NUCLEIC ACID INSIGHTS

DELIVERY AND FORMULATION

SPOTLIGHT

EXPERT INSIGHT

Challenges and advances of the stability of mRNA delivery therapeutics

Jin Zhai, Trystin Cote, and Yupeng Chen

mRNA therapeutics have garnered significant attention in the biomedical realm, showing immense potential across a spectrum of applications from COVID-19 to cancer treatments. Their ability to trigger precise protein expression, particularly in genome editing, is pivotal in minimizing off-target effects. At the core of mRNA therapy lies a dual-component system, comprising the mRNA itself and a delivery vehicle. The breakthrough success of novel COVID-19 vaccines has catapulted lipid nanoparticles to prominence as the preferred delivery vehicle. However, despite their US FDA approval and efficacy, lipid nanoparticles face a significant challenge: poor stability at room temperature, which limits their applications in various geographic regions with disparities in infrastructure and technology. This review aims to dissect the issue of stability inherent in lipid nanoparticles and other mRNA delivery platforms such as polymer-based materials and protein derivative materials. We herein endeavor to unravel the factors contributing to their instability and explore potential strategies to enhance their stability. By doing so, we provide a comprehensive analysis of the current landscape of mRNA delivery systems, highlighting both their successes and limitations, and paving the way for future advancements in this rapidly evolving field.

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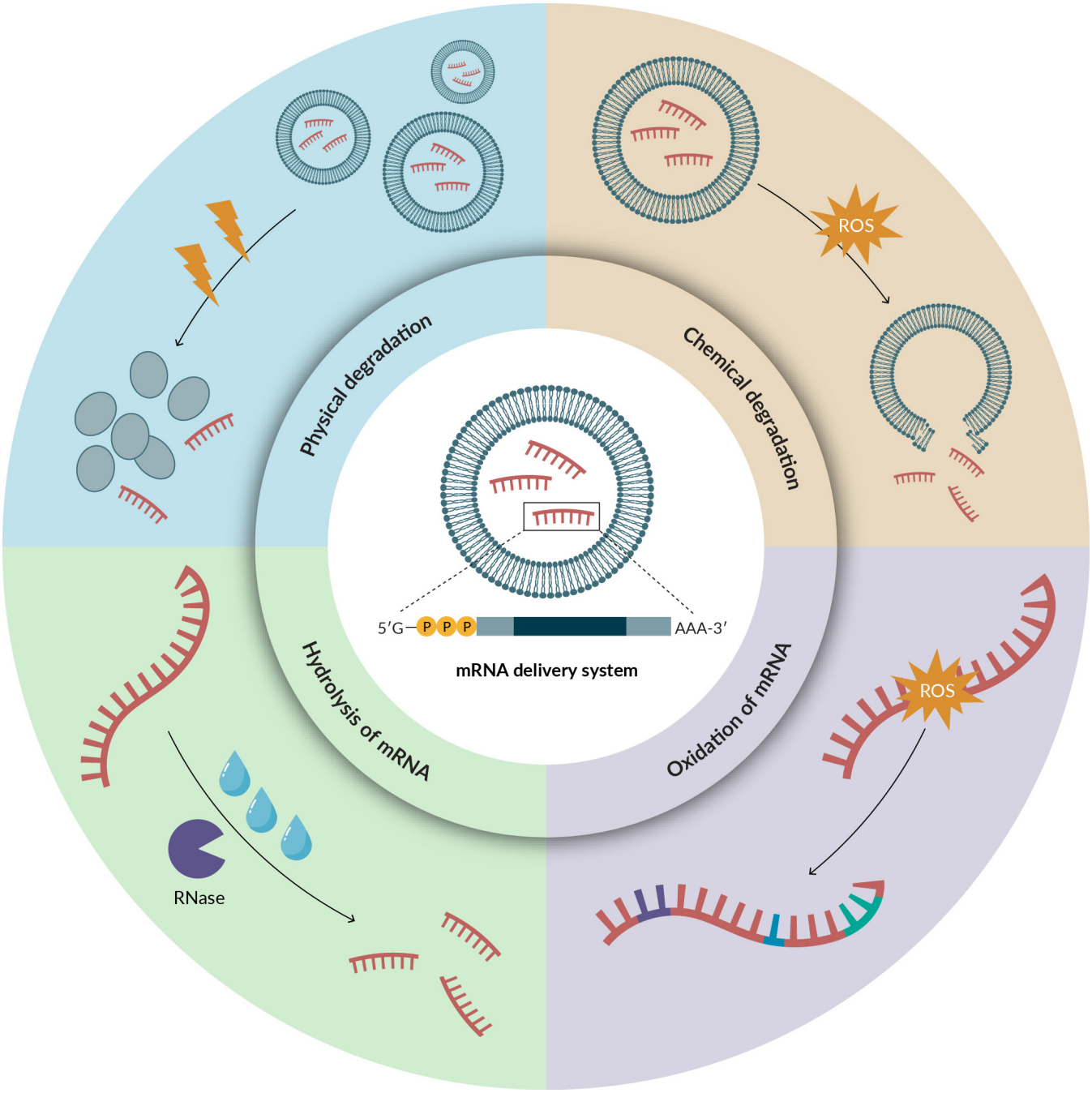
GRAPHICAL ABSTRACT



INTRODUCTION

In the evolving field of genetic medicine, mRNA has emerged as a transformative tool, indicating a new era of therapeutic strategies [1]. The critical role of mRNA as a transient mediator between DNA and proteins provides a unique platform for treating and preventing disease, supporting the concept of the mRNA application as a therapeutic agent [2].

