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Chemical engineering strategies to enhance mRNA–LNP stability for therapeutic applications

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The inception of mRNA vaccines for COVID-19 has catalyzed a transformative shift in the field of vaccination, offering expeditious, scalable, and potent countermeasures to a global health emergency. Despite significant advances, mRNA remains inherently unstable under physiological conditions due to its susceptibility to degradation by ubiquitous ribonucleases and physicochemical factors, making its storage, transport and clinical application challenging. This review explores the critical determinants influencing mRNA stability and discusses how chemical engineering strategies are suited to enhance mRNA stability, including 5′ cap modification, poly(A) tail engineering, optimization of untranslated regions, as well as coding sequence refinements, reversible 2′-OH acylation, the development of circular RNA constructs and self-amplifying RNA systems. We also discuss efforts towards mRNA immunogenicity regulation and advanced mRNA delivery systems, along with progress in storage and transport solutions, which have further contributed to addressing stability concerns. Finally, we discuss the remaining challenges in clinical translation and provide forward-looking perspectives on emerging mRNA-based technologies.

1. Introduction

Recent advances in mRNA therapy have revolutionized modern medicine, exemplified by the rapid development of COVID-19 vaccines and promising applications in cancer immunotherapy.[1] Innovations in nucleotide chemistry like pseudouridine modification and lipid nanoparticles (LNPs) have enhanced delivery efficiency and reduced immunogenicity, enabling mRNA to transiently express therapeutic proteins at high levels.[2] mRNA's versatility now extends to protein supplementary therapy and gene editing, offering solutions for previously untreatable genetic disorders.[3] Despite these breakthroughs, the inherent instability of mRNA due to rapid enzymatic degradation and hydrolysis profoundly impacts cellular processes by governing the duration of protein expression, thereby influencing the effectiveness and safety of mRNA-based therapies.[4–7] It necessitates cold-chain storage, complicates dosing regimens, and restricts therapeutic protein yields, particularly in systemic applications.[8,9] Enhancing stability is critical to prolonging mRNA half-life *in vivo*, minimizing manufacturing or logistical burdens, and ensuring consistent therapeutic effects, especially for chronic conditions requiring sustained protein expression.[5]

In the realm of mRNA vaccines, enhanced stability can mitigate degradation during storage and delivery, potentially simplifying logistical challenges associated with cold-chain requirements.[10] Furthermore, prolonged mRNA stability within target cells can extend protein expression, thereby enhancing both the duration and potency of immune responses triggered by vaccines, crucial for bolstering protective immunity against infectious diseases.[11] Likewise, delivering mRNA with a prolonged lifespan to express therapeutic protein production can prolong the therapeutic window by sustaining protein expression.[12] This capability not only enhances therapeutic efficacy but also holds promise for reducing dosing frequencies, thereby improving patient compliance and treatment outcomes. Fundamentally, mRNA stability refers to its resilience against enzymatic degradation pathways within cells and extracellular environments.[13] This property is intricately regulated by RNA-binding proteins (RBPs), untranslated regions (UTRs), and secondary structures that influence mRNA susceptibility to decay.[14] Advancements in strategies to enhance mRNA stability encompass a range of approaches, including optimization of the mRNA structure and the incorporation of modified nucleotides or chemical modifications into mRNA sequences to confer resistance to degradation.[14] Additionally, delivery systems such as LNPs are employed to

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shield mRNA from enzymatic attack and facilitate efficient cellular uptake, promoting sustained protein expression.[15] While COVID-19 mRNA vaccines have demonstrated success, ongoing efforts to enhance mRNA stability remain crucial for addressing the logistical challenges and expanding the mRNA technology to a broader range of diseases.[16–18]

This review focuses on chemical engineering strategies aimed at improving the stability of mRNA–LNP formulations, a critical factor for the success of mRNA-based therapeutics. We cover the basic mechanisms that mediate mRNA decay and outline current technique progress, with exemplary studies, to demonstrate how advances in mRNA–LNP chemical engineering have improved its stability and assisted successful therapies, spanning nucleotide chemical modifications, codon optimization, terminal and backbone engineering, and improved mRNA construction (circRNA), which collectively enhance mRNA integrity and translation efficiency. We then explore LNP formulation advancements, such as the development of low-immunogenicity LNPs and the design of thermostable LNPs for enhanced stability and performance. Innovations in storage and handling, including lyophilization and spray drying, are also discussed for their role in extending shelf life and preserving therapeutic functionality. Finally, we address the challenges of clinical translation and highlight emerging developments in mRNA design and delivery that enhance therapeutic precision and durability. Together, these strategies form a cohesive framework for engineering more stable, effective, and scalable mRNA–LNP therapeutics.

2. Determinants of mRNA stability

mRNA stability influences gene expression outcomes, reflecting a balance between intrinsic molecular features and cellular or environmental factors that determine degradation rates and protein production. This section outlines key determinants: primary sequence, ribonuclease (RNase) activity, chemical degradation and thermal stress, which are essential for advancing mRNA-based therapeutics.

2.1. Intrinsic sequence and structural determinants

The structural integrity and stability of mRNA molecules are fundamentally determined by their nucleotide sequence and structural features. Sequences enriched in guanine–cytosine (GC) pairs exhibit greater stability due to the stronger triple hydrogen bonds formed, compared to the dual bonds in adenine–uracil pairs.[19] However, excessive GC content can form rigid secondary structures that impede translation initiation or recruit degradation machinery.[20] Intramolecular structures, such as stem-loops and hairpins, can either shield mRNA from ribonuclease cleavage by masking vulnerable sites or promote decay by serving as recognition motifs for degradation complexes, depending on their sequence context and position.[21,22] Codon usage also plays a critical role in mRNA stability, as codons aligned with abundant transfer RNA (tRNA) pools enhance translation elongation rates, thereby stabilizing mRNA by reducing ribosome stalling and exposure to nucleases.[23]

2.2. RNases

RNases are pivotal in modulating mRNA stability by catalysing the cleavage of phosphodiester bonds, facilitating RNA degradation and regulation. RNases are ubiquitous across organisms and tissues, contributing to cellular homeostasis by regulating RNA turnover and maturation.[24] RNases are categorized into exoribonucleases, which degrade RNA from the 5′ or 3′ termini, and endoribonucleases, which cleave RNA internally. Cellular protective mechanisms, including 5′ capping, 3′ polyadenylation, RNA duplex formation, and ribonuclease inhibitors, mitigate RNase activity to preserve mRNA integrity.

2.2.1. Exoribonucleases. Exoribonucleases degrade mRNA processively from either the 5′ or 3′ end, playing a central role in eukaryotic mRNA decay *via* deadenylation-dependent or independent pathways.[25] In deadenylation-dependent decay, the poly(A) tail is shortened by deadenylases, triggering 5′ → 3′ degradation by the XRN1 exoribonuclease following DCP1/DCP2-mediated decapping, or 3′ → 5′ degradation by the exosome complex.[26,27] Deadenylation-independent pathways, such as nonsense-mediated decay (NMD), target mRNAs with premature termination codons (PTCs) for rapid 5′ → 3′ degradation without prior deadenylation. The nonstop decay (NSD) pathway further eliminates transcripts lacking stop codons, preventing aberrant protein synthesis. NSD involves ribosome stalling at the 3′ end, followed by 3′ → 5′ exosomal degradation mediated by the Ski7 protein[28] (Fig. 1A). Poly(A)-binding protein cytoplasmic 1 (PABPC1) is a key regulator, protecting poly(A) tails from deadenylases like poly(A)-specific ribonuclease (PARN) through steric hindrance and recruiting factors for translation and mRNA export.[29,30] Conversely, RNA helicases (such as those associated with AU-rich elements) can displace PABPC1, accelerating deadenylation and mRNA decay.[31,32] These opposing mechanisms ensure precise control over mRNA stability.

2.2.2. Endoribonucleases. Endoribonucleases cleave mRNA internally, generating unprotected fragments susceptible to further exoribonucleolytic degradation. These enzymes, often components of complexes like the exosome, are critical in pathways such as endonuclease-mediated decay (EMD)[33] and NMD,[34] which eliminate defective transcripts harbouring PTCs. Key endoribonucleases, including Argonaute proteins (AGO) and SMG6, mediate sequence-specific cleavage, while non-coding RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) guide mRNA degradation or translational repression[35,36] (Fig. 1B). RNase H enzymes maintain genome stability by removing harmful RNA/DNA hybrids that form during defective mRNA biogenesis processes such as transcription, splicing, export and degradation.[37] The interplay of mRNA sequences, secondary structures, and RBPs tightly regulates endonuclease activity, ensuring selective degradation of aberrant transcripts.

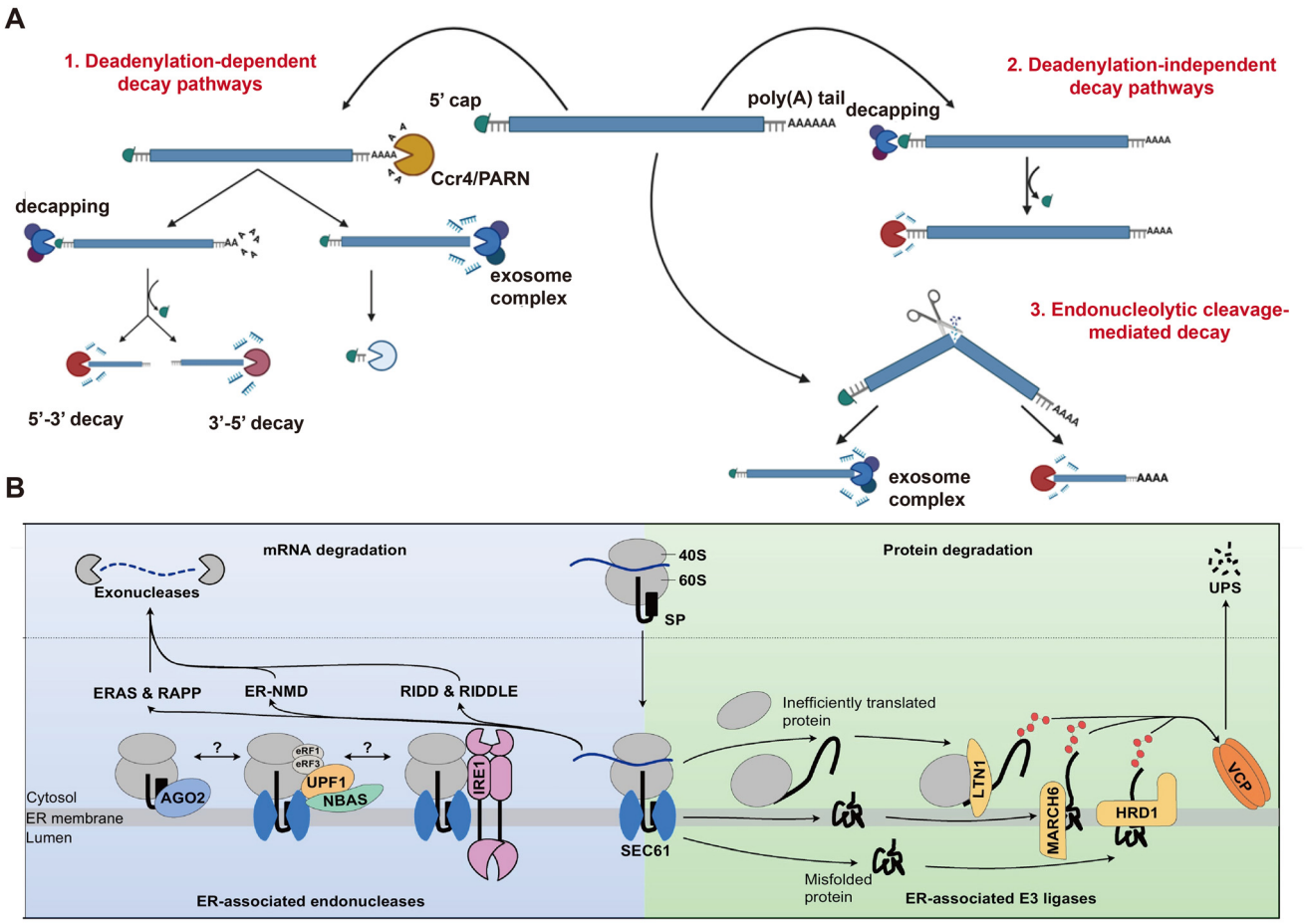

Fig. 1 RNases mediate mRNA decay. (A) Mechanisms of exoribonucleases: (1) deadenylation-dependent or (2) deadenylation-independent mRNA decay and (3) endonuclease-mediated mRNA decay. Graphics were created with BioRender.com (access date: March 29, 2023). (B) Model of endoplasmic reticulum (ER)-associated RNA decay pathways and their integration with ER quality control (ERQC). Reproduced with permission from ref. 36. Copyright 2024, Elsevier.

