Review | Received 27 November 2024; Accepted 17 March 2025; Published 21 March 2025
https://doi.org/10.55092/exrna20250006

RNA modifications in mammals: from basic research to biotechnological application

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Highlights:

- RNA modifications, like base, ribose, and phosphate modifications, regulate gene expression by altering RNA structure, stability, and function.
- Discusses impact of RNA modifications on gene expression, stability, and functionality in RNA-based therapies.
- Summarizes recent advances in RNA modification applications for mRNA vaccines, siRNA, ASOs, and CRISPR-Cas9.
- Highlights challenges and future trends in RNA modification technologies for therapeutic development.
- Emphasizes potential of combining machine learning and RNA modifications for optimized drug design.

Abstract: RNA, a crucial molecule in protein synthesis and gene expression regulation, plays an essential role in organisms. RNA modifications, acting as epigenetic marks, subtly adjust the structure, stability, and function of RNA, thereby regulating gene expression and exerting profound effects on cellular functions and organismal health. These natural modifications, together with RNA editing that alters nucleotide sequence of mRNA, constitute the epitranscriptome, which is vital for cellular metabolism. With the rapid advancement of biotechnology, RNA-based therapies and technologies have emerged as a frontier in biotech research. Various RNA drugs, including small interfering RNA (siRNA), antisense oligonucleotides (ASO), mRNA vaccines, and the small guide RNA (sgRNA)

required for CRISPR gene editing, have been continuously developed. It has been widely demonstrated that RNA modifications can alter the physicochemical properties of RNA, enhance resistance to nucleases, reduce immunogenicity, and optimize *in vivo* functionality, leading to their extensive application in RNA-related biotechnologies. Furthermore, the variety of RNA modifications has expanded beyond natural modifications with the invention of an increasing number of artificial modifications. This review delves into the common types of RNA modifications, including base, ribose, and phosphate modifications, discussing their impact on RNA structure and how these modifications influence the biological characteristics of RNA. The current applications of these modifications in the biotechnology field are summarized, highlighting their significance in RNA-based therapies.

Keywords: RNA modification; mRNA vaccine; ASO; siRNA

1. Introduction

RNA serves as a crucial bridge for the transfer of genetic information from DNA to proteins in biological processes. Post-transcriptional modifications of RNA are widespread in nature and can influence RNA structure, stability, and interactions with proteins. These modifications play a significant role in processes such as RNA splicing, translation, and degradation, thereby regulating gene expressions [1–4]. Since the 1950s, scientists have identified various chemical modifications in RNA (Figure 1), including pseudouridine [5,6], N^6-methyladenosine [7], and 5-methylcytosine [8]. As the number of discovered chemical modifications in cellular RNA increases, and post-transcriptional RNA editing that alters mRNA nucleotides is identified, the field of epitranscriptomics has emerged within nucleic acid biology, providing a new perspective for understanding the complex regulatory mechanisms [4,9,10]. Further research has also shown that RNA modifications are associated with the onset and progression of various diseases. Dysregulated RNA modifications can disrupt the corresponding gene expression regulatory mechanisms, leading to cellular metabolic disorders and potentially contributing to neurological diseases or cancer [1,11].

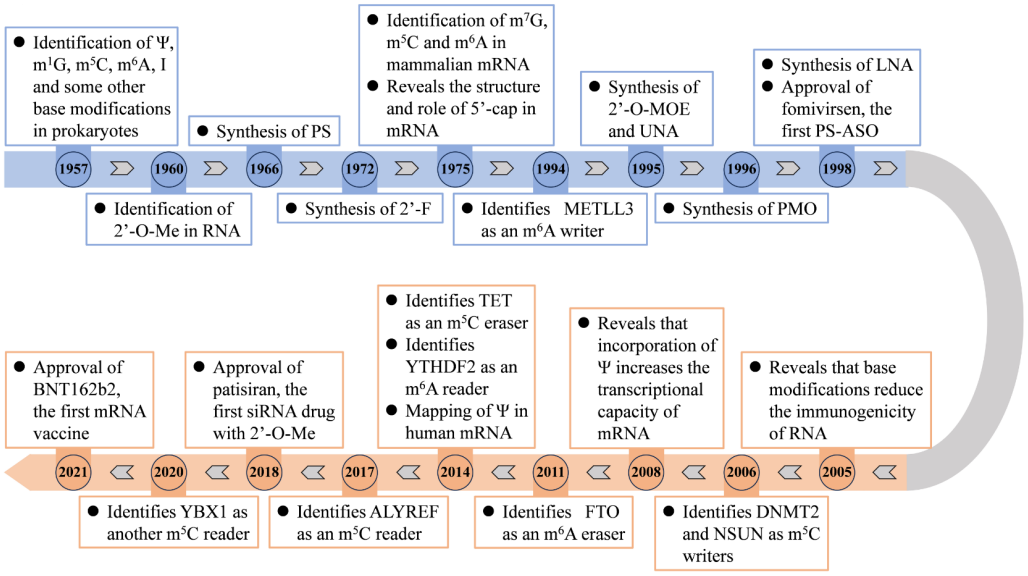

Figure 1. The historical milestone event in RNA modification. Discoveries of natural and artificial RNA modifications are shown in the time line.

With the continuous progress of biotechnologies, the utilization of RNA has become more diverse and sophisticated. An increasing number of RNA-based drugs, such as small interfering RNA (siRNA), antisense oligonucleotides (ASO), and mRNA vaccines, have emerged [12–14]. CRISPR gene editing also relies on small guide RNA (sgRNA) for targeted guidance [15]. Since 1960, the spectrum of RNA modifications has expanded beyond naturally occurring ones (Figure 1). A wide range of non-natural modifications, including locked nucleic acids (LNA) [16] and phosphorothioate (PS) [17], have been developed and utilized. Chemical modifications of the components of ribonucleotides, such as the bases, ribose, and phosphate groups, can alter the physicochemical properties of RNA. For example, pseudouridine and N^1-methylpseudouridine modifications can reduce the immunogenicity of exogenous mRNA [18,19], while the introduction of LNA and PS modifications into ASOs and siRNAs can enhance their stability [17,20]. Therefore, in the research and development of RNA-based biotechnologies and therapeutic strategies, selecting the optimal chemical modifications to improve the functionality of exogenous RNA is becoming an increasingly important consideration. Recent advances in site-specific modification technologies have made it possible to precisely target and modify specific nucleotides within RNA molecules. Techniques such as CRISPR-based RNA editing and chemically assisted RNA modification have enabled researchers to introduce modifications at defined positions, thereby enhancing the stability, functionality, and therapeutic potential of RNA-based drugs [21,22].

