UTRs, located on either side of the coding sequence, regulate both mRNA stability and translation [8]. This study aims to review and critically evaluate the structural elements of nucleoside-modified mRNA, namely the

5' cap, poly(A) tail, and UTRs, in terms of their influence on mRNA vaccine stability, translational efficiency, and immune modulation. Furthermore, it explores the feasibility and potential of diverse optimization strategies, thereby providing a theoretical foundation and technical guidance for the future design and industrial-scale production of safer and more efficient mRNA vaccines.

2. Classes of Nucleotide Modifications and Associated Biological Functions

2.1. Nucleotide Modifications and mRNA Translation

Nucleotide modifications have been demonstrated to enhance mRNA protein expression while reducing immunogenicity. Common types include 2'-O-methylation, N1-methylpseudouridine (m1Ψ), and 5-methylcytidine (m5C). In 2005, Karikò and colleagues reported that RNA containing nucleotide modifications exhibited lower immunogenicity compared with unmodified RNA. They further showed that pseudouridine (ψ) modification significantly improved protein production, such as increasing erythropoietin yields. More recent studies have revealed that phosphorothioate modification can further accelerate protein synthesis [9].

2.2. 2′-O-Methylation and Immune Evasion

The 2'-O-methyl modification represents a critical feature of the mRNA cap structure, enabling viral RNA to mimic host mRNA and thereby evade immune detection. Certain cytoplasmic viruses rely on their own viral methyltransferases to carry out N-7 and 2'-O methylation. In one study using Japanese encephalitis virus as a model, researchers generated a mutant strain lacking 2'-O-methylation activity. While this mutant retained replicative capacity, it became highly sensitive to interferons and IFIT proteins. In vivo, it displayed an attenuated phenotype, induced potent humoral and cellular immune responses, and conferred complete protection against lethal viral challenge. These findings highlight the feasibility of exploiting 2'-O-methylation–deficient strains for live-attenuated vaccine development [10]. In summary, 2'-O-methylation allows viral RNAs to disguise themselves as host mRNAs to evade immune clearance; loss of this modification renders viruses vulnerable to interferon-mediated restriction, resulting in attenuation suitable for vaccine design.

2.3. N1-Methylpseudouridine in mRNA Vaccines

N1-methylpseudouridine (m1Ψ) has been extensively employed in RNA-based therapeutics and mRNA vaccines, including COVID-19 vaccines where uridine residues are replaced with m1Ψ. This modification markedly reduces innate immune activation, enhances mRNA stability, and promotes higher protein output. In vitro studies suggest that m1Ψ can modulate ribosome dynamics and, under certain conditions, induce low levels of +1 frameshifting. Similar to pseudouridine, it may influence translation speed, tRNA selection, and protein folding [11]. Widely adopted in mRNA vaccines, m1Ψ improves protein expression primarily by dampening innate immune recognition. However, it may slow translation, change tRNA use, and lead to minor frameshift events, which means it could affect how accurately proteins are made and folded. Therefore, we need to thoroughly evaluate these impacts, which is important for improving the design of mRNA drugs and vaccines.

2.4. 5-Methylcytidine in Viral Replication

5-methylcytidine (m5C) is another vital RNA modification that can control viral replication and gene expression. Research has shown that HBV expression is linked to RNA methylation. Removing *NSUN2* reduces viral expression. In contrast, removing *TET2* increases expression. Two key m5C sites were identified on HBV RNA. These sites are *C2017* and *C131*. Mutations at these sites lower both viral replication and expression. The mechanism is also clear. *NSUN2* stabilizes HBV RNA through m5C modification. At the same time, the HBV core protein raises *NSUN2* levels. This creates a positive feedback loop that promotes viral replication. Together, these results show that m5C helps HBV replication by keeping RNA stable. This reveals a new epigenetic regulation mechanism in HBV biology [12].