2.3. Physicochemical factors

Physicochemical factors such as ionic fluctuations, oxidative conditions, and pH variation can significantly compromise mRNA integrity. These factors destabilize mRNA by disrupting its secondary and tertiary structures, which are essential for maintaining its functional conformation. In addition, they can cleave the phosphodiester backbone of the mRNA molecule, leading to fragmentation and loss of coding potential. Such structural damage reduces the stability of the mRNA, making it more susceptible to degradation both *in vitro* and *in vivo*. As a result, the efficiency of translation is diminished, leading to lower protein expression levels. Together, these effects limit the durability and effectiveness of mRNA-based technologies, posing challenges for their use in both research and therapeutic applications.

2.3.1. Hydrolytic degradation. Unlike DNA, mRNA contains a reactive 2′-hydroxyl (2′-OH) group on ribose, which promotes intramolecular transesterification *via* alkaline catalysis. The 2′-OH turns into 2′-O^- and acts as a nucleophile attacking the adjacent phosphodiester bond which triggers strand cleavage or reversible isomerization. This nucleophilic attack cleaves the RNA chain by breaking phosphodiester linkages and turns into 2′,3′-cyclic phosphodiester and 5′-OH cleavage products, thereby reducing mRNA stability.[38,39] This process is modulated by multiple factors. Buffers such as imidazole, morpholine and carboxylates can catalyse mRNA strand cleavage and isomerization,[40] as can metal ions including Mg^{2+}, Zn^{2+} and Fe^{3+} (Fig. 2B and C).[41–43] Trace metal contamination during buffer preparation can destabilize mRNA. Additionally, hydrolysis rates increase under alkaline pH and thermal stress, highlighting the importance of storing mRNA at low temperatures (−20 °C to −70 °C) and maintaining a neutral pH (~7).

2.3.2. Oxidative degradation. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and the capacity of cellular antioxidant defences. mRNA is highly vulnerable to ROS such as hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), superoxide (O_2^{-}), and singlet oxygen ($^1O_2$), which are generated from ionizing radiation, photolysis, or metal-catalysed redox reactions. ROS alters mRNA turnover through multiple mechanisms, including base oxidation and strand breaks. For example, ROS can induce oxidative damage, thus producing by-products such as 8-oxo-G,

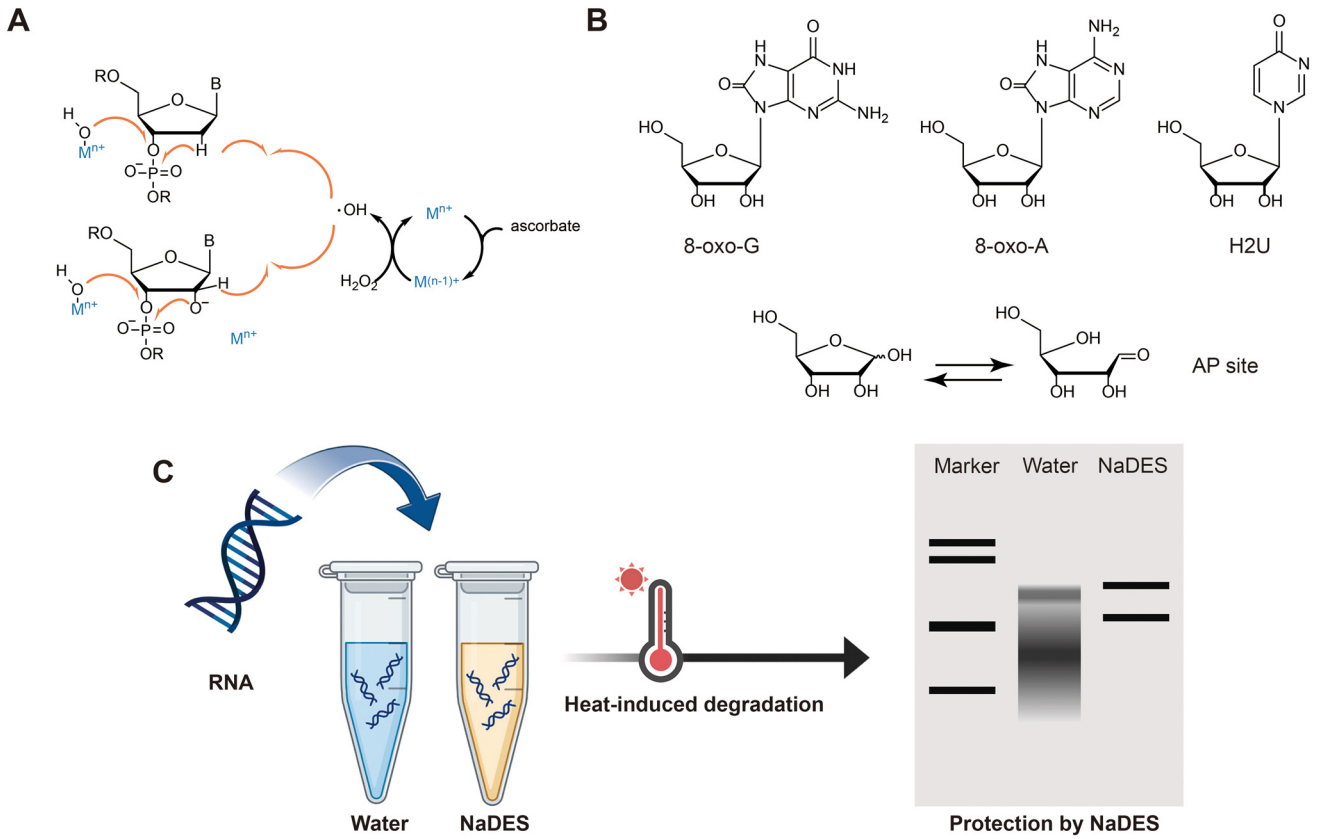

Fig. 2 Chemical factors and thermal stress that affect mRNA stability. (A) Summary of hydrolytic and oxidative cleavage pathways for nucleic acids, promoted by metal complexes. Reproduced with permission from ref. 49. Copyright 2018, Elsevier. (B) Structures of oxidative RNAs: 8-oxo-G (8-oxo-7,8-dihydro-guanosine), 8-oxo-A (8-oxo-7,8-dihydro-adenosine), H2U (4-pyrimidinone ribofuranoside), and AP site (apurinic/apyrimidinic site). Reproduced with permission from ref. 44. Copyright 2021, the Authors. (C) Natural deep eutectic solvents protect RNA from thermal-induced degradation. Reproduced with permission from ref. 50. Copyright 2023, Elsevier.

8-oxo-A and an abasic site, which then cause mutagenesis.[44] In addition to mRNA damage, oxidation also impairs the function of RBPs like HuR and AUF1, reducing their affinity for target transcripts and destabilizing mRNAs. Oxidative lesions within mRNA also disrupt recognition by decay mechanisms such as the exosome and RISC, leading to aberrant stability and dysregulated gene expression.[45] This destabilization plays a significant role in disease pathogenesis. For example, ROS-induced degradation of tumour suppressor or DNA repair transcripts facilitates tumorigenesis, while impaired stability of neuronal survival mRNAs exacerbates cell loss in neurodegenerative diseases.[46] Small molecules, such as antioxidants and RNA-stabilizing agents, may restore mRNA integrity under oxidative stress.[47] Modulating RBP or microRNA activity also offers potential to counteract ROS-mediated dysregulation.[48] These strategies may also pave the way for novel interventions in oxidative stress-driven diseases.

2.3.3. Impact of pH. mRNA is highly sensitive to pH, which influences both structural integrity and susceptibility to degradation.[51] Under acidic conditions, base protonation disrupts hydrogen bonding and base stacking, leading to the unfolding of secondary structures and increased vulnerability to hydrolytic cleavage of the phosphodiester backbone.[51] Alkaline environments, on the other hand, promote non-enzymatic cleavage reactions that further compromise mRNA integrity.[38] Optimizing the physicochemical parameters of mRNA–LNP systems is also essential for maximizing their therapeutic potential, with buffer pH emerging as a key determinant of formulation quality and biological performance. Recent studies have emphasized the importance of buffer pH during mRNA–LNP formulation and storage, showing that an aqueous phase pH of 4 enhances encapsulation efficiency and cellular expression due to stronger electrostatic interactions between mRNA and ionizable lipids.[51] This results in uniform particle formation and stable delivery, as demonstrated by consistent protein expression in AML12 cells and correlated with *in vivo* liver expression in mouse models. Moreover, pH adjustments during formulation significantly influence biodistribution and hepatic mRNA expression following both intramuscular and intravenous administration.[51] Notably, mRNA–LNPs formulated at pH 4 and 5 exhibit superior transfection efficiency and long-term stability due to their inverse hexagonal structure, which is thermodynamically favoured and less prone to phase transitions.[52] As pH increases above the pK_a, the structure shifts to a kinetically trapped lamellar phase, leading to a loss of transfection efficiency over time. However, lower pH also

increases aggregation propensity, indicating a trade-off between structural and colloidal stability.[52] These findings underscore the critical role of pH control throughout LNP preparation, with direct implications for mRNA stability, delivery efficiency, and therapeutic performance.

2.3.4. Thermal stress. Temperature critically influences mRNA structure integrity. Elevated temperatures accelerate molecular motion and promote the disruption of secondary and tertiary structures, such as stem-loops and pseudoknots, thereby exposing vulnerable regions to hydrolytic and oxidative attack. These thermal effects can also enhance the rate of spontaneous cleavage at labile sites, including those near modified nucleotides or single-stranded regions. Circular dichroism studies show that mRNA adopts an A-form helix at ~4 °C, shifting toward a Z-form at physiological temperatures (~37 °C), with potential impacts on protein interactions, ribosome binding and translational efficiency.[39] Conversely, low temperatures preserve mRNA integrity by stabilizing the structure and reducing enzymatic activity.[17] However, excessive cooling can impair translation by slowing initiation and elongation due to reduced ribosome mobility and altered tRNA dynamics.[53] Prolonging mRNA stability by thermal control is critical for applications such as vaccine packaging, storage and transportation.

Overall, mRNA stability is determined by the combined influence of its intrinsic sequence and structural features, susceptibility to RNase-mediated degradation, and sensitivity to different physicochemical stresses. These factors collectively indicate current challenges in the preservation of mRNA molecules. Understanding these determinants is therefore essential for optimizing mRNA design, improving formulation strategies, and enhancing the reliability and performance of mRNA-based therapeutics. The stability of mRNA–LNP therapeutics can be enhanced through interventions at multiple hierarchical levels, ranging from molecular sequence and chemical modification to RNA structural design and formulation engineering (Table 1). In the next sections, we discuss mRNA engineering strategies and advanced delivery vehicles developed to address stability challenges across different stages of the mRNA lifecycle.

3. Engineering strategies for enhancing mRNA–LNP stability

Given the diverse mechanisms that promote mRNA decay, recent advances in mRNA sequence design and modification provide powerful tools for prolonging protein expression, enhancing biostability and reducing immunogenicity of mRNA, including 5′ cap modifications, poly(A) tail optimization, nucleoside substitution, codon optimization, secondary structure tuning, and UTR engineering.

3.1. Sequence engineering of mRNA molecules

The coding sequence (CDS) dictates protein synthesis and significantly influences mRNA stability. Codon optimization, favouring codons paired with abundant tRNAs, minimizes ribosome stalling, reducing mRNA exposure to degradation pathways.[54] Incorporating stabilizing motifs into CDS, such as stem-loop structures or RBP-binding sequences, within the coding region can enhance their resistance to exonucleases.[55] In addition to prolonged mRNA translation, CDS and UTR sequence optimizations are used with structure-based rules to mitigate mRNA hydrolytic degradation. Besides, it demonstrated that chemically modified nucleotides in CDS and UTR, such as N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), pseudouridine (Ψ), 5-methylcytidine (m^5C), and N^4-acetylcytidine (ac^4C), have been validated to improve mRNA stability (Fig. 3A).