This review summarizes the types and functions of some widely used RNA modifications, and highlights their recent advances in biotechnological applications. It also discusses the challenges in this field and future trends, aiming to illustrate the broad prospects of RNA modifications and provide potential ideas for the development of new technologies.

2. Classification of RNA modifications

2.1. Base modifications

Base modifications mainly include the substitution or isomerization of natural bases. Karikó and Weissman demonstrated that introducing appropriate base modifications into RNA can reduce its susceptibility to degradation and immunogenicity [23]. The Nobel Assembly awarded the 2023 Nobel Prize in Physiology or Medicine to these two researchers for their pioneering work in mRNA vaccine technology development. Here, we provide an overview of three common types of RNA base modifications: pseudouridylation, cytosine methylation, and adenine methylation.

2.1.1. Pseudouridylation

Pseudouridine (Ψ) is the most prevalent type of base modification in natural RNA and is often referred to as the “fifth nucleotide” due to its high abundance in total RNA. It was the first RNA modification to be discovered [5]. Pseudouridine is a rotational isomer of uridine, formed by breaking the N1-C1’ bond between uridine and ribose, followed by a 180° rotation around the N3-C6 axis and the formation of a new C5-C1’ bond between the base and the ribose (Figure 2). The isomerization process of pseudouridine biogenesis is mainly catalyzed by the pseudouridine synthase (PUS) family. Some PUS can directly recognize and catalyze the isomerization of ribonucleotide, while most require the formation of a ribonucleoprotein (RNP) complex with Box H/ACA RNA to function [24–26].

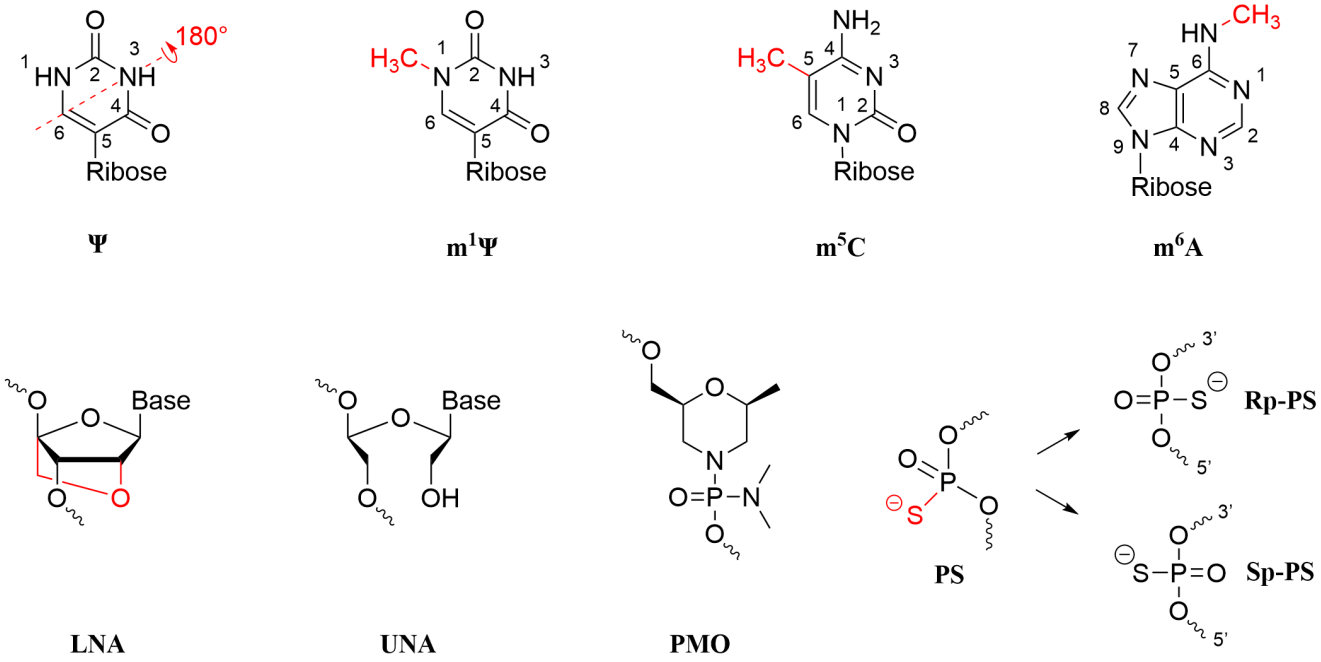

Figure 2. Chemical structure of some modifications mentioned in this review. The upper four are natural base modifications, and the lower four are synthesis modifications. Ψ, pseudouridine. $m^1Ψ$, N^1-methylpseudouridine. m^5C, 5-methylcytosine. m^6A, N^6-methyladenosine. LNA, locked nucleic acid. UNA, unlocked nucleic acid. PMO, phosphorodiamidate morpholino. PS, phosphorothioate. Rp and Sp, the two stereoisomers of PS in R and S configuration. The two configurations are shown in Fischer projection. The numbering of Ψ and $m^1Ψ$ follows the numbering rule of uridine.

Pseudouridine modifications are essential for RNA structure and function. This modification is widely distributed in non-coding RNAs such as tRNA, rRNA, and snRNA, with many sites being highly conserved and often located at functional regions of these RNAs [27]. The recognition region of snRNA U2 contains several pseudouridine sites that are closely related to RNA splicing function [28]. Studies have shown that knockdown or knockout of *PUS10* can lead to impaired processing of pri-miRNA [29]. Additionally, telomerase RNA contains pseudouridine sites that are closely associated with its stability [30]. Pseudouridine is also an important post-transcriptional modification affecting mRNA splicing and translation, with its function depending on its position in the mRNA [24,27]. Pseudouridine modifications can also alter codon specificity. Replacing U with Ψ in a stop codon can inhibit the recognition by release factors (RFs) and convert the stop codon into a sense codon [31]. Alterations in the activity or expression levels of certain pseudouridine synthases can lead to diseases and even cancer [11]. For example, the inactivation of dyskerin pseudouridine synthase (DKC1) results in dyskeratosis congenita [32]. Abnormal DKC1 expression is also associated with the development and progression of various cancers. Notably, in some cancer types, such as breast cancer [33] and chronic lymphocytic leukemia [34], DKC1 expression is downregulated, while in others, such as colorectal cancer [35] and prostate cancer [36], it is upregulated. Therefore, the role of DKC1 in cancer likely depends on the specific tissue and cancer type, and further research is needed to elucidate these mechanisms [26].