3. Optimization of mRNA Structure

The 5' cap structure is an important feature of eukaryotic mRNA. It is involved in many cell processes, including RNA splicing, export from the nucleus, recruitment of translation initiation factors, and protection against 5'-end exonuclease degradation. Many mRNA studies rely on efficient and reliable preparation and modification of 5'-capped RNA [13]. For mRNA vaccines, the 5' cap must be seen as a "self" feature, so a natural CAP-1 structure is needed. Abnormal caps (CAP-0) or uncapped mRNAs (5'ppp/5'pp) can be detected by pattern recognition receptors like RIG-I and IFIT. This triggers type I interferon production and promotes mRNA degradation. The 5' cap also recruits translation initiation factors and may help form a closed-loop RNA structure, regulating translation efficiency [14]. Therefore, in vaccine preparation, it is important to maximise capping efficiency. This reduces uncapped or wrongly capped mRNAs, which could cause over-activation of innate immunity [15]. Studies show that RNA methyltransferases can make site-specific modifications at the 5' cap with non-natural cofactors. These include N2 or N7 methylation of guanine and N6 methylation of the first adenosine. Such modifications improve the function and controllability of mRNA. When combined with click chemistry, optochemical methods, and high-throughput sequencing, these approaches can label mRNA, study protein interactions, and regulate immune responses. They also guide the design of stable and controllable long mRNAs [16].

Apart from the 5' cap, the polyadenylate tail (poly(A) tail) is a key factor in determining mRNA stability and translation efficiency. By protecting mRNA from exonuclease attack, it extends the half-life, strengthens stability, and facilitates translation. Poly(A)-binding protein (PABP) binds simultaneously to translation initiation factors (such as eIF4G and eIF4E) and the 5' cap, creating a circular structure that harmonizes stability with translation. In addition, the length of the poly(A) tail has a significant impact: tails shorter than 20 nucleotides inhibit translation, while medium- to long-length tails (120-300 nucleotides) in dendritic and T cells significantly enhance stability and translational efficiency. However, genome-wide studies have revealed that highly translated mRNAs often carry short poly(A) tails, suggesting that optimal poly(A) length is cell-type dependent and should be tailored to specific contexts to optimise translation [17]. For example, researchers have synthesised mRNAs with chemically and topologically modified poly(A) tails, which prolong protein expression and enhance overall expression levels [18].

Untranslated regions (UTRs) also play a pivotal role in mRNA stability and translation, including both 5' UTRs and 3' UTRs. These regions regulate ribosome recruitment, translation initiation efficiency, and mRNA lifespan, thereby exerting a critical influence on the expression of exogenous mRNAs. Effective mRNA design should preserve regulatory elements in the 5' UTR to promote ribosome binding while avoiding inhibitory motifs such as uORF or IRE. This increases antigen protein expression. The 3' UTR regulates stability and translation through AREs, MREs, and the poly(A) tail. Thus, removing negative elements and improving stability and translation are critical. Recent studies created new 5' UTRs, such as 5UTR05, through combinatorial screening and combined them with different 3' UTRs to enhance exogenous mRNA translation. Results showed that 5UTR05 drove protein expression at levels comparable to the highly active 5' UTR in mRNA-1273, used in COVID-19 vaccines. Further tests revealed that pairing 5UTR05 with IGHG2 or mtRNR1 3' UTRs greatly increased translation efficiency. These findings demonstrate that UTR combination design is a powerful approach to boost mRNA translation [19].

4. Conclusion

mRNA vaccines are advancing rapidly. Compared with traditional vaccines, they are more flexible, faster to produce, and able to code for complex antigens. Adding nucleotide modifications (such as N1-methylpseudouridine and 5-methylcytidine) and improving the 5' cap, poly(A) tail, and UTRs can increase stability, boost protein yield, and reduce innate immune response. These improvements raise clinical effectiveness and extend their use to cancer, chronic viral infections, and new infectious diseases.

However, challenges remain. Long-term safety is not yet clear, translation may vary, and there may be risks to protein folding or immune balance. Future studies should carefully test the effects of modifications, design personalised strategies for different diseases, and build scalable production systems. Continued innovation in nucleotide chemistry and structural engineering will be important for developing safe, efficient, and widely applicable mRNA vaccines.

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