The 5′ and 3′ UTRs regulate mRNA stability and translation through interactions with RBPs and miRNAs. A well-studied example is the human α-globin mRNA whose 3′ UTR contains a C/U-rich stability element. This sequence supports the

Table 1 Integrated summary of mRNA–LNP stability optimization strategies

Optimization dimension	Strategy examples	Primary stabilization target	Key benefit	Representative trade-offs
Sequence-level engineering	• Codon usage optimization • GC content tuning • UTR redesign	• Intrinsic mRNA degradation and translation efficiency	• Improves basal stability without altering formulation	• Highly context-dependent; limited protection during storage
Chemical modification of nucleotides	• Ψ, $m^1Ψ$, m^5C, m^6A • Modified cap structures	• RNase resistance and immune evasion	• Clinically validated; scalable IVT incorporation	• Possible effects on fidelity and epitranscriptomic regulation
Backbone and end-structure protection	• Reversible 2′-OH acylation • Optimized poly(A) tails	• Hydrolytic and exonuclease-mediated degradation	• Strong protection during handling and delivery	• Added synthetic and deprotection complexity
RNA structural optimization	• Circular RNA • Self-amplifying RNA	• End-dependent degradation pathways	• Dramatically prolonged RNA persistence	• Larger size or reduced translational efficiency
Lipid composition engineering	• Ionizable lipid head-group tuning • Helper lipid selection	• Encapsulation stability and intracellular release	• Improves delivery efficiency and *in vivo* stability	• Susceptible to oxidation or chemical degradation
Formulation-level stabilization	• Oxidation-resistant lipids • Stabilizing excipients	• Chemical and colloidal stability of LNPs	• Enhances shelf life and storage robustness	• Formulation complexity and scale-up challenges
Storage and logistics strategies	• Lyophilization • Solid-state delivery formats	• Long-term chemical degradation	• Enables cold-chain-independent distribution	• Reconstitution and process optimization required

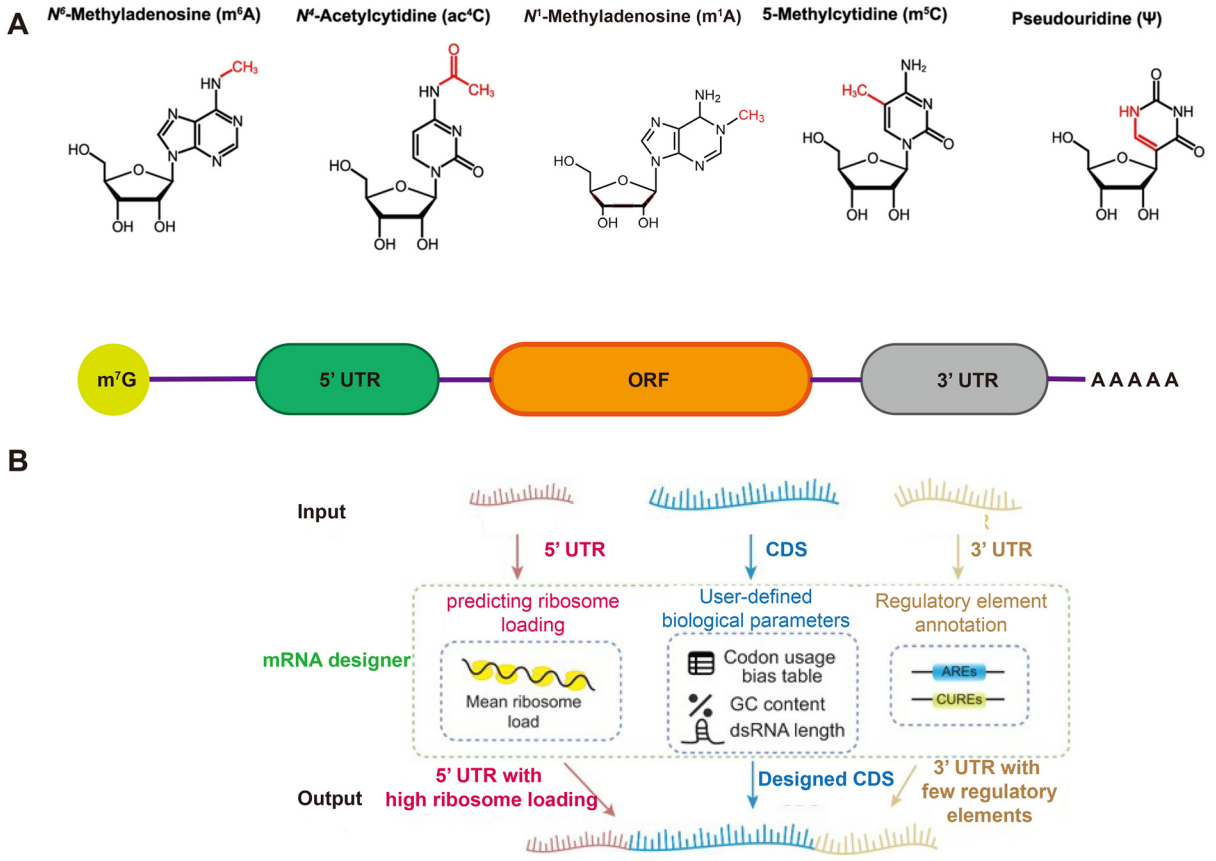

Fig. 3 CDS/UTR optimization RNA modification and AI-assisted sequence design enhance mRNA translation capacity. (A) RNA modifications in mRNA. Adapted with permission from ref. 56. Copyright 2020, Nature Portfolio. (B) mRNAdesigner optimizes mRNA CDS and UTRs to enhance stability and translation efficiency. Reproduced with permission from ref. 57. Copyright 2025, the Authors, Oxford University Press.

assembly of a protective messenger ribonucleoprotein complex that prevents deadenylation and protects the transcript from endonucleolytic and exonucleolytic attacks.[58] In addition, AU-rich elements (AREs) within the 3′ UTR offer another route for enhancing mRNA stability. AREs are typically associated with transcript degradation through their interaction with destabilizing RBPs. It has been demonstrated that, by modifying the sequence, spacing, or density of AREs, their destabilizing effects can be attenuated, leading to a prolonged mRNA half-life.[59]

An AI-assisted mRNA structure design uses advanced computational tools to optimize coding sequences and untranslated regions. A striking AI algorithm, UTR-LM, which is pretrained on multi-species 5′ UTRs, can achieve up to 60% higher translation efficiency and identify unannotated internal ribosome entry sites (IRES), enabling targeted translation for personalized medicine.[60] In addition, to optimize the CDS of mRNA, another AI tool, LinearDesign was developed to improve codon optimality by screening the mRNA sequence with the lowest minimum-free-energy (MFE) and balancing it with the codon adaptation index (CAI) using a combined objective function, MFE $-\lambda|p|\log$ CAI. LinearDesign optimizes mRNA sequences in 11 minutes, increasing the half-life by up to 2 to 3-fold compared to unoptimized mRNA. The generated COVID-19 mRNA vaccine can boost antibody titres 128-fold higher than unoptimized mRNA.[61] However, this algorithm lacks a free energy model with *N*1-pseudouridine, resulting in reduced effectiveness and potential mismatch of mRNA. Moreover, current 5′ UTR design strategies explore only a minuscule fraction of the vast sequence space, lacking comprehensive evaluation against a broader diversity of their synthetic or engineered counterparts. Thus, Zhang *et al.* established a novel method named "GEMORNA", which optimizes mRNA with CDS and UTR, respectively. As a result, GEMORNA-generated CDS 5′ UTR and 3′ UTR reveal stronger and long-lasting expression, respectively. GEMORNA's deep generative models design mRNAs with up to 15.9-fold higher protein expression and 4-fold increased antibody responses, applied to therapeutic proteins like insulin for diabetes or monoclonal antibodies for cancer.[62] Furthermore, Mo *et al.* introduced an open-access platform that can optimize all parts of mRNA sequences called "mRNAdesigner".[57] It optimizes coding sequences and 5′/3′ UTRs by balancing the codon adaptation index, rare codon usage, GC content, unpaired nucleotide ratios, and structural features like maximum stem length. The optimized 5′ and 3′ UTR design achieves a 2.32-fold increase in RSV F protein expression compared to the unoptimized mRNA, while maintaining broad cross-species adaptability and inducing strong antigen-specific immune responses in mice

(Fig. 3B). Overall, these AI algorithms leverage strategies like GC content modulation and pseudouridine modifications to minimize ribonuclease-mediated degradation, ensuring robust performance in vaccine development.[63] However, developing AI models that generalize well across diverse sequences and biological contexts remains difficult. Balancing multiple design objectives while avoiding unintended off-target effects also presents significant hurdles. Finally, integrating experimental feedback to refine AI algorithms continues to be an ongoing challenge in this rapidly evolving field.

3.2. Chemical modification of mRNA molecules

Reversible 2′-OH acylation, also called "cloaking", which promotes 2′-ester modification, protects mRNA from degradation by attaching acyl groups to the 2′-hydroxyl of ribose sugars, shielding reactive sites from nucleases and thermal stress.[57] Electrophilic compounds such as acylimidazoles and isatoic anhydrides have been developed to selectively react with the 2′-OH groups of mRNAs. The reactivity of 2′-OH acylation is influenced by the RNA structural conformation, stereochemistry, accessibility of RNA and adduct stability, with unpaired regions and small loops of RNA exhibiting the highest reactivity.[64] This modification is reversible, allowing controlled restoration of mRNA functionality. The acyl groups can be removed within cells through an "uncloaking" step, restoring the 2′-OH groups by 2′-ester hydrolysis and thereby reactivating full mRNA functionality for translation and gene editing. Known as the ester hydrolysis catalyst, nucleophiles could catalyse the removal of 2′-OH acylation, including imidazole, pyridine and tris. Notably, tris buffer can promote efficient hydrolysis of *N*,*N*-dimethylglycine (DMG) esters under physiological conditions[65] (Fig. 4A and B). Steric effects and the chemical design of the reagents also influence reaction efficiency. Ongoing advances have introduced new classes of reagents, including sulfonyltriazoles and 2-mercaptopyridine, which support site-specific 2′-OH modification and enhance RNA cellular uptake by thiol exchange reactions on the cellular membrane. These developments are expanding the range of possibilities for RNA research and therapeutic applications[66] (Fig. 4C–E). Furthermore, RNA "caging" strategies using photolabile or chemically removable acyl groups enable precise temporal control of mRNA activity, as demonstrated in CRISPR-Cas9 systems.[67] This method inhibits sgRNA activity after RNA 2′-OH acylation cloaking, with partial restoration of sgRNA guided Cas9 DNA cleavage upon tetrazine "uncloaking" treatment while recovering natural 2′-OH, receiving downregulated GFP expression levels. This reversible 2′-OH acylation-based backbone modification represents a promising strategy in

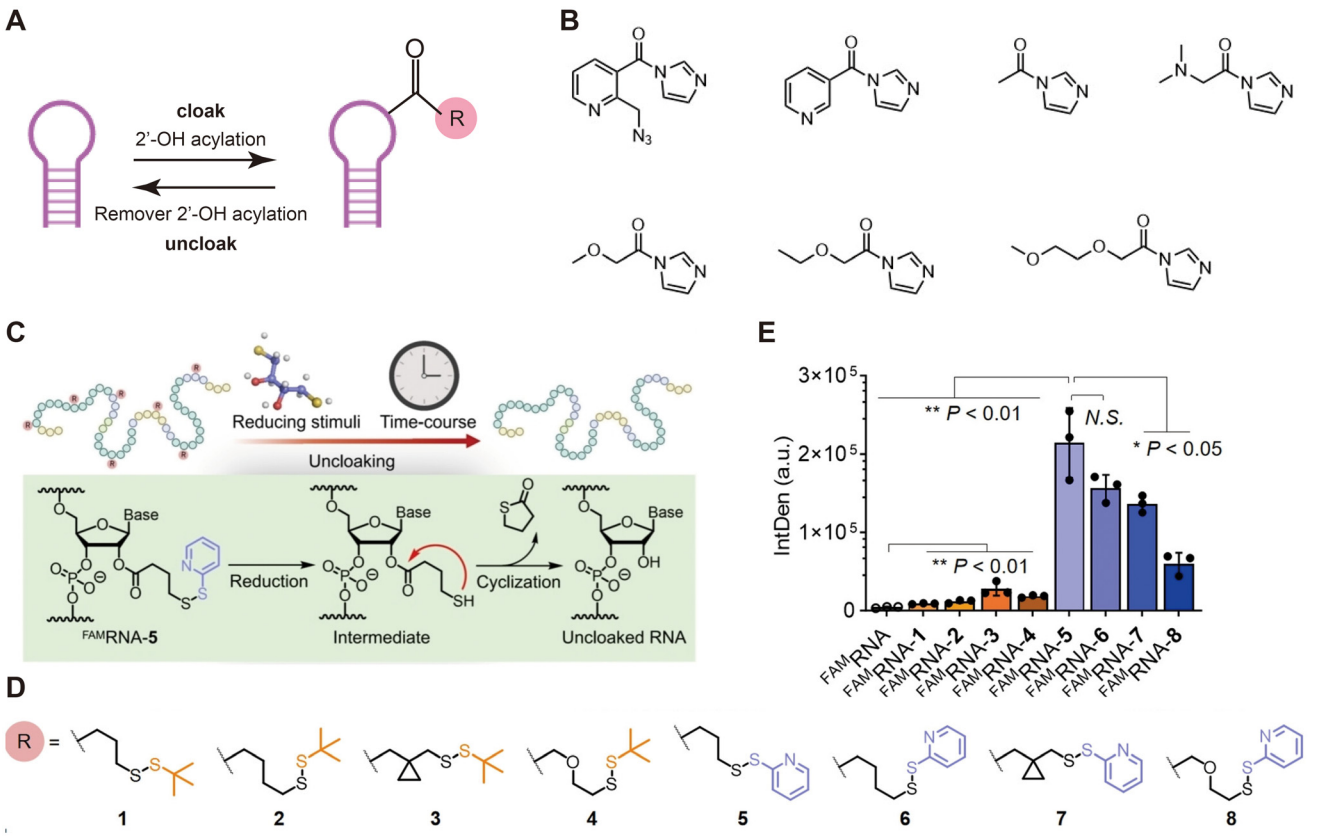

Fig. 4 Reversible 2′-OH acylation protects mRNA. (A) Reversible 2′-OH acylation protects mRNA from RNase digestion and deprotects in cells recovering translation.[56] (B) Acylimidazole reagent analogs. (C, E) Redox-responsive acylation and cyclization-mediated removal enhance RNA cellular uptake. (D) Acyl group insertion into RNA 2′-OH. Reproduced with permission from ref. 66. Copyright 2024, the Authors, Wiley-VCH GmbH.

enhancing RNA stability, while the reversibility and specificity are crucial for restoring original RNA function and enabling specific region targeting in future mRNA drug development.