The dysregulation of DKC1 can indeed affect overall pseudouridine levels in cells. DKC1 is a key enzyme responsible for the pseudouridylation of rRNA and other non-coding RNAs, and its inactivation or abnormal expression can lead to a reduction in pseudouridine levels, particularly in rRNA and

telomerase RNA [32]. This reduction in pseudouridine levels can impair ribosome function and telomere maintenance, contributing to cellular dysfunction and cancer progression [33]. Furthermore, the role of different Box H/ACA RNAs in guiding DKC1 functions is crucial. Box H/ACA RNAs are small nucleolar RNAs (snoRNAs) that guide DKC1 to specific uridine residues in target RNAs, ensuring precise pseudouridylation [37]. Different Box H/ACA RNAs can direct DKC1 to modify distinct RNA substrates, thereby regulating various cellular processes. For instance, the Box H/ACA RNA SNORA42 has been shown to guide DKC1 to modify specific sites in rRNA, which is essential for ribosome biogenesis and function [38]. Dysregulation of these Box H/ACA RNAs can lead to aberrant pseudouridylation patterns, further contributing to disease states [39].

N^1-methylpseudouridine ($m^1\Psi$) is a derivative of pseudouridine (Ψ). In eukaryotes, it is formed by the methylation of Ψ by Nucleolar Essential Protein 1 (Nep1) [40,41]. $m^1\Psi$ was first identified in rRNA in 1978 [42], and its presence in tRNA was discovered later in 2012 [43]. However, the function of $m^1\Psi$ in natural RNA remains to be further investigated. Even so, the significance of $m^1\Psi$ in mRNA vaccine has been proved, as it reduces the immune stimulation and increases the translation level [19].

2.1.2. Cytosine methylation

5-methylcytosine (m^5C) is a conserved and common mark in various types of RNA. In mRNA, m^5C is mainly distributed in the coding regions and 3'-UTR. It is also associated with the structure and stability of tRNA and rRNA, thereby influencing the accuracy of the translation process [44,45]. In mammalian cells, cytosine methylation in RNA is related to m^5C methyltransferases, including DNMT2 and NSUN families of enzymes [46]. Additionally, m^5C modifications can be removed by demethylases, such as the TET enzyme family, which oxidize m^5C to 5-hydroxymethylcytosine [45,47]. The recognition of m^5C in RNA is mediated by RNA-binding proteins such as ALYREF [48] and YBX1 [49]. The reading of m^5C on RNA affects RNA transport and translation, thus regulating gene expression and further influencing cellular processes such as differentiation and circadian rhythms [44,45]. Proteins from the DNMT2 and NSUN families show abnormal expression in various cancer cells, leading to cellular carcinogenesis by affecting the function of tRNA and rRNA [26]. For example, in skin cancer where *NSUN2* is upregulated, the m^5C modifications by NSUN2 can maintain the stability of tRNA, and knocking out *NSUN2* inhibits protein translation in mouse stem cells, thereby suppressing cancer progression and making the cells hypersensitive to cytotoxic stress [50].

2.1.3. Adenine methylation

Adenine can be methylated at N1 and the amino group on C6, respectively forming N^1-methyladenosine (m^1A) and N^6-methyladenosine (m^6A). m^1A modifications are distributed in the 5'-UTR of mRNA [51]. Under physiological conditions, m^1A introduces a positive charge and prevents adenine from forming Watson-Crick hydrogen bonds, altering base-pairing interactions. Consequently, this modification can influence the secondary structure of RNA and its interaction with proteins [52]. Recent studies have highlighted the functional relevance of m^1A in the 5'-UTR of mRNA. For instance, m^1A in the 5'-UTR has been shown to enhance translation efficiency by promoting ribosome binding and initiation complex formation [53]. Additionally, m^1A modifications in the 5'-UTR can regulate mRNA stability and decay, thereby influencing gene expression levels [54]. These findings underscore the critical role of m^1A in

modulating mRNA function and cellular processes. m^6A modifications are abundant in mRNAs, mainly located in the coding regions and 3'-UTRs, where they are associated with post-transcriptional modifications [55]. m^6A does not affect Watson-Crick base pairing, but m^6A: U pairs are less stable than A: U pairs [56]. Unpaired m^6A residues have stronger stacking interactions than unmodified adenines, which can enhance the thermal stability of single-stranded RNA [57]. m^6A modification is reversible, with its formation controlled by the methyltransferase complex consisting of METTL3 and METTL14, and the deletion regulated by FTO [58,59]. Several studies have reported associations between m^6A modifications on mRNA and cancer, suggesting that m^6A-related genes may serve as potential therapeutic targets [60,61]. A study has shown that METTL3 is overexpressed in acute myeloid leukemia (AML) cells, identifying it as a key gene involved in AML cell proliferation [62]. METTL14 has also been reported to exert oncogenic effects by modulating m^6A modifications on *MYC* or *MYB* mRNAs [63]. Moreover, m^6A modifications can regulate miRNA maturation. Studies have shown that m^6A modifications on pri-miRNAs by METTL3 and METTL14 promote the process of pri-miRNA to pre-miRNA through the DGCR8 mechanism [64,65]. Relevance between the m^6A-mediated miRNA maturation and progression of cancers has been confirmed. For example, METLL3-dependent miR-17-92 maturation and METLL14-dependent miR-17-5p maturation respectively reduce the tolerance of gastric cancer and promote the tolerance of colorectal cancer [65,66]. But m^6A modifications' downstream effects on gene expression remain unclear.

2.2. Ribose modifications

Ribose modifications play a crucial role in the design of non-coding RNA therapeutics, including siRNAs and ASOs. These modifications introduce steric hindrance, thereby inhibiting nuclease-mediated hydrolysis and thus enhancing RNA stability. A common strategy involves modifying the ribose at the C2' position. Substitutions such as 2'-O-methylation (2'-O-Me), 2'-O-methoxyethylation (2'-O-MOE), and 2'-fluoro (2'-F), are widely employed to improve RNA's thermal stability and nuclease resistance [67,68]. The 2'-O-methyl nucleotides (Nm) are prevalent in natural cells, typically found in the 5' cap structure (cap1 and cap2) of mRNAs and in non-coding RNAs like tRNAs and rRNAs. In eukaryotes, tRNAs are methylated by tRNA methyltransferase family (Trm), and rRNAs are methylated through a snoRNA-depended mechanism via methyltransferase fibrillarin (FBL) [69]. In human cells, two methyltransferases catalyze the 2'-O-methylation of mRNA cap, namely CMTr1 and CMTr2, which respectively promote methylation of cap1 and cap2 [70]. Capping at the 5' end is an essential step in the post-transcriptional modification of endogenous mRNAs. The 5' cap serves multiple vital functions, including protecting the mRNA from nuclease degradation, providing recognition tags, and facilitating translation initiation [71]. In recent years, research interest on mRNA 2'-O-Me modification has expanded beyond the 5' cap structure. With development of sensitive and high-throughput Nm-sequencing techniques, Nm sites in internal mRNA are mapped [72,73]. Though several studies reported that the rRNA methyltransferase FBL can also methylate specific nucleotides on mRNA and increase mRNA stability [74,75], whether other enzymes contribute to mRNA methylation and how these modifications influence gene expression is not clear yet. In the context of RNA therapeutics, 2'-O-Me modifications are frequently utilized to boost binding affinity to target nucleic acids, increase nuclease resistance, and mitigate immunological stimulation [67,76]. The 2'-O-MOE modifications, an enhanced derivative of

2'-O-Me, demonstrates increased affinity and nuclease resistance, playing a significant role in the development of ASO drugs [77–79].

Another category of ribose modifications involves the bridging of the C2' with another carbon site, usually C4' or C5', to introduce a bicyclic structure. A prime example is locked nucleic acid (LNA), which is formed by linking the 2'-O and 4'-C of the ribose with a methylene group (Figure 2) [16]. LNA modifications "lock" the RNA into a 3'-endo conformation, significantly enhancing its affinity and resistance to degradation by most nucleases [20,80,81]. Despite this, LNA-modified RNAs remain compatible with RNase H and RNA-induced silencing complex (RISC), rendering LNA a suitable tool for ASO and siRNA designs [82,83]. Regarding cancer treatment, new delivery strategies for LNA-based miRNA inhibitors are being developed. Nanoparticle-conjugated LNA-miRNA inhibitors can improve delivery to tumor cells, reducing off-target effects. For example, in one study, researchers utilized LNA antagonists to inhibit miR-222 in B chronic lymphocytic leukemia cell lines, and the results demonstrated a significant reduction in cell viability [84], strongly indicating the potential value of LNA inhibitors in cancer treatment. Recently, LNA has also been incorporated into miRNA inhibitor due to its steric blockage function [85], showing potential in cancer therapies such as miR-21 inhibitor against melanoma [86] and miR-92a inhibitor against endometrial cancer [87]. However, some studies have suggested that LNA modifications may increase the risk of hepatotoxicity [67,82]. Recent research has expanded the applications of LNA. In gene editing, LNA-modified guide RNAs (gRNAs) enhance the cleavage efficiency of the CRISPR-Cas system. For example, a study by Hendel *et al.* [88] demonstrated that LNA incorporation in gRNAs increased the stability of the gRNA-Cas9 complex, leading to more precise targeting at specific genomic loci.

Additionally, certain modifications alter the entire ribose ring structure. Two notable examples are unlocked nucleic acid (UNA), which is formed by opening the ribose ring, and phosphorodiamidate morpholino oligonucleotide (PMO), which replaces the ribose ring with a morpholino ring (Figure 2). In contrast to LNA, UNA slightly reduces the thermal stability of RNA duplexes but increases their flexibility [89], positioning as a potential candidate for siRNA development [81]. PMO modifies both the sugar ring and the phosphate group of nucleotides. In PMO, the ribose ring is replaced by a morpholino ring, and the phosphodiester linkage is substituted with a phosphorodiamidate linkage [90,91]. Due to its neutral charge, PMO is less recognizable by nucleases and less likely to interact with other intracellular proteins, thus reducing side effects. However, this incompatibility also precludes the use of PMO in therapeutics based on RNase H cleavage [92,93]. PMO is now utilized in the development of steric blocking ASO drugs for multiple clinical applications [67,77]. Recent advancements focus on delivery and combination therapies. Cellpenetrating peptides (CPPs) are being used to conjugate with PMO for more efficient cell entry. A study described new methods for the parallel chemical synthesis of peptide-PMO conjugates [94]. This work demonstrated the enhanced delivery of PMO-CPP conjugates into cells relevant to Duchenne muscular dystrophy research, which is also highly relevant for the delivery of PMO into neurons in the context of neurodegenerative diseases. The findings are crucial as efficient cell entry of PMO can potentially improve the targeting of neurodegenerative disease-related RNAs. PMO is also being explored in combination with other nucleic-acid-based therapies. As reported [95], the combination of different nucleic-acid-based agents can offer enhanced therapeutic efficacy. When PMO is combined with siRNAs, it can block decoy RNAs, thereby enhancing siRNA-mediated gene silencing. This combinatorial approach has shown improved treatment efficacy in cancer models. By leveraging

the unique properties of each nucleic-acid-based agent, the combination therapy can potentially overcome the limitations of single-agent therapies and provide more effective treatment options for various diseases.

In comparison to non-coding RNA therapeutics, ribose modifications in mRNA therapeutics are less common. The ribose modifications previously mentioned generally inhibit RNA recognition by enzymes, which may disrupt mRNA translation. In mRNA therapeutics, ribose modifications typically involve 2'-O-Me at the 5' end to form a 5' cap structure. For example, COVID-19 mRNA vaccines incorporate a type I cap structure to protect the mRNA from degradation [96]. The role of the 5' cap in mRNA vaccines will be discussed in greater detail later.

2.3. Phosphate modifications

Phosphate modifications, primarily introduced through biotechnological means and not occurring naturally in animal cells, are exemplified by phosphorothioate (PS) modification. These modifications replace one of the non-bridging oxygen atoms in the phosphate group with a sulfur atom (Figure 2). Invented in 1966, PS modifications were found to enhance nucleotide resistance to phosphatases [97]. They significantly improve the metabolic stability of nucleic acids, although they may reduce affinity for complementary nucleic acids [98]. Incorporating PS modifications into the RNA backbone can also enhance the transport and uptake of oligonucleotide therapeutics within the body [99] and does not interfere with the interaction between ASOs and RNase H [17,67]. These advantageous properties have made PS a highly promising modification, leading to its rapid integration into ASO development. PS can be classified into two stereoisomers based on the chirality of the phosphorothioate linkage: Rp and Sp (Figure 2). The Rp isomer forms more thermostable RNA complexes, while the Sp isomer shows greater resistance to nucleases [100,101]. Therefore, balancing the ratio of Rp and Sp isomers is crucial for achieving optimal outcomes. Moreover, a study has shown that a 3'-SpSpRp-5' sequence promotes more efficient RNase cleavage compared to random stereochemistry [100], suggesting that specific combinations of PS stereoisomers can generate additional steric effects.

The non-bridging oxygen atoms in the phosphate group can also be substituted with other atoms or groups, including boranophosphate (PB) [102], alkyl phosphonates [103], and phosphotriesters [104]. Compared to PS, PB modifications exhibit better nuclease resistance and lower cytotoxicity but reduce the thermostability of RNA duplexes [102,105]. Combining PB and PS modifications may be a viable strategy to enhance the biological activity of ASOs [106]. Alkyl phosphonates and phosphotriesters are neutral groups that inhibit protein binding and reduce cytotoxicity [103,104], making them promising for applications. Additionally, there are modification strategies that replace the entire phosphate group with other groups, such as substituting the phosphodiester linkage with an amide bond [107], which enhances the affinity of ASOs or siRNAs for their RNA targets [108]. The synthetic methods for the aforementioned chemical modifications are more complex than those for PS, and further research is required, limiting their widespread application. Nevertheless, they remain potential options for RNA drug design.