3.3. Structure optimization of mRNA molecules

3.3.1. Poly(A) tail engineering. The poly(A) tail, typically consisting of 200 to 250 adenine nucleotides, plays a significant role in stabilizing mRNA. However, gradual shortening of the poly(A) tail by 3′-exonucleases ultimately leads to mRNA decay. Advances in chemical and structural engineering of poly(A) have been demonstrated as effective strategies to enhance mRNA stability. One established approach is incorporating chemically modified nucleotides into the poly(A) tail. It turns out that adenosine analogs containing 2′-*O*-methyl or 2′-*O*-methoxyethyl groups on the ribose and thiophosphate linkage can improve resistance to exonuclease while maintaining translation compatibility.[68] Cleavage and polyadenylation are critical steps in the maturation of mRNA. AAUAAA serves as both a polyadenylation signal and a cleavage signal, mediating cleavage of 10–30 nucleotides downstream. Therefore, positioning the AAUAAA motif within the poly(A) tail can prevent the generation of RNA isoforms and truncated proteins while also improving polyadenylation efficiency and enhancing mRNA stability.[69] Further innovations involve the installation of azido groups on poly(A) tails through enzymatic extension using yeast poly(A) polymerase. These azido-modified mRNAs allow bioorthogonal click chemistry reactions for fluorescent labelling for real-time visualization while maintaining intact coding and UTRs in living cells[70,71] (Fig. 5A).

Chemical modification and structural enhancement of the poly(A) tail have significantly improved its performance. Messenger-oligonucleotide conjugated RNAs (mocRNAs), in which modified oligonucleotides are ligated to the 3′ end of mRNAs, protect the poly(A) tail from exonuclease degradation while preserving 2- to 4-fold protein translation increment[72] (Fig. 5A). Another engineering strategy through the generation of branched poly(A) tails has been demonstrated to enhance mRNA stability by reducing mRNA degradation with the deadenylation-dependent decay pathway and increase protein expression *via* more cytoplasmic poly(A) and PBP-1 binding and translation initiation complex formation compared to traditional single poly(A) tails. *In vivo* experiments demonstrated that branched mRNAs exhibited approximately twice the half-life compared to unmodified mRNA and sustained protein expression for up to 14 days, while the single poly(A)-tailed reporter mRNA only remained for two days (Fig. 5B). The study successfully demonstrated efficient multiplexed genome editing using sgRNAs with branched poly(A) tails, targeting the metabolic genes *Pcsk9* and *Angptl3* in the mouse liver at lower mRNA administration doses, highlighting its therapeutic potential.[73] Despite the encouraging results, the complicated manufacturing process may hinder its further large-scale production.

3.3.2. 5′ cap modification. 5′ cap modification (*e.g.*, m^7G) can protect mRNA from 5′ exonucleases and decapping enzymes and facilitate translation initiation by forming an RNA–eukaryotic translation initiation factor (eIF4F) complex. The canonical cap remains vulnerable to enzymatic cleavage, which can lead to premature degradation and loss of transcript integrity. Recent research indicated that endogenous mRNA caps carry different 5′-terminal nucleotides and additional modifications like methylations (2′-*O*-methylation and m^6A) on the first transcribed nucleotide. In addition to screening endogenous 5′ caps, Sikorski *et al.* reported a novel method to produce trinucleotide 5′ cap analogs and ligate them on mRNAs by T7 RNA ligase, analysing protein expression levels. Notably, 2′-*O*-methylation at the first transcribed nucleotide (A) reveals significantly higher protein expression.[74] Interferon-induced protein with tetratricopeptide repeats (IFIT) can recognize and accommodate the heterogeneous 5′ cap structure, out-compete cap-binding with eIF4F, and inhibit the formation of a translation initiation complex. Furthermore, m^6A_m and 2′-*O*-methyl A, the earliest developed "self-tag" caps, reveal lower IFIT binding affinity compared to eIF4F, specifically contributing to escape from IFIT-compatible binding.[75] However, the exact mechanisms by which these methyl modifications enhance translation efficiency or promote immune evasion remain unclear, and studying cap–protein interactions may help elucidate the underlying mechanisms.

Other advances include a photoreactive cap analog which contains two light-sensitive groups and multiple chemical modification sites for mRNA real-time labelling in cells[76] (Fig. 6A). These analogs allow precise crosslinking with translation regulators such as eIF4E and Dcp2, enabling the study of cap–protein interactions within complex assemblies. Another novel analog, AvantCap (N^6-benzyl-substituted cap), has been demonstrated to be resistant to demethylation by FTO and improve mRNA quality by enhancing purification, reducing double-stranded RNA content, and eventually increasing 6-fold protein expression in both cultured cells and animal models. The development of hydrophobic photocaged cap analogs enables capping efficiency analysis and mRNA purification by reversed-phase HPLC that separates capped and uncapped mRNA with reversible hydrophilicity–hydrophobicity differences, achieving nearly 100% capping efficiency even for Cap-2-type transcripts.[77] Stereo-controlled synthesis of phosphorothioate nucleotide analogs offers a powerful strategy to precisely modulate mRNA stability and biological activity. This study introduces a modular, reagent-based platform for the stereocontrolled and scalable synthesis of stereopure nucleoside α-thiodiphosphates and α-thiotriphosphates, including both symmetrical and unsymmetrical dinucleoside thioisosteres.[78]

As the limiting step of mRNA translation, the translation initiation factor forming a capacity increase could enhance the total protein expression level and improve its short half-life and translation capacity. Chen *et al.* demonstrated a novel strategy named ligation-enabled mRNA–oligonucleotide assembly (LEGO®) *via* innovative topological designs.[79] They screened several chemtopological nucleotides as 5′ cap and 5′ UTR, revealing two modifications: locked nucleic acid (LNA) N^7-methylguanosine modification on the cap and 5 × 2′

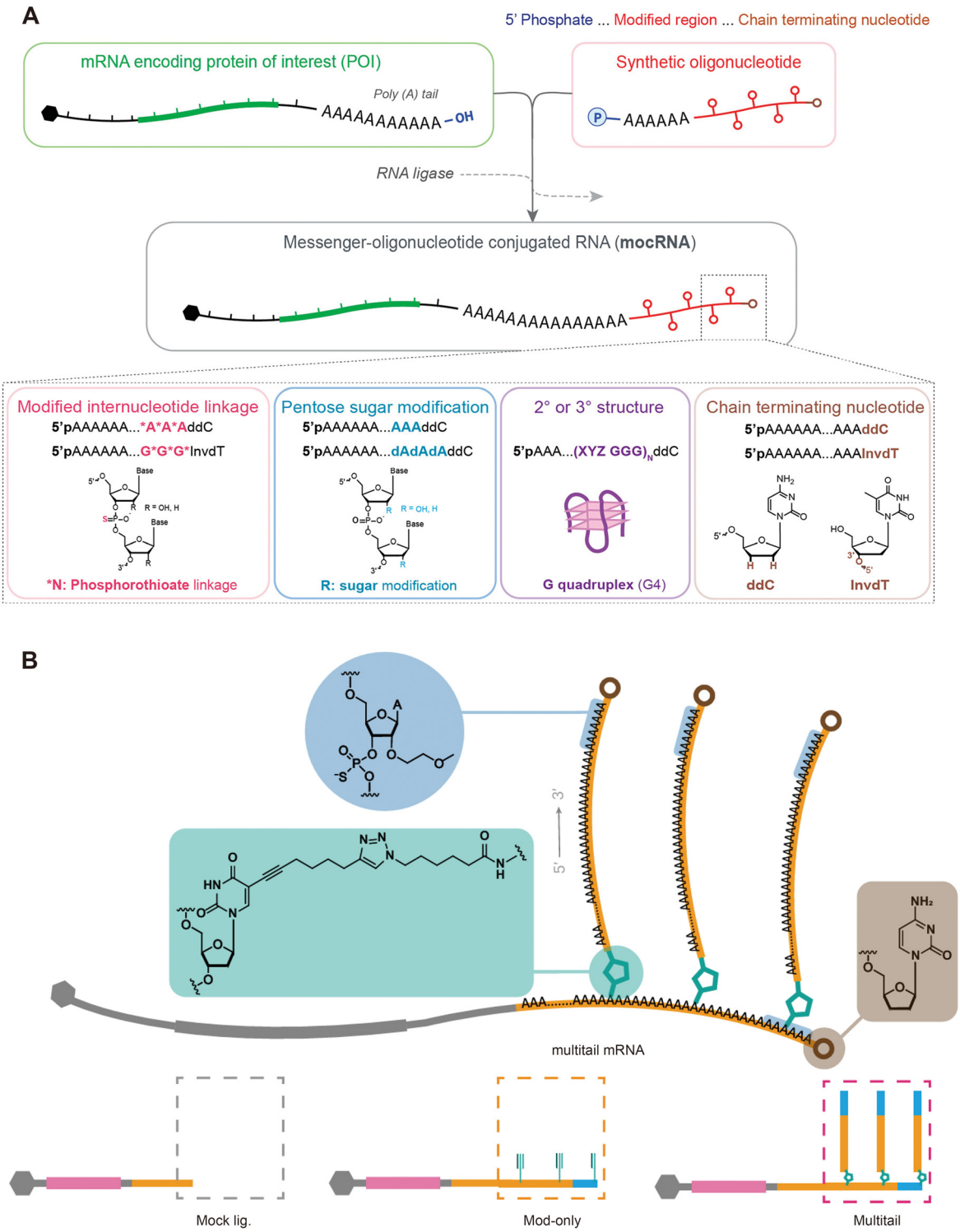

Fig. 5 Poly (A) tail modification enhances mRNA stability and promotes translation conformation initiation. (A) Modified mocRNA construction *via* T4 RNA ligase mediated RNA ligation.[72] Reproduced with permission from ref. 72. Copyright 2022, the Authors. (B) Branched poly(A) modification enhances translation capacity by improving biostability and PABP protein binding affinity. Reproduced with permission from ref. 73. Copyright 2024, Springer Nature.