3. RNA modifications in biotechnology field

3.1. mRNA vaccines

mRNA vaccines furnish immune cells with the genetic blueprint of antigen proteins, enabling the production of antigen fragments recognized by the immune system. This recognition triggers immune responses, conferring protection against specific viruses, infectious diseases or even cancer. Structurally, mRNA vaccines mirror natural mRNAs, including a 5' cap structure, 5' untranslated region (5'-UTR), an open reading frame encoding desired antigens, a 3' untranslated region (3'-UTR), and a polyadenylate tail. Once inside the cell, the mRNA vaccine serves as a template for the translation of the encoded antigenic proteins, activating the adaptive immune response and ultimately resulting in the desired vaccine effect [14,109]. The first mRNA influenza vaccine, developed in the 1990s, successfully induced cytotoxic T lymphocytes (CTLs) with anti-influenza capabilities in mice [110]. In 2017, Pardi *et al.* formulated an mRNA vaccine against the Zika virus [111], which has since advanced through Phase I clinical trials [112]. The recent SARS-CoV-2 pandemic has seen the emergency production support for numerous mRNA vaccines, including Pfizer's BNT162b2 [113] and Moderna's mRNA-1273 [114], which have significantly contributed to combating COVID-19 and garnered increased attention from the academic community and the public.

Ψ and $m^1Ψ$ are the most extensively utilized base modifications in mRNA vaccines. In 2005, Karikó and Weissman demonstrated that replacing U with Ψ in exogenous mRNA alters its secondary structure, thereby inhibiting the activation of Toll-like receptors within cells and significantly reducing mRNA immunogenicity [23]. Later, it was found that $m^1Ψ$ modifications enhance mRNA translation more effectively than Ψ modifications, while also diminishing immunogenicity and cytotoxicity [19,115]. The majority of recent mRNA vaccines have adopted $m^1Ψ$ in place of U, with the COVID-19 mRNA vaccines being the most notable examples. Pfizer's BNT162b2 vaccine, which employs T7 RNA polymerase and incorporates $m^1Ψ$-TP as raw materials, replacing all U residues in the 5'-UTR, 3'-UTR, and coding sequence of the mRNA with $m^1Ψ$, thereby enhancing the vaccine's stability and translation activity [96].

In addition to Ψ and $m^1Ψ$, m^5C can also increase the stability and translation activity of mRNA [19,116,117]. Currently, m^5C modifications are predominantly utilized in the development of cancer mRNA vaccines [118]. Verbeke *et al.* administered m^5C/Ψ-modified mRNAs, along with the TLR agonist MPLA, to mice and they observed that this combination enhanced the vaccine's safety and translation activity, effectively inducing T cell immune responses [119]. Wang *et al.* developed a co-delivery strategy that combines m^5C-modified mRNAs with Ψ-modified siRNAs. They demonstrated that the mRNA vaccine induced robust T cell immunity and humoral immune responses in a C57BL/6 mouse model with B16F10 melanoma, effectively inhibiting tumor growth [120]. Andries *et al.* demonstrated that mRNA with both m^5C and $m^1Ψ$ modifications exhibited better translation activity and lower cytotoxicity *in vitro* transfection experiments compared to mRNA with only m^5C or Ψ modifications [19]. Although the difference in activity was less pronounced in mouse experiments, it still suggested that the $m^5C/m^1Ψ$ combination possesses greater therapeutic potential.

m^6A modifications have also been considered as an alternative for base modifications in mRNA vaccines. Starostina *et al.* demonstrated that incorporating 20% m^6A into fully Ψ-modified mRNA vaccines significantly reduces mRNA cytotoxicity without compromising translation activity [121]. However, research on the effectiveness and safety of m^6A modifications in mRNA vaccines is still limited,

and the advantages m^6A has shown are relatively modest. As mRNA vaccines incorporating Ψ and $m^1Ψ$ with proven effectiveness have been extensively developed, m^6A has not been widely adopted yet.

Apart from base modifications, it is also critical to equip mRNA vaccines with certain structural elements, such as 5' cap and poly (A) tail. 5'-capping is an essential step in mRNA vaccines design, as 5' cap structure is required for protecting mRNA from nuclease, facilitating translation and reducing immunogenicity [71,122]. In mRNA vaccine development, vaccinia capping enzymes are frequently used to add various types of 5' caps to the mRNA, rendering exogenous mRNA more similar to natural mRNAs [123]. Poly (A) tail also effects mRNA stability and translation. In natural mRNA, poly(A) tails with different length dynamically regulate gene expression. In mRNA vaccine, poly (A) tails are added into mRNA by enzymatic polyadenylation or *in vitro* transcription, enhancing the longevity of the vaccine [122,124]. In addition, codon optimality determines mRNA stability by effecting translation elongation [125]. Hence, codon optimization is another crucial factor for mRNA vaccine, and this process is usually accomplished in silico. Recently, Zhang *et al.* introduced a novel algorithm LinearDesign for codon optimization, and profoundly increase the half-life and codon usage in mRNA vaccine for COVID-19 and varicella-zoster virus [126]. These strategies not only enhance the stability of mRNA vaccines in cellular environments but also improve their efficiency in inducing immune responses, providing a powerful tool for mRNA vaccine development.

3.2. Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short synthetic nucleic acids that can specifically bind to target mRNAs through Watson-Crick base pairing. The mechanisms of ASOs are mainly categorized into two types: steric hindrance to block protein translation, and the recruitment of nucleases, such as RNase H1, to degrade target RNA and modulate gene expression [13,127]. Unmodified oligonucleotides are rapidly degraded by nucleases in the internal environment and within cells. Consequently, extensive chemical modifications are often incorporated into ASOs to enhance stability, reduce immune responses, and minimize off-target toxicity. In ASOs, the major modifications involve phosphate modifications and ribose modifications, such as PS and PMO, while base modifications are less frequently used. Table 1 lists some of the chemical modifications included in FDA-approved ASO drugs, while some ASO drugs in development phase are listed in Table 3.

Table 1. Approved ASO drugs.