O-methyl modification on the 5′ untranslated region, which respectively enhanced RNA–eukaryotic translation initiation factor (eIF4E–eIF4G) binding, ribosome recruitment and decapping enzyme resistance. Moreover, two chemtopological 5′ caps called "multi-caps" revealed 10-fold more protein and dramatically improved antibody responses in SARS-CoV-2 vac-

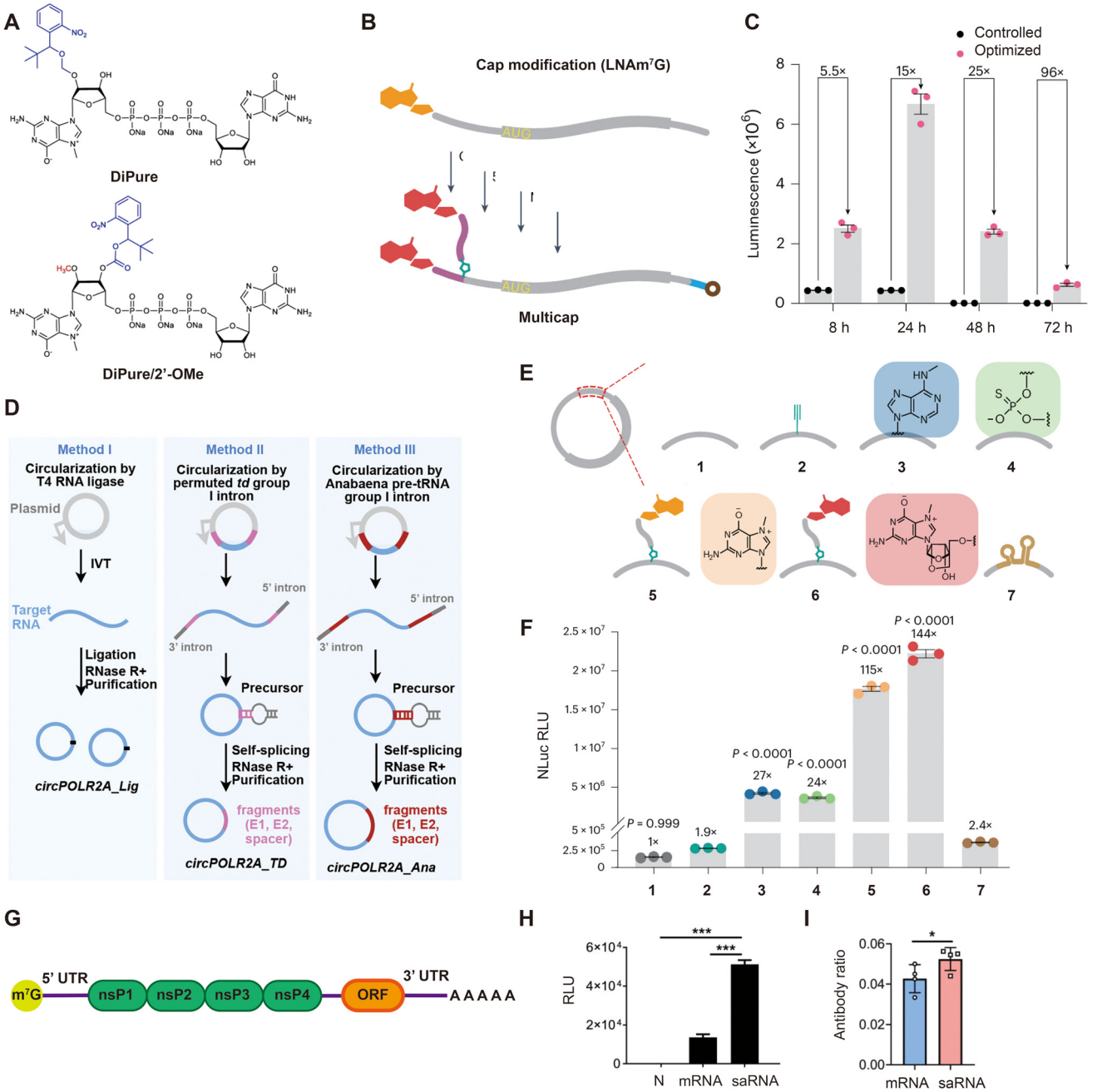

Fig. 6 5′ cap modification, circRNA and saRNA enhance mRNA translation and protein expression. (A) Reversible photocage 5′ cap enables controllable mRNA translation. (B and C) Multi-cap modified mRNA enhances 10-fold mRNA translation compared with a single cap *in vivo*.[76] Reproduced with permission from ref. 79. Copyright 2024, Springer Nature. (D) Several strategies for mRNA circularization. Copyright 2022, Elsevier; 2022, Springer Nature. (E and F) A modified 5′ cap crosslinked into circRNA enhances protein expression. Reproduced with permission from ref. 79. Copyright 2024, Springer Nature. (G) The sequence structure of saRNA. (H) saRNA, also called repRNA, prolonged protein expression and (I) showed much compatible antibody expression. Reproduced with permission from ref. 80. Copyright 2023, the Authors.

cination models, generating 17-fold higher antibody titres after a single dose (Fig. 6B and C).

3.3.3. Circular RNA. Circular RNAs (circRNAs) possess a covalently closed-loop structure that lacks both 5′ and 3′ ends. CircRNAs are synthesized *in vitro* through enzymatic ligation with T4 RNA ligase, ribozyme-mediated circularization (*e.g.*, permuted intron–exon (PIE) or *trans*-ribozyme-based circularization (TRIC)), Group II intron splicing and chemical ligation, producing stable and covalently closed RNAs for therapeutic and research applications[81–84] (Fig. 6D–F). CircRNAs are highly resistant to exonuclease-mediated degradation due to the circular structure. This unique structure grants circRNAs exceptional stability, with an average cellular half-life exceeding 18 hours while that of the linear is 4 to 7.4 hours.[85] Another

critical advantage of circRNAs is their relatively low innate immunogenicity like retinoic acid-inducible gene I (RIG-I) and toll-like receptors (TLRs) compared to unmodified linear mRNAs. Endogenous circRNAs could bind 16–26 nt imperfect RNA to form duplexes and act as inhibitors of double-stranded RNA (dsRNA)-activated protein kinase (PKR) related to innate immunity.[86] This reduced immune stimulation is particularly valuable for mRNA-based protein replacement and supplementary therapies, where sustained expression with minimal immune activation is preferred.[87]

CircRNAs are also capable of supporting robust protein expression due to their enhanced biostability and IRES/UTR optimization. Litke *et al.* demonstrated that circRNAs delivered *via* LNPs in mice produced 5 to 15-fold higher protein levels than linear mRNAs over 7 days, attributed to stability and sustained translation.[83] Furthermore, the 5′ UTR, 3′ UTR and IRES elements could be optimized by inserting HRV-B3 into Apt-eIF4G to enhance circRNA expression by up to 224-fold compared to unoptimized circRNAs.[83] When equipped with IRESs, circRNAs facilitate cap-independent translation and have been shown to outperform linear mRNAs *in vivo.*[88] For example, a circRNA encoding the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein generated strong immune responses in rhesus macaques. This circRNA-based vaccine induced higher levels of neutralizing antibodies than conventional mRNA vaccines.[89]

To address the limited translation efficiency of IRES-dependent circRNAs, two innovative cap-based strategies were employed: either introducing an m^7G cap that covalently ligated on RNA oligonucleotide by T4 RNA ligase mediated RNA ligation to form circRNA by method I (Fig. 6D), or a non-covalent strategy in which an m^7G cap containing RNA oligonucleotide formed a dsRNA complex with circRNA. Covalent modification of the m^7G cap significantly enhances translation and further reduces immune activation, especially when used in combination with N^1-methylpseudouridine ($N^1m\Psi$).[90] While non-covalent hybridization of the circular RNA with an m^7G cap-containing oligonucleotide improved translation efficiency by more than 50-fold compared to circRNA only and enabled rolling-circle-type protein synthesis that provides long repeating peptides and enhances protein production.[91] Introducing LNA N^7-methylguanosine multicaps *via* method I allows circRNAs to utilize cap-dependent translation, harnessing the robust eIF4E–eIF4G machinery without foreign sequences, enhancing 3.7-fold antibody production and higher immune responses.[79] Although circRNA is emerging as a robust RNA tool, further understanding and investigation of delivery carriers are required to achieve effective targeted delivery.

3.4. Self-amplifying RNA

Self-amplifying RNA (saRNA) is derived from RNA viruses, encoding non-structural proteins (nsP1–4) for replication machinery and replacing structural protein genes with immunogen-encoding genes, enabling amplified and prolonged antigen expression[80] (Fig. 6G–I). However, the large size and complex secondary structures of saRNA render it susceptible to enzymatic degradation, limiting stability and efficacy. Sequence optimization, including codon adjustment and GC content modulation, minimizes destabilizing secondary structures that attract ribonucleases.[92] In murine models, m^5C-modified saRNA elicited antibody titres 5-fold higher than wild-type saRNA and 121-fold higher than N^1-methylpseudouridine-modified mRNA at low doses, conferring complete protection against lethal SARS-CoV-2 infection.[93] A current phase 1 trial of the m^5C-modified saRNA vaccine encoding SARS-CoV-2 reported a 3-fold increase in IgG titres, highlighting its potential to extend antigen expression and mitigate interferon-mediated suppression.[93,94] The saRNA-based rabies mRNA vaccine RBI-4000, administered at one-tenth the dose of the commercial RabAvert® vaccine, elicited a significantly higher antibody response and maintained titres above the 0.5 IU mL^{-1} threshold with slower or no decay over a period exceeding six months.[95]

Replication of saRNA generates excessive amounts of dsRNA, which triggers strong innate immune responses. The formation of dsRNA during saRNA replication activates innate immune pathways, including TLRs, RIG-I, and stimulator of IFN genes (STING), triggering type I IFN and inflammatory cytokine production. To mitigate saRNA immunogenicity, modified nucleotide analogs and high-performance liquid chromatography were used to remove double-stranded RNA. Incorporation of modified nucleotides, such as pseudouridine and 5-methylcytidine (m^5C), can also enhance translational efficiency and reduce innate immune activation.[93]

In essence, mRNA engineering approaches enhance stability by addressing the molecular and environmental mechanisms that drive transcript decay. Optimization of CDS and UTR sequences improves codon usage, structural accessibility, and interactions with RNA-binding proteins, thereby reducing nuclease susceptibility and supporting sustained translation. Chemical nucleoside modifications further strengthen molecular integrity and reduce innate immune activation. Engineering of the poly(A) tail and the 5′ cap protects key terminal regions, prolonging mRNA half-life and enhancing translation initiation. Emerging approaches such as reversible backbone modification, circularization, and self-amplifying architectures introduce structural solutions that extend durability, promote higher protein output, and decrease immunogenicity. Together, these strategies demonstrate how rational design can exert precise control over mRNA stability and substantially enhance the therapeutic potential of mRNA technologies. However, while engineered mRNA molecules offer clear advantages in improving mRNA stability and protein production, their increased structural complexity often comes at the cost of reduced manufacturing yield, challenges in achieving high purity and limited scalability.[96,97] Addressing these limitations will require advances in mRNA manufacturing, particularly the development of efficient enzymatic toolkits—such as polymerases, ligases, and capping enzymes—that are compatible with diverse chemical modifications and complex RNA architectures. In parallel, new purification strategies, including chromatographic materials capable of selectively distinguish-

ing circular RNA from linear RNA species, will be essential for achieving high purity without compromising yield and scalability.

3.5. Advanced mRNA–LNP formulation development

LNPs help protect mRNA from degradation, promote cellular uptake, and support endosomal escape, all of which are essential for effective protein expression. However, there is still a need to improve LNPs due to several ongoing challenges. One major issue is immunogenicity, where certain lipid components can provoke unwanted immune responses that reduce therapeutic effectiveness and limit repeat dosing.[98] Another concern is lipid oxidation, which can produce reactive byproducts that compromise both the safety and stability of the mRNA payload.[98] Additionally, LNPs often suffer from poor thermal stability, requiring strict cold-chain storage that complicates distribution and access, especially in low-resource environments.[99] Advancing the design of delivery materials by creating more stable lipid formulations, incorporating protective additives, or exploring alternative carriers is key to overcoming these limitations and fully realizing the potential of mRNA-based therapies.