Name (Approval time)	Chemical modifications	Indication	References
Eteplirsen (2016)	PMO	DMD	[128,129]
Nusinersen (2016)	2'-O-MOE, PS, m^5C	Spinal muscular atrophy	[130,131]
Inotersen (2018)	2'-O-MOE, PS	ATTRv	[132]
Milasen (2018)	2'-O-MOE, PS, m^5C	Neuronal ceroid lipofuscinosis 7	[133]
Golodirsen (2019)	PMO	DMD	[134]
Viltolarsen (2020)	PMO	DMD	[135]
Casimersen (2021)	PMO	DMD	[136,137]

Table 1. *Cont.*

Name (Approval time)	Chemical modifications	Indication	References
Eplontersen (2023)	2'-O-MOE, PS	ATTRv	[138]
Tofersen (2023)	2'-O-MOE, PS, m^5C	Amyotrophic lateral sclerosis	[139]
Olezarsen (2024)	2'-O-MOE, PS, m^5C	Familial chylomicronemia syndrome	[140]

PMO, phosphorodiamidate morpholino oligonucleotide. 2'-O-MOE, 2'-O-methoxyethyl. PS, phosphorothioate. m^5C, 5-methylcytosine. DMD, Duchenne muscular dystrophy. ATTRv, hereditary transthyretin amyloidosis.

3.3. Small interfering RNA

Small interfering RNA (siRNA) is a class of small double-stranded RNA molecules capable of mediating gene silencing through the mechanism of RNA interference. Inside the cell, siRNA engages proteins such as Argonaute-2 and Dicer to assemble the RISC. Within this complex, one strand of the siRNA is discarded, while the other strand aligns with the target mRNA through complementary base pairing and leads to the mRNA's cleavage by RISC. Unlike ASOs, RISC can dissociate after cleaving one mRNA molecule and then proceed to bind and cleave additional mRNAs. This "catalytic" mechanism enables siRNAs to repress gene expression even at low concentrations [141]. Though gene silencing by siRNA requires full complementary between siRNA and the target mRNA, off-target effect is possible, proposing that balance between affinity and specificity is an important consideration when designing siRNA drugs [142]. And chemical modification is an effective strategy.

Incorporation of RNA modifications in siRNA drugs is strategically designed to diminish immunogenicity, enhance stability, and decrease off-target toxicity. As shown in Table 2, the predominant modifications in siRNAs encompass ribose modifications, including 2'-O-methylation (2'-O-Me), 2'-O-methoxyethylation (2'-O-MOE), and locked nucleic acid (LNA), alongside phosphate modifications like phosphorothioate (PS) [12,141]. In the initial phases of development, siRNA drugs often featured partial modifications. For example, patisiran, an siRNA drug for treating hereditary transthyretin-mediated amyloidosis (ATTRv), only incorporates 2'-O-Me modifications on a small number of nucleotides [143]. With technological advancements, the majority of contemporary siRNA drugs now favor comprehensive modifications of both RNA strands, such as inclisiran [144] and nedosiran [145]. Base modifications are less commonly applied in approved siRNA drugs, suggesting that their identification and application may represent a promising avenue for future research. As shown in Table 3, siRNA drugs are being applied to an increasing number of diseases, demonstrating broader therapeutic potential.

Table 2. Approved siRNA drugs.

Name (Approval time)	Chemical modifications	Indication	References
Patisiran (2018)	2'-O-Me	ATTRv	[143]
Givosiran (2019)	PS, 2'-O-Me, 2'-F	Acute hepatic porphyrias	[146]
Inclisiran (2020)	PS, 2'-O-Me, 2'-F	Hypercholesterolemia	[144]
Lumasiran (2020)	PS, 2'-O-Me, 2'-F	Primary hyperoxaluria type 1	[147]

Table 2. *Cont.*

Name (Approval time)	Chemical modifications	Indication	References
Vutrisiran (2022)	PS, 2'-O-Me, 2'-F	ATTRv	[148]
Nedosiran (2023)	PS, 2'-O-Me, 2'-F	Primary hyperoxaluria type 1	[145]

2'-O-Me, 2'-O-methyl. PS, phosphorothioate. 2'-F, 2'-fluoro. ATTRv, hereditary transthyretin amyloidosis.

Table 3. Some ASO and siRNA drugs in clinical trials.

Type	Name	Company	Chemical modifications	Indication	Status	References
ASO	Brogidirsen	Nippon Shinyaku	PMO	DMD	Phase 1/2	[149]
	Pelacarsen	Novartis & Ionis	2'-O-MOE, PS	Cardiovascular disease	Phase 3	[150]
	Donidalorsen	Ionis	2'-O-MOE, PS	Hereditary Angioedema	Phase 3	[151]
	Bepirovirsen	GSK	2'-O-MOE, PS	Chronic hepatitis B	Phase 2	[152]
siRNA	Zilebesiran	Alnylam	2'-O-Me, 2'-F	Hypertension	Phase 2	[153]
	Fitusiran	Alnylam & Sanofi	PS, 2'-O-Me, 2'-F	Haemophilia A & B	Phase 3	[154]
	Olpasiran	Eli Lilly	PS, 2'-O-Me, 2'-F	Cardiovascular disease	Phase 2	[155]
	Plozasiran	Arrowhead	Not available	Hyperlipidemia	Phase 2	[156]

Clinical trials listed in this table refer to the phases that the drug has completed. PMO, phosphorodiamidate morpholino oligonucleotide. 2'-O-MOE, 2'-O-methoxyethyl. PS, phosphorothioate. 2'-O-Me, 2'-O-methyl. 2'-F, 2'-fluoro. DMD, Duchenne muscular dystrophy.

3.4. RNA aptamer

RNA aptamers are single-stranded oligonucleotides with certain tertiary structures that exhibit high specificity and affinity for target molecules, ranging from small organic compounds to proteins. The broad scale of target types makes RNA aptamers capable in various scenarios requiring molecular recognition, including but not limited to disease treatment, disease diagnosis, and pollutant detection [157]. Most RNA aptamers are generated through systematic evolution of ligands by exponential enrichment (SELEX), a standard method involving iterative processes of incubation, separation and amplification [157,158].

RNA modification can be introduced into aptamers before SELEX for increasing the structure variety, or after SELEX for enhancing the properties of selected aptamers (Table 4) [158]. For example, post-SELEX incorporation of 2'-F and 2'-O-Me can improve nuclease resistance of aptamers, which have been utilized in the first RNA aptamer drug pegaptanib [159]. Gruenke *et al.* built ribose modified libraries of pyrimidines with 2'-F, 2'-O-Me and 2'-NH_2 modifications for SELEX, and obtained a 2'-F modified RNA aptamers with high affinity to HIV-1 reverse transcriptase [160]. Other types of modifications, including base and phosphate modifications, can also be brought into RNA aptamers to improve the affinity and stability [158,161]. Besides, employing L-RNA, the mirror-image nucleotide of natural RNA, for RNA aptamers is another novel strategy to enhance the stability and selectivity. Several L-RNA aptamers showed promising prospect in clinical trial [162]. In 2023, FDA approved the

second RNA aptamer drug avacincaptad pegol, which was modified with 2'-F and 2'-O-Me, indicating that the RNA aptamer is still a promising therapy and chemical modification plays an irreplaceable role in aptamer.