3.5.1. Engineering LNPs with reduced immunogenicity. LNPs inadvertently activate pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), due to their positive charge and amphiphilic structure, leading to the release of proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α).[100] Notably, the amine headgroups of ionizable lipids have been shown to directly engage TLR4 and CD1d, driving structure-dependent immune activation.[101] The release of proinflammatory cytokines contributes to a sustained inflammatory environment that can negatively impact mRNA therapeutics.[102] These cytokines promote the expression of RNases, which degrade the mRNA payload before it can be translated, thereby reducing its therapeutic potential. In addition, prolonged or excessive immune activation may lead to immune exhaustion and impaired innate immune function, further undermining the stability and overall effectiveness of mRNA-based treatments.[102,103] This interplay between inflammatory signalling and translational output represents a central determinant of mRNA vaccines, underscoring the need to balance immune activation and protein expression. The inflammatory environment also interferes with the cellular translation machinery. Inflammatory signalling pathways, particularly those involving IFNs, can suppress translation by modifying key regulatory proteins such as eIF2α or by altering ribosomal function.[104] Recent evidence indicates that excessive endosomal membrane damage during LNP-mediated endosomal escape triggers cytosolic danger sensing pathways that amplify inflammation.[105] This suppression reduces the efficiency of protein synthesis from the delivered mRNA. Additionally, pre-exposure to LNPs has been shown to induce long-term immunological changes, including reduced adaptive immune responses and altered resistance to infections, which may indirectly affect the cellular environment and its capacity to support mRNA translation.[102]

PEG-lipids are employed to improve colloidal stability, reduce aggregation, and prolong circulation time in the bloodstream by minimizing opsonization and clearance by the immune system. Yet, their repeated use presents notable safety concerns since PEG-lipids can provoke the accelerated blood clearance effect and induce anti-PEG antibodies, which can reduce therapeutic efficacy and increase immunogenicity upon subsequent administrations.[106] These immune responses pose challenges for chronic or multi-dose treatments, limiting the clinical utility of PEG–LNP formulations. Upon replacing poly (ethylene glycol) (PEG) lipids with poly(carboxybetaine) (PCB) lipids, a zwitterionic alternative that offers improved biocompatibility and reduced immune recognition is obtained. This substitution enhanced mRNA transfection efficiency and therapeutic protein expression by improving endosomal escape and particle stability.[107] *In vitro* studies show higher protein expression levels in transfected cells, while *in vivo* data reveal sustained therapeutic effects with minimal immune activation. Immunogenicity assessments, including cytokine profiling and anti-polymer antibody titres, confirm that PCB-LNPs elicit significantly lower immune responses compared to PEG-LNPs.[107] These findings highlight PCB-lipids as a promising alternative for safer and more effective mRNA delivery, particularly in applications requiring repeated dosing.[107] Additionally, reduced immunogenicity in mRNA–LNP vaccines can be achieved by adjusting the PEG lipid ratio and phospholipid compositions, which significantly influence immune cell interactions and cytokine responses. Beyond headgroup and helper lipid composition, the degree of tail unsaturation in ionizable lipids has also been shown to critically regulate membrane fusion, endosomal escape efficiency,[108] and LNP immunogenicity. The study demonstrated that LNP H, composed of 0.5% PEG lipid, 10% DSPC, 50% ionizable lipid, and 38.5% cholesterol, enhanced humoral immunity while maintaining a favourable safety profile, likely due to improved mRNA delivery and reduced innate immune activation. In comparison, LNP Q, the benchmark formulation with the same components but 1.5% PEG lipid, and LNP W, which also contains 1.5% PEG lipid but substitutes DSPC with DOPS, showed lower protein synthesis and reduced cytokine induction. Zwitterionic phospholipids like DSPC were less immunostimulatory, while anionic phospholipids such as DOPG and DOPS promoted stronger CD8+ T cell responses and elevated IL-1β levels, indicating enhanced adjuvanticity.[109] These outcomes, supported by distinct cytokine profiles, demonstrate that fine-tuning LNP composition can direct immune responses for tailored vaccine applications.

Moreover, NC-TNPs are a novel class of mRNA delivery vehicles that emulate the structural and biophysical properties of natural cell membranes, markedly attenuate TLR-driven signalling and cytokine production (*e.g.*, IFN-β and IL-1β)[110] (Fig. 7A and B), while avoiding the immunogenicity associated with conventional cationic or ionizable LNPs. Similar strategies employing endogenous anti-inflammatory lipids have been shown to suppress innate immune activation while maintaining nucleic acid delivery efficiency.[111] An *in vitro* study

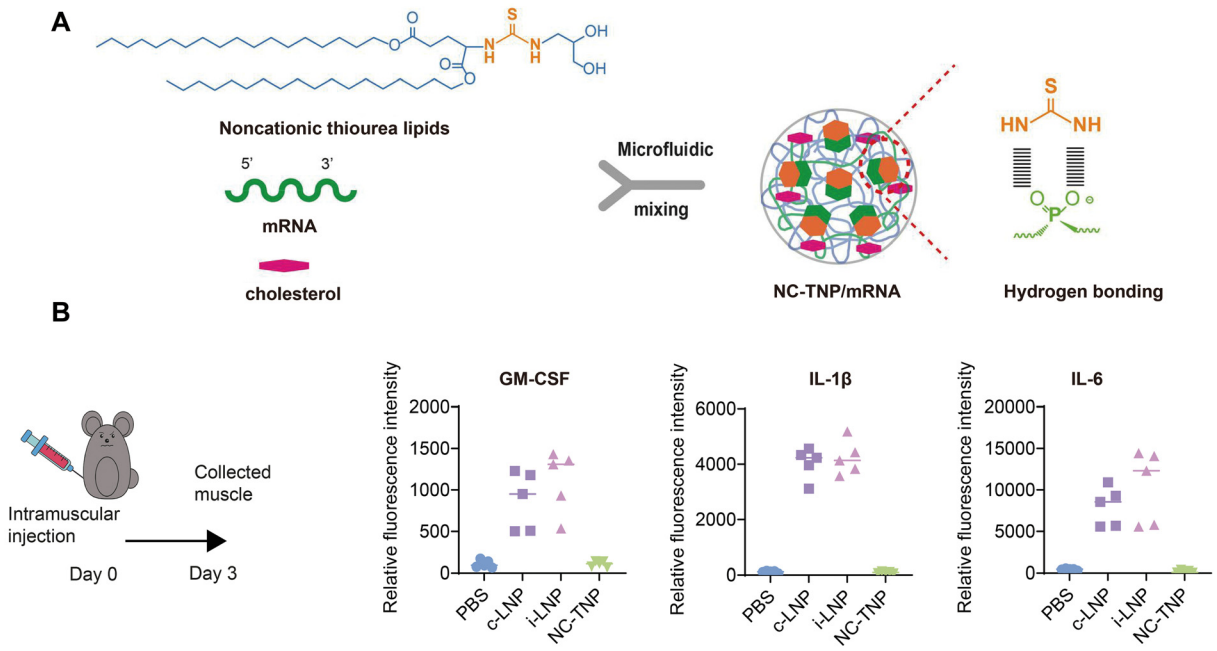

Fig. 7 Examples of LNPs with reduced immunogenicity. (A) Noncationic thiourea lipids with lower cationic charge showed lower immunotoxicity. The schematic shows the preparation of noncationic thiourea lipid nanoparticles (NC-TNPs) through microfluidics. (B) NC-TNP elicited lower levels of proinflammatory cytokines which were similar to the PBS groups. Reproduced with permission from ref. 110. Copyright 2023, PNAS.

using the DC2.4 cell line showed that NC-TNPs enhanced mRNA transfection efficiency, reduced TLR activation, and lowered cytokine release compared to traditional LNPs.[110] They also effectively encapsulate various nucleic acids and protect them from nuclease degradation. In mouse models, NC-TNPs exhibited minimal immunotoxicity, avoided the accelerated blood clearance effect seen with PEGylated systems, and preferentially accumulated in the spleen, promoting strong $CD8^+$ T cell and antibody responses.[110] These findings position NC-TNPs as a safer and more effective alternative to benchmark mRNA delivery platforms for repeated and long-term therapeutic applications.[110] On the other hand, SS-cleavable and pH-activated lipid-like materials (ssPalm) offer enhanced mRNA stability and immunogenic control through a dual-responsive hydrophilic head that contains pH-sensitive tertiary amines for endosomal escape and disulfide bonds for cytoplasmic release. Among these, ssPalmE combined with the α-helical cationic peptide KALA (ssPalmE-KALA) demonstrates reduced baseline immunogenicity while enabling strong, antigen-specific immune activation.[112] *In vitro*, BMDCs transfected with ssPalmE-KALA (with vitamin E scaffold lipids) produced high levels of IFN-β and IL-6 only in the presence of mRNA, confirming that the carrier itself does not trigger unintended immune responses. This activation was stronger than that observed with ssPalmM-KALA (with myristic acid-based lipids) and EPC-KALA (with egg phosphatidylcholine as the lipid) and was comparable to DNA transfection. *In vivo*, BMDCs transfected with OVA-encoding mRNA using ssPalmE-KALA induced robust tumour suppression in mice, while control groups receiving Luc-mRNA or PBS showed minimal immune response. Compared to the highly immune-stimulative ssPalmE, which is unsuitable for mRNA delivery due to its inflammatory potential, ssPalmO provides a safer and more effective alternative.[113] ssPalmO features self-degradable disulfide bonds and phenyl ester linkers that break down into oleic acid, helping suppress inflammation. *In vitro* and *in vivo* mouse studies showed that mRNA–LNPssPalmO significantly reduced cytokines like IL-6 and IL-1β and minimized adverse effects such as fever and weight loss compared to SM-102-based LNPs.[113] When combined with DOPC helper lipids, ssPalmO improved mRNA release and protein expression while maintaining low inflammation. HA-LNP ssPalmO also induced IFN-γ-producing CD4+ T cells and offered cross-protection against heterologous influenza strains without neutralizing antibodies.[113] RNA-seq analysis confirmed its suppression of inflammatory gene expression, highlighting ssPalmO's potential for safe and effective mRNA vaccine delivery.

3.5.2. Thermostable LNPs. Thermal stress increases lipid membrane fluidity and permeability, leading to destabilization of LNPs. This disruption compromises mRNA integrity by facilitating cargo leakage, impairing the bilayer structure, and accelerating degradation processes such as hydrolysis and oxidation.[114–116] These effects are particularly problematic during storage, transport, and formulation processes, where temperature fluctuations are common.[117] Moreover, maintaining colloidal stability under thermal stress is challenging, as elevated temperatures can induce particle aggregation or fusion, altering size distribution and surface characteristics. Such changes not only reduce delivery efficiency but also

heighten the risk of immunogenicity and off-target effects.[106,118] Addressing these challenges requires careful lipid composition tuning, incorporation of stabilizing agents, and rigorous thermal profiling to ensure consistent performance across clinical and logistical conditions.

To enhance the thermostability of mRNA-loaded LNPs without compromising delivery efficacy, researchers have engineered novel ionizable lipids through diverse structural strategies. One approach involved the rational design of an α-branched lipid library with a fixed CL4 headgroup, where 32 lipids were synthesized *via* alkylation reactions to explore structure–activity relationships. Findings revealed that molecular symmetry and carbon chain length significantly influenced LNP viscosity, encapsulation efficiency, and thermal stability, with a critical microviscosity threshold of 120 mPa s identified for optimal performance.[119] Another strategy introduced DOG-IM4, a lipid featuring an imidazole headgroup and a polyoxyethylene spacer, which maintained mRNA integrity for up to six months at 4 °C and 25 °C, and induced strong immune responses in mice and macaques using influenza HA mRNA, retaining potency even after one year (Fig. 8A and B).[120] A third innovation focused on esterase-triggered decationizable quaternium lipidoids, particularly AMB-POC18, which preserved positive charge during storage but reversed to negative upon esterase exposure, facilitating efficient mRNA release. AMB-POC18 LNPs demonstrated superior *in vitro* transfection efficiency (>68%) after 3 days at 4 °C and selectively targeted antigen-presenting cells in the spleen *in vivo*, eliciting robust immune responses against melanoma.[121] Collectively, these studies underscore the importance of the lipid architecture, charge dynamics, and microviscosity in designing thermostable LNPs, offering promising avenues for long-term mRNA vaccine storage and targeted immunotherapy.

Microneedle-based technologies are emerging as a transformative approach to vaccine delivery. One microneedle patch focuses on thermostable mRNA vaccine administration using

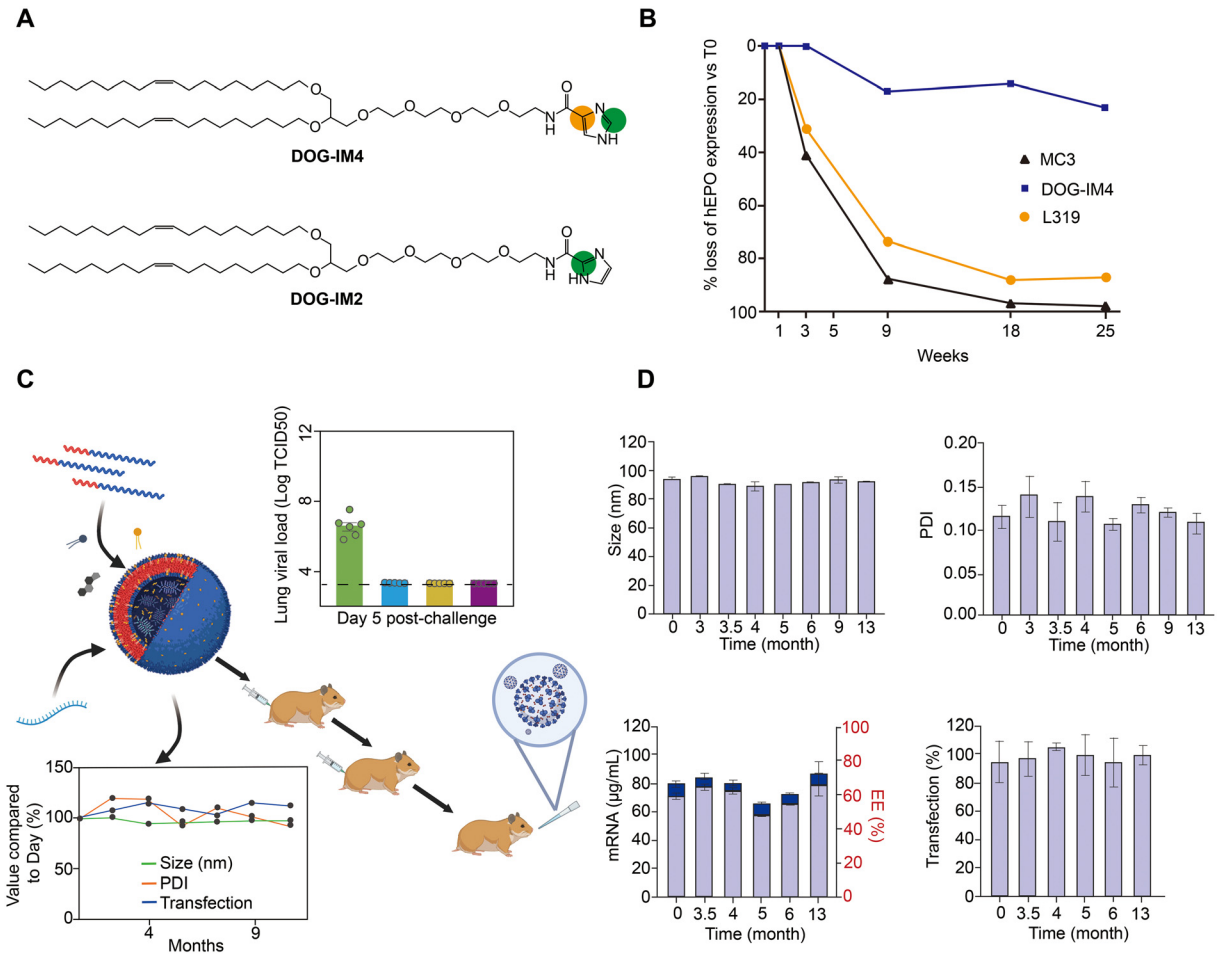

Fig. 8 Examples of LNPs with higher thermostability. (A) Thermostability of mRNA/LNPs could be improved by imidazole modified lipids. Structures of imidazole modified lipids, DOG-IM4 and DOG-IM2. (B) Long-term human erythropoietin (hEPO) expression level of DOG-IM4 after storing at 4 °C for different times. Reproduced with permission from ref. 120. Copyright 2022, Elsevier. (C) Combining block copolymer polybutadiene-*b*-poly (ethylene glycol) (PBD-*b*-PEO) with LNPs to produce thermostable block copolymer nanoparticles (BNPs). Schematic of BNPs with high thermostability. (D) Particle size, PDI, total mRNA, encapsulated mRNA, encapsulated efficiency and transfection efficiency of BNPs across 13 months. Reproduced with permission from ref. 125. Copyright 2025, American Chemical Society.

LNPs embedded in a dissolvable polymer matrix, which demonstrated preserved bioactivity *in vitro* and strong immune responses in mice, with enhanced protein expression *via* intradermal application (Fig. 9A–C).[122] These patches remained stable for six months at room temperature and one month at 37 °C, and were consistently produced using an automated microneedle vaccine printer.[122] Another study integrates NIR fluorescent microparticles for invisible on-patient medical record-keeping (OPMR), with *in vitro* validation confirming no compromise to LNP–mRNA integrity (Fig. 9D).[123] *In vivo* pig studies showed deep dermal dye deposition and high signal retention over 10 weeks, while immunization trials in mice and pigs demonstrated non-inferior efficacy.[123] Shelf-life testing in rats confirmed sustained mRNA activity for three months at room temperature, and biocompatibility assessments revealed minimal inflammation, no fibrosis, and gradual dye clearance, supporting the safety and practicality of this dual-function platform.

The use of copolymers in mRNA delivery systems significantly enhances thermostability by forming protective matrices that shield mRNA from hydrolytic and thermal degradation, maintaining structural integrity and translational activity even under elevated temperature conditions.[124] Block copolymer integration, specifically using PBD-*b*-PEO, significantly enhances LNP thermostability. Hybrid BNPs that combine PBD-*b*-PEO with ionizable lipids, cholesterol and helper lipids achieve exceptional stability, with luciferase mRNA–BNPs remaining intact for over one year at 4 °C (ref. 125) (Fig. 8C and D). Replacing DMGyield000 with PBD-*b*-PEO in Onpattro-like formulations yields stable BNPs that maintain a uniform size, low polydispersity, and 90% encapsulation efficiency for up to 13 months at 4 °C, outperforming conventional LNPs prone to aggregation, mRNA leakage, and reduced transfection efficiency.[125] Immunization studies *in vivo* by delivering COVID-19 mRNA vaccine using PBD-*b*-PEO induced durable immune responses, and protective efficacy against

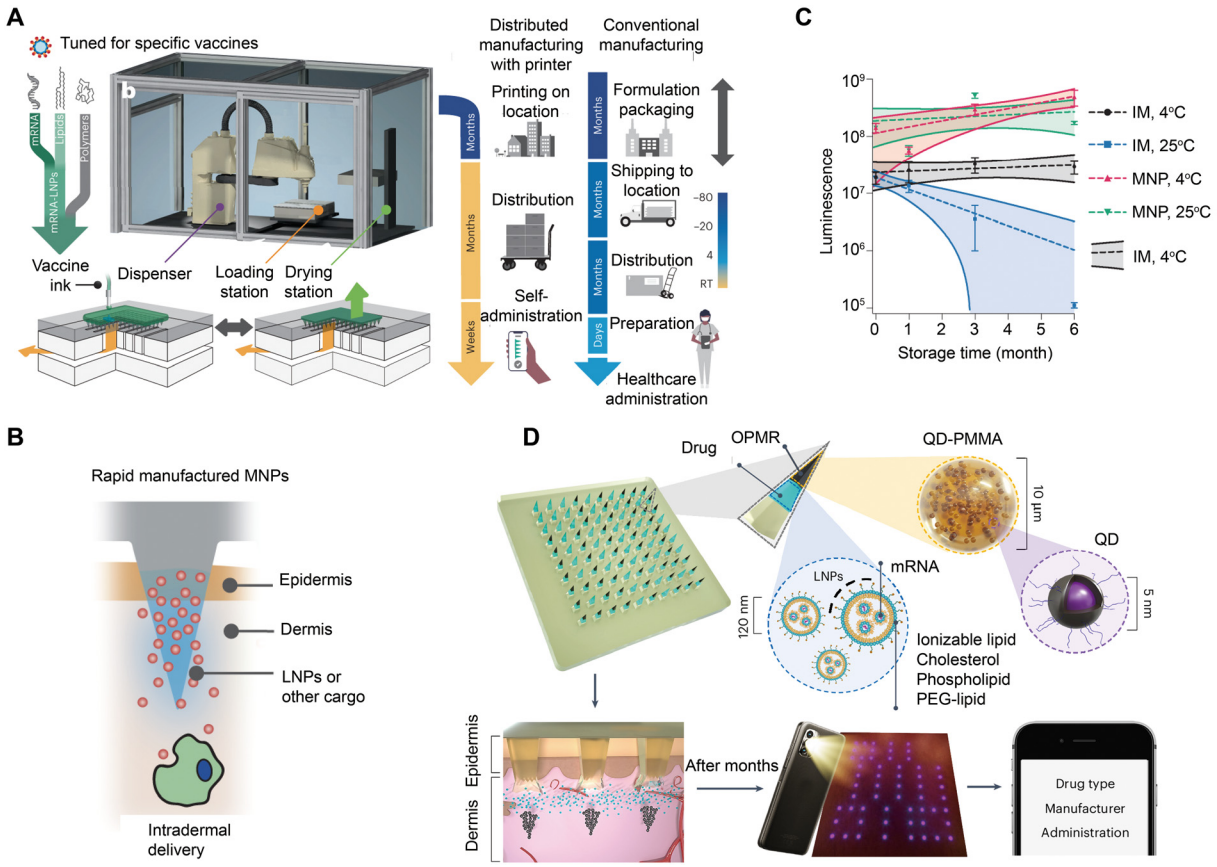

Fig. 9 Examples of LNPs in microneedles with higher thermostability. (A) Customizable modular inks composed of mRNA, lipids, and polymers enable microneedle-based vaccine printing. These formulations can be dried for remote distribution, supporting decentralized vaccine production. (B) Microneedles dissolve upon application, releasing mRNA–LNP vaccines directly into the intradermal layer for effective delivery. (C) The PVP : PVA matrix preserves mRNA–LNP integrity within microneedle patches, enabling robust protein expression even after six months of storage at ambient temperature. Reproduced with permission from ref. 122. Copyright 2023, the Authors, Springer Nature America, Inc. (D) Near-infrared (NIR) fluorescent quantum dot dye encapsulated with PMMA microparticles is co-delivered with mRNA-loaded lipid nanoparticles *via* microneedles supported by a dissolvable polymer base. Upon application, the dye microparticles are embedded into the dermis in a specific pattern encoding medical data, while the mRNA–LNPs are taken up by immune cells to trigger an immune response. NIR patterns are captured and decoded on a display for medical information retrieval. Reproduced with permission from ref. 123. Copyright 2025, the Authors.

SARS-CoV-2, maintaining immunogenicity and avoiding anti-PEG antibody boosting even after 24 weeks of storage.

Despite these advances, achieving a balance between mRNA–LNP stability and biocompatibility is complex, as the new lipid components may induce toxicity or immune responses. Additionally, ensuring consistent manufacturing quality, scalability, and storage stability of LNPs remains difficult.[126,127] Addressing these challenges is essential to improve mRNA protection, reduce dosage requirements, and enhance the safety profile of LNP-based therapeutics.

3.5.3. Metal-assisted mRNA–LNP stabilization. Emerging evidence indicates that coordination interactions between metal ions and mRNA or LNP components constitute a promising alternative strategy to improve the stability and *in vivo* performance of mRNA therapeutics. For example, zinc-coordinated ionizable lipids can form tight yet reversible coordination with mRNA phosphate groups, yielding LNP formulations with enhanced encapsulation stability and significantly higher *in vivo* translation compared to conventional SM-102-based LNPs.[128] Building on this concept, Zn^{2+}-coordinated LNP platforms have also been developed that combine metal ion coordination with novel lipid compositions to improve organ-specific targeting and immunogenicity, demonstrating both enhanced mRNA delivery efficiency and immune response.[129]

Additionally, metal ion-mediated mRNA enrichment strategies, such as Mn^{2+}-mRNA complexes subsequently coated with lipid shells, have been shown to substantially increase mRNA loading capacity and cellular uptake, thereby enhancing vaccine potency and reducing lipid-associated toxicity.[130] Beyond lipid-based systems, metal–organic nanoparticle platforms incorporating divalent metal ions also provide robust mRNA stabilization and efficient *in vivo* protein expression, highlighting the potential of metal coordination chemistry as a versatile tool to complement existing mRNA–LNP technologies.[131]

3.5.4. Storage and transport of mRNA–LNP formulations. Cryopreservation at ultra-low temperatures, typically below −70 °C, is widely used for long-term storage. For instance, Moderna's mRNA-1273 vaccine can be stored for up to six months at −20 °C, while Pfizer–BioNTech's BNT162b2 must be stored at −70 °C.[132] These strict temperature requirements pose significant challenges in regions with limited cold-chain infrastructure. To address this limitation, alternative preservation strategies such as lyophilization (freeze-drying) and encapsulation within protective matrices or nanoparticles have been developed to extend shelf life and improve stability during storage and transport.

Lyophilized mRNA–LNP vaccines have shown exceptional thermal stability, retaining structural integrity and immunogenic function for up to six months at room temperature (25 °C). However, instability in any form poses challenges for storing mRNA–LNPs in aqueous buffers as wet formulations prior to lyophilization. External factors such as buffer pH and storage temperature can further influence stability. As a result, it is crucial to optimize lyophilization parameters, including buffer composition, cycle duration, and temperature, to preserve the physicochemical integrity of LNPs during storage.[133] Lyophilization was carried out using a freeze dryer by freezing samples at −45 °C, drying them in two stages, and then sealing the vials with nitrogen before storing them at various temperatures for stability testing. This advancement helps overcome cold-storage challenges and improves vaccine accessibility in underserved regions. Both preclinical and clinical studies of lyophilized SARS-CoV-2 mRNA vaccines have demonstrated strong immune responses, including robust antibody generation and T-cell activation. The lyophilization process preserves the vaccine's physicochemical properties and immunogenicity up to 12 weeks at room temperature or 24 weeks at 4 °C while maintaining efficacy (Fig. 10A–D). Notably, booster doses resulted in a 253-fold increase in Omicron-neutralizing antibodies. This lyophilization approach marks a key advancement in making global distribution of mRNA vaccines more practical and efficient.[133]

Additionally, drying methods such as spray drying and vaporization, which are commonly used in stabilizing protein-based vaccines, are now being investigated for mRNA formulations.[134] These techniques may enable long-term storage under ambient conditions, which can help overcome distribution challenges. To support large-scale manufacturing, applying Quality by Design principles with an emphasis on critical quality attributes and process parameters can optimize these drying technologies. Such innovations in stabilization strategies hold the potential to extend vaccine shelf life, reduce distribution barriers, and increase global access to mRNA-based immunizations. Spray drying or freeze drying of mRNA-loaded lipid-like nanoparticles (LLNs) with cryoprotectants such as sucrose or trehalose can help maintain particle size and support *in vitro* delivery efficiency. However, structural changes during drying and reconstitution may reduce *in vivo* efficacy due to altered interactions with serum proteins. Optimizing cryoprotectant concentrations and processing conditions is therefore essential to achieve both long-term stability and effective biological performance[134,135] (Fig. 10E and F).