Table 4. RNA aptamers that completed Phase 2 trial.

Name	Chemical modifications	Indication	Status	References
Pegaptanib	2'-F, 2'-O-Me	Age-related macular degeneration	Approved in 2004	[159]
Avacincaptad pegol	2'-F, 2'-O-Me	Age-related macular degeneration	Approved in 2023	[163]
Olaptesed pegol	L-RNA	Chronic lymphocytic leukemia, glioblastoma	Phase 1/2	[164,165]
Emapticap pegol	L-RNA	Type 2 diabetes	Phase 2a	[166]

Clinical trials listed in this table refer to the phases that the drug has completed. 2'-O-Me, 2'-O-methyl. 2'-F, 2'-fluoro.

3.5. CRISPR-Cas9

Gene editing technology is a modern biotechnological approach that involves precise addition, deletion, or reprogramming of specific DNA sequences within an organism's genome. This technology has significantly propelled biological research and has been widely applied in both laboratory experiments and clinical trials. A significant breakthrough in gene editing technology is the emergence of the CRISPR-Cas9 system (CRISPR: Clustered regularly interspaced short palindromic repeats, Cas: CRISPR-associated), which originates from the adaptive immune system in bacteria. The CRISPR system uses RNA (including crRNA and tracrRNA) to guide Cas endonucleases to specific DNA sequences for site-specific cleavage [167]. In the CRISPR-Cas9 gene editing technology, the Cas9 protein forms a RNP with a designed single-guide RNA (sgRNA), targeting and cleaving the specified DNA site [15,168]. The CRISPR-Cas9 system has rapidly become a central focus in gene editing research due to its high efficiency, simplicity, and programmability. However, as CRISPR-Cas9 editing derives from bacteria, it is possible to stimulate human immunological response, inducing Cas9-reactive T cells [169]. Furthermore, a major challenge for CRISPR-Cas9 is the off-target effect [170]. The off-target effect is a mechanistic defect caused by mismatch between sgRNA and genomic DNA, and RNA modification on sgRNA is a promising solution.

RNA modification techniques can be applied to sgRNAs to enhance the editing efficiency of the CRISPR-Cas9 system [171]. Hendel *et al.* introduced 2'-O-Me, 2'-O-methyl-3'-phosphorothioate (MS) and 2'-O-methyl-3'-thiophosphonoacetate (MSP) modifications in the three nucleotides at the 3' and 5' ends of sgRNA, demonstrating that these modifications could increase sgRNA stability and reduce off-target effects of CRISPR [172]. Yin *et al.* modified the invariant part of the sgRNA with 2'-O-Me and 2'-F, showing that these modifications could enhance the stability of the sgRNA without affecting its ability to bind with Cas9 [173]. Additionally, a study reported that LNA modifications in specific regions of the crRNA can reduce off-target effects by up to 23,000-fold. They also introduced another novel bridged nucleic acid BNA^{NC} (2', 4'-BNA^{NC}[N-Me]) which improved crRNA specificity more effectively than LNA, suggesting that bridged ribose modification might be a useful tool to improve the specificity of CRISPR cleavage [174]. Furthermore, replacing RNA residues with DNA residues is another

modification option for gRNA. Substituting the first 10 ribonucleotides at the 5' end of the crRNA with deoxyribonucleotides can effectively reduce off-target effects and lower synthesis costs [175].

4. Conclusion

RNA modifications are pivotal mechanisms for regulating gene expression, as they alter the structure, stability, and function of RNA molecules, playing crucial roles in biological processes. Base modifications, such as pseudouridylation, cytosine methylation, and adenine methylation, have been shown to change the physicochemical properties of RNA, enhance its resistance to nucleases, reduce immunogenicity, and optimize its functionality within cells [1–3]. Ribose modifications, such as 2'-O-Me and LNA, improve the thermostability and nuclease resistance of RNA, thereby facilitating the *in vivo* transport and uptake of RNA therapeutics [76,80]. Phosphate modifications, such as PS, further enhancing the stability and functionality of RNA [17].

Emerging studies highlight the importance of understanding RNA modifications not merely as isolated alterations but as components of a dynamic regulatory network. For instance, while $m^1\Psi$ and m^5C independently enhance mRNA stability and translation efficiency, their combined use in mRNA vaccines has been shown to synergistically reduce immunogenicity while amplifying antigen production [19,115]. These findings underscore the potential for multi-modification strategies to optimize RNA functionality, though systematic studies mapping these interactions remain sparse. A study by Karikó *et al.* [176] demonstrated that pseudouridine modifications in mRNA not only enhance translation efficiency but also reduce immune activation, providing a foundation for understanding how combined modifications like Ψ and m^5C can synergistically improve RNA-based therapeutics.

The selection of RNA modifications must align with the therapeutic context: 1) Pseudouridylation *vs.* Cytosine Methylation: In the development of mRNA vaccines, the selection of RNA modifications needs to be closely aligned with the therapeutic context. Pseudouridylation and cytosine methylation have different application emphases. Pseudouridine is highly favored in mRNA vaccines due to its dual functions of reducing innate immune activation and enhancing translation efficiency [18,19], whereas m^5C is often prioritized in cancer vaccines due to its ability to stabilize mRNA and enhance immunogenicity. Regarding cancer vaccines, although it cannot be simply concluded that m^5C is more advantageous than other modifications, the m^5C modification has unique effects in stabilizing mRNA and enhancing immunogenicity. For example, Verbeke *et al.* combined mRNAs containing both m^5C and Ψ modifications with the TLR agonist MPLA, and demonstrated a promising anti-tumor immune effect in melanoma models. This study indicates that the strategy of combining m^5C modification with other modifications and TLR agonists in the design of cancer vaccines has potential application value, providing new ideas for optimizing the immune effect of cancer vaccines [119]. 2) Base *vs.* Ribose Modifications: For siRNA, ribose modifications like 2'-O-Me and LNA are critical for nuclease resistance and reducing off-target effects [67], while base modifications (e.g., m^6A) are more relevant in ASOs to modulate protein-RNA binding. The FDA-approved siRNA drug inclisiran exemplifies this principle, utilizing 2'-O-Me and PS modifications to achieve long-term cholesterol reduction [144]. 3) Phosphate Modifications: PS linkages remain indispensable for ASO pharmacokinetics due to their resistance to serum nucleases and improved tissue penetration [17]. However, recent advances in stereochemistry-controlled PS isomers (e.g., 3'-SpSpRp sequences) have further optimized their efficacy and safety [100].