Maintaining consistent particle size, moisture content, and reconstitution properties are critical for the application of drying technologies. Additionally, the current drying processes can expose mRNA to stress conditions like heat or shearing, risking structural damage. Overcoming these challenges requires developing protective excipients and optimized protocols to ensure long-term stability, ease of storage, and consistent performance of dried mRNA formulations.

In addition to physical and enzymatic degradation pathways, chemical instability within the LNP formulation itself can compromise mRNA integrity. Current studies demonstrated that ionizable lipids, particularly those containing tertiary amines, are susceptible to oxidation and hydrolysis during storage or processing, leading to the generation of reactive aldehyde species. These aldehydes, derived *via N*-oxide intermediates of the ionizable lipid, can form covalent lipid–mRNA adducts, resulting in decreased mRNA activity and impaired translation.[136,137] The mechanistic model for mRNA–

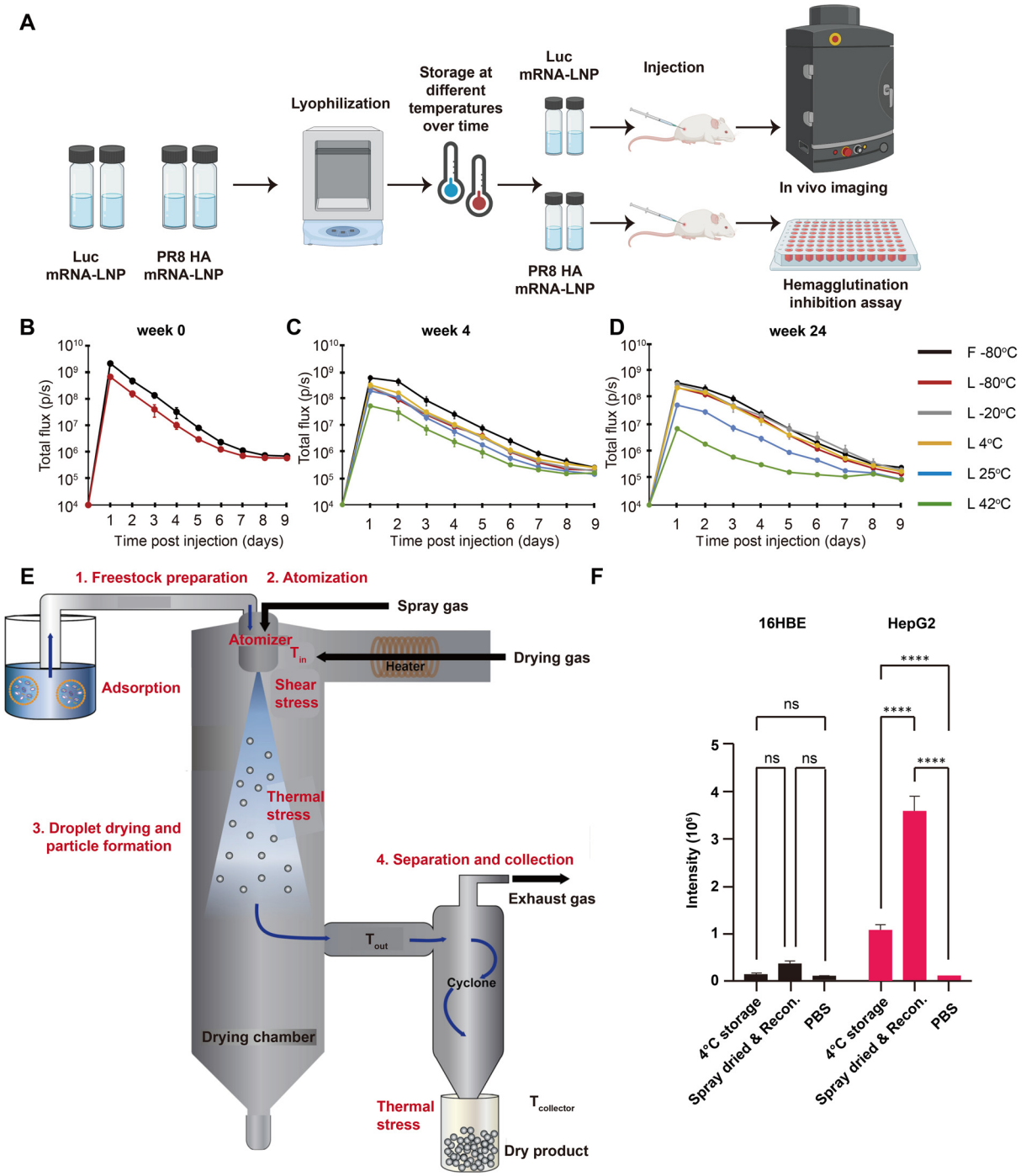

Fig. 10 Examples of storage methods to enhance the stability of LNPs. (A) Schematic of workflow synthesis and testing of lyophilized LNPs. Quantification of bioluminescent signals after IM injection of frozen and reconstituted lyophilized Luc mRNA–LNPs stored after 0 (B), 4 (C) or 24 weeks (D) of storage. Reproduced with permission from ref. 133. Copyright 2022, Elsevier. (E) The workflow of the spray drying process. (F) Quantification of green fluorescent protein (GFP) fluorescence after transfecting HepG2 and 16HBE cells with LNPs processed by spray drying and stored at room temperature and liquid LNPs stored at 4 °C. Reproduced with permission from ref. 135. Copyright 2023, Elsevier.

lipid adduction proposes that mRNA phosphate groups brought into proximity with oxidized lipid peroxide species can undergo covalent modification by aldehyde cleavage products.[138] These findings highlight the importance of minimizing lipid oxidation and *N*-oxide formation during formulation and storage to maintain mRNA stability. Additionally, the development of antioxidant lipids may provide an alternative for advancing next generation delivery systems.

Delivery vehicle optimization contributes to mRNA stability by protecting the transcript from degradation, modulating immune responses, and preserving functional integrity under variable physiological and logistical conditions. Engineering LNP compositions can reduce immunogenicity, limit inflammation-driven RNase induction, and support efficient translation, while alternative lipid chemistries and biomimetic carriers further minimize innate immune activation and enhance biocompatibility. Thermostable lipids mitigate temperature-induced leakage and structural disruption, extending the shelf life of mRNA–LNP formulations and reducing dependence on the cold-chain infrastructure. Advances in storage technologies, including lyophilization and emerging drying approaches, address practical barriers to distribution without compromising biological activity. Overall, mRNA stability is not determined solely by payload optimization but also by the physicochemical and immunological environments shaped by the delivery system, making material optimization essential for realizing the full clinical and global potential of mRNA therapeutics.

4. Clinical translation challenges

The therapeutic potential of mRNA is significant, yet its clinical application faces critical hurdles. The inherent instability of mRNA remains a primary challenge. Advances in synthetic nucleoside modifications and purification techniques have improved stability and reduced immune activation, but achieving an optimal balance of half-life, translational efficiency, and immune evasion requires further refinement. Unlike DNA-based therapies, mRNA avoids genomic integration, reducing risks of insertional mutagenesis and enhancing safety. However, its transient expression, lasting hours to days, necessitates repeated dosing for chronic conditions. Strategies like optimized UTRs, chemically modified nucleotides, and circular RNA formats are being explored to extend expression without compromising efficiency. Safety concerns persist, as repeated dosing may trigger innate immune responses, leading to inflammation or reduced efficacy. LNP delivery systems also pose risks of local or systemic toxicity. Comprehensive preclinical safety assessments and long-term monitoring are critical to ensure efficacy and tolerability across diverse clinical settings.

Producing clinical-grade mRNA involves complex processes, including *in vitro* transcription (IVT), purification to remove immunogenic impurities like double-stranded RNA, and LNP formulation, all under stringent Good Manufacturing Practice (GMP) standards. While mRNA vaccine production has demonstrated scalability, extending this to diverse therapeutic applications, especially personalized or repeat-dosing treatments, remains challenging. Consistent production of long, chemically modified mRNA sequences requires robust processes to ensure batch-to-batch consistency in capping efficiency, poly (A) tail length, and purity.[139] Artificial intelligence (AI) is increasingly utilized to optimize mRNA sequence design by minimizing immunogenic motifs and enhancing ribosomal engagement, though excessive structural constraints may impair translation.[140]

Regulatory challenges complicate mRNA therapeutic development, particularly regarding LNP excipients. The FDA mandates rigorous characterization of encapsulation efficiency, nanoparticle stability, and manufacturing reproducibility.[141] Next-generation delivery systems, such as targeted LNPs and exosomal carriers, enhance tissue specificity but introduce additional regulatory complexities. Combining mRNA with gene-editing technologies like CRISPR further necessitates adaptable regulatory frameworks. The novelty of mRNA therapeutics requires evolving guidelines to address unique manufacturing, delivery, and clinical challenges. Standardized quality control, potency assays, and safety assessments are essential for product consistency and patient safety. Collaborative efforts among industry, regulators, and academia are crucial to establishing harmonized standards and accelerating clinical translation.

5. Conclusion and perspectives

In summary, mRNA technology stands at the forefront of innovation, poised to reshape the landscape of modern medicine. From its role in preventing infectious diseases through robust vaccine development to enabling personalized cancer treatments, mRNA has demonstrated unprecedented versatility. Moreover, its capacity to facilitate the production of therapeutic proteins holds immense promise for treating a spectrum of diseases. Moreover, despite emerging engineering strategies in advancing mRNA technology, the development of a successful mRNA drug from bench to bedside is still encountering numerous challenges as this workflow involves a chain of costly, complex and intricate operations. Therefore, further novel technologies should be considered to yield significant advancements in mRNA therapeutics.

Recently AI has revolutionized mRNA therapeutic design by leveraging machine learning and large-scale datasets to optimize sequences for stability, translational efficiency, and safety. Advanced models eliminate destabilizing motifs, optimize codon usage, and refine UTRs to enhance ribosome recruitment and protein expression. AI also aids in optimizing mRNA structures to avoid translation-hindering secondary structures and LNP formulations for targeted delivery and minimal toxicity. However, AI-assisted LNP design still encompasses untapped areas for further increase, promising to enhance the targeted delivery capabilities of mRNA therapeutics. In addition to brain-targeted mRNA delivery, LNPs need intrathecal injections with low efficiency, and using machine learning on existing datasets could enable the screening and optimization of more precise brain-penetrating systems, potentially overcoming the blood–brain barrier challenges.[142]

DNA/RNA barcoding technology is also accelerating mRNA development, especially for delivery system screening and

identification, enabling high-throughput *in vivo* screening of mRNA formulations,[143] enabling fast *in vivo* screening of mRNA–LNP formulations on minimal animal samples by tagging mRNA payloads with unique barcodes, allowing simultaneous evaluation of multiple structures and formulations with high efficiency. This approach identifies delivery vehicles with specific tissue tropism, such as lung-targeting LNPs for respiratory disease. However, current research has revealed the variations of mRNA delivery and expression across species, posing significant challenges for translational research and clinical development. The design and application of barcoding systems in humanized models or large animals will provide valuable insights for understanding interspecies differences, which should be essential to enhance the efficacy and safety of mRNA treatments in humans.

In conclusion, diverse high-efficiency methods have markedly enhanced mRNA therapeutics efficiency, targeting precision, and sustained efficacy. These innovations enable prolonged protein expression for chronic disease management and more effective targeted therapies.

Author contributions

Ze Zhang: writing – original draft and review & editing; Yen Hui Ong, Bangda Fan and Bowei Yang: writing – original draft; Yi Yan Yang: conceptualization and review; and Qianqian Ni: conceptualization and writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included, and no new data were generated or analysed as part of this review.

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