Despite these advances, critical questions remain unresolved: 1) Interplay Between Modifications: How do multiple modifications co-occurring on the same RNA molecule influence its structure and function? For example, does Ψ in the coding region interfere with m^6A-mediated mRNA decay pathways? Recent studies have revealed that the co-occurrence of Ψ and m^6A on RNA molecules can lead to intricate crosstalk, where Ψ may alter RNA secondary structure or interfere with m^6A-binding proteins, potentially suppressing m^6A-mediated mRNA decay pathways and enhancing mRNA stability [53,177]. 2) Long-Term Effects: The long-term biological effects of RNA modifications, such as Ψ and m^6A, remain a critical concern in therapeutic applications. Rigorous preclinical studies and extended clinical monitoring are essential to evaluate potential risks, including immune activation and unintended protein aggregation [178]. Prospective clinical trials monitoring modified RNA therapeutics over extended periods are urgently needed. 3) Context-Specific Rules: Predictive models to guide modification selection (e.g., Ψ for immunogenicity reduction *vs.* m^5C for stability) are lacking, necessitating large-scale comparative studies. Although RNA modification technologies have been widely applied in the development of mRNA vaccines, siRNAs, and ASOs, several key challenges and issues remain in fully harnessing the advantages of RNA modifications.

Firstly, the function mechanisms of many natural RNA modifications remain to be fully explored, which limits their further application. Currently, the base modifications most commonly used in RNA therapeutics are Ψ, $m^1Ψ$, and m^5C, while other base modifications have been less studied for their effects on exogenous RNA. Other natural base modifications, such as m^6Am, ac^4C, I, and m^7G, also influence the structure and function of RNA [1,2] and theoretically have the potential for biotechnological applications. In addition, there is interplay between certain modifications. For example, Xiang *et al.* discovered that m^6A depletion increases A-to-I editing through promoting association of adenosine deaminase acting on RNA ADAR [179]. Further research is needed for a comprehensive interaction network of RNA modifications. The location of most modifications is not limited to specific classes of RNA, and many RNA modification proteins target both coding and non-coding RNAs. The same RNA modification may have different functions in different classes of RNAs. For some RNA modifications that are present at low levels, there is currently a lack of high-resolution detection methods. Developing efficient and accurate techniques to determine the types and distribution of RNA modifications *in vivo* may become a promising research direction [180,181]. A comprehensive understanding of the functions, mechanisms, and regulatory signaling networks of natural RNA modifications will help to better utilize RNA modifications to enhance the efficacy of exogenous RNA and aid in expanding the repertoire of RNA modifications available for human use.

Furthermore, safety concerns are also a crucial consideration in the development of RNA modification technologies. In 2024, Mulroney *et al.* revealed that COVID-19 mRNA vaccines containing $m^1Ψ$ might cause ribosomal frameshifting, resulting in the translation of aberrant proteins [182]. However, by this time, the mRNA vaccines had already been widely inoculated, suggesting that some side effects caused by chemical modifications in RNA therapeutics may not be fully evident during clinical trials, and long-term monitor is necessary. Currently, most drug studies are conducted in animal models rather than in humans, which means it is not yet fully determined whether the RNA modifications in these drugs will have the same efficacy and safety profiles in the human body [183]. Due to the complex recognition mechanisms of RNA modifications within cells, it is essential to thoroughly consider the potential off-target effects of RNA modifications. This suggestion aims to prevent the special RNA

modifications carried by exogenous RNA from interfering with normal gene expression regulation or signaling pathways. Additionally, for non-natural chemical modifications, such as specific ribose and phosphate modifications, it is crucial to extensively study their interactions with intracellular proteins and other natural nucleic acids to minimize their cytotoxicity.

How to utilize the diverse array of RNA modifications for drug design more effectively is a promising research direction as well. Currently, RNA-based therapies have widely exploited RNA modifications, and many approved nucleic acid drugs incorporate multiple types of chemical modifications together. By combining different chemical modifications and determining their optimal sequences and positions within the RNA, it is possible to further optimize pharmacological properties and reduce potential risks. Besides, given that RNA modifications might show different functions in different RNA context, selecting the appropriate modifications for RNA-based therapies according to specific application requirements is a critical consideration in drug development. With the large diversity of RNA modifications, there are a huge quantity of potential selections and possible combinations of modifications for one single RNA sequence, making it impractical to compare them all through wet-lab experiments. Using molecular stimulation techniques to model modified RNAs, or employing artificial intelligence to screen for the optimal modification schemes in a high throughput way, may be a feasible approach to addressing this challenge. Machine learning methods have been employed in the design of mRNA vaccines with consideration of certain base modifications [184]. In the future, similar approaches may be applicable to the design of other RNA-based therapies, fully considering various chemical modifications and their combinatorial effects.

In conclusion, future research in RNA modifications should prioritize three key directions to unlock their full potential in biotechnology and medicine. First, systems-level analyses, such as high-throughput screening and single-molecule sequencing, can map modification combinations and their functional outcomes. For example, foundational work by Karikó and Weissman demonstrated how nucleoside modifications (e.g., pseudouridine) reduce immunogenicity while enhancing mRNA stability, providing a critical framework for therapeutic design [23]. Second, AI-driven design can accelerate the optimization of RNA modifications. Recent studies have leveraged machine learning to predict codon usage and modification patterns, significantly improving mRNA vaccine efficacy [185]. Third, standardized frameworks are essential to ensure safety and scalability. Collaborative initiatives like the Epitranscriptomics Consortium can harmonize evaluation protocols, as exemplified by clinical trials of personalized mRNA cancer vaccines [186]. By advancing these priorities, RNA modification technologies will drive transformative breakthroughs in disease treatment and precision medicine.

Acknowledgments

Funding: This work was supported by grants from the National Natural Science Foundation of China (No.32401274), the CAMS Innovation Fund for Medical Sciences (No. CIFMS-2021-I2M-5-015), the Excellent Postdoctoral Program of Jiangsu Province (2023ZB697) and Natural Science Foundation of Jiangsu Province (BK20230786).

Conflicts of interests

The authors declare no conflicts of interest.

Authors' contribution

Conceptualization, Z.F. and H.G.; methodology, Z.F. and Y.L. (Yongle Liu); software, Y.L. (Yihang Li); validation, Y.L. (Yihang Li), Y.L. (Yongle Liu) and Z.F.; formal analysis, Y.L. (Yihang Li); investigation, Y.L. (Yihang Li) and Y.L. (Yongle Liu); resources, Y.L. (Yongle Liu); data curation, Y.L. (Yihang Li); writing—original draft preparation, Y.L. (Yihang Li) and Y.L. (Yongle Liu); writing—review and editing, Z.F. and Y.L. (Yongle Liu); visualization, Z.F. and Y.L. (Yongle Liu); supervision, Z.F. and H.G.; project administration, H.G.; funding acquisition, H.G. All authors have read and agreed to the published version of the manuscript.

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