Nucleic acid therapy

Nucleic acid treatments are designed to use the body's own cellular machinery in order

to fight disease. They work by introducing specific DNA or RNA sequences into cells to complement defective genes, silence harmful genes, or provide instructions for the production of therapeutic proteins [3]. This strategy differs from conventional medicines, which often only suppress symptoms without altering the underlying disease pathology [4]. As such, the benefits of nucleic acid therapy are that it may provide a more targeted, effective, and potentially curative approach, in particular for conditions where traditional treatments do not work [5].

However, overcoming important biological challenges such as stability and delivery is essential for the full potential of nucleic acid therapeutics. Nucleic acids, in particular mRNA, are inherently unstable molecules that are prone to rapid degradation in the extracellular environment [6]. Due to the already present difficulty of transporting these negatively charged macromolecules across the cell membrane and into the cytoplasm, instability of the delivery system or mRNA will only create additional barriers to achieving therapeutic effects [7]. These limitations have necessitated the development of mRNA delivery platforms in order to ensure mRNA integrity from the point of administration to its eventual translation within target cells.

mRNA delivery vehicles

The delivery of mRNA to target cells is one of the key elements for the efficient use of mRNA as a therapeutic tool. The development of efficient delivery vehicles for mRNA is essential, as these carriers need to protect mRNA from enzymatic degradation, facilitate cellular uptake, and ensure its release into the cytoplasm, where it can be transformed into functional proteins, all while minimizing potential immunogenic and off-target effects [8]. Numerous strategies for mRNA delivery have been developed, such as lipid-based materials, polymers, protein derivatives, and inorganic particles [9, 10].

Among current strategies of delivery vehicles, lipid nanoparticles (LNPs) have gained prominence, especially highlighted by their successful application in COVID-19 mRNA vaccines [11]. LNPs encapsulate the mRNA in a lipid structure, providing protection against nuclease degradation and enhancing cellular uptake. They are usually composed of ionizable lipids, phospholipids, PEGylated lipids, and cholesterol, and play a crucial role in endosomal escape, ensuring that mRNA reaches the cytoplasm [12]. In addition, the modular nature of LNPs makes it possible to optimize their size, charge, and lipid composition with the goal of improving targeting and reducing immunogenicity. Despite their advantages, the LNPs are confronted with challenges specifically due to their inability to target tissues beyond the liver and potentially due to a diminished long-term safety profile [13–15].

Another popular class of delivery vehicles is polymeric nanoparticles, including biodegradable polymers such as polylactic-glycolic acid or naturally occurring polymers such as chitosan [16, 17]. These particles may be designed to have controlled release characteristics and can be adapted for the purpose of targeting specific types of cells [18]. In addition, they provide a degree of flexibility in terms of cargo capacity and allow for the delivery of not only mRNA, but also some molecules such as siRNA or CRISPR Cas9 components [19].

Protein derivatives as a strategy for mRNA delivery includes exosomes and peptide-based systems. Exosomes are small vesicles that are naturally secreted by cells with inherent targeting capabilities. They can be loaded with mRNA and used as delivery vehicles, potentially reducing immunogenic responses [20]. Peptide-based delivery systems involve the development of peptides that bind to mRNA and facilitate its entry into cells [9]. These systems are still in the early stages of development, but their biocompatibility and targeting potential are promising.

In addition to carriers made of organic materials, inorganic nanoparticles have emerged as promising alternatives for mRNA delivery. These particles, typically made of materials such as gold, silica, or calcium phosphate, are known for their stability, uniformity in size, and ease of surface modification. For example, in order to facilitate cellular uptake and targeted delivery, gold nanoparticles can be functionalized with a variety of ligands [21]. In addition, mRNA can be released from gold nanoparticles in response to external stimuli such as light due to the intrinsic photothermal properties of gold nanoparticles [22]. However, potential cytotoxic effects and issues with biocompatibility and effective degradation after therapeutic use are among the challenges faced by inorganic nanoparticles.

Despite these promising mRNA delivery platforms, the quest to optimize the stability of delivery systems still continues. Challenges remain in preventing delivery system degradation and achieving long-term stability, efficacy, and safety.

MECHANISMS OF mRNA DELIVERY SYSTEM DEGRADATION

An important aspect of achieving clinical translation of mRNA therapeutics is not only the delivery of the mRNA to the target cells, but also the stabilization of the entire delivery system (including both the delivery platform and the mRNA itself) before its function is achieved. Understanding the potential degradation mechanisms of these systems is critical to ensure safe, long-term storage and maximize stability.

Physical degradation of delivery platforms

Physical degradation of drug-loaded delivery systems refers to damage to the mRNA delivery system due to mechanical or thermal stress, including aggregation and leakage of cargo. For lipid nanoparticles and polymeric nanoparticles, physical degradation can occur during storage, transportation, and handling, where temperature fluctuations or mechanical agitation cause the particle structure to break down. The breakdown compromises the integrity of the encapsulated mRNA, making it susceptible to enzymatic degradation. The stability of LNPs is significantly dependent on the storage temperature, while the pH level of the solution is less critical in storage conditions [23]. In addition, lipoplexes, cationic liposome complexes, are unstable in solution and form aggregates during long-term storage at room temperature [24]. Even some commercially available liposome formulations demonstrate physical instability in aqueous solutions because of encapsulated solute leakage and aggregation during long-term storage [25].

Chemical degradation of delivery platforms

Chemical degradation is a change in the chemical structure of the delivery system or mRNA itself. In lipid-based systems, this includes oxidation or hydrolysis of lipid components, which can affect the particle's ability to protect and transport mRNA. The oxidation of lipids occurs at the double bonds of unsaturated fatty acids, which provide sites where radicals can easily form when exposed to reactive oxygen species (ROS) [26]. This oxidation can critically impair structural integrity, potentially precipitating the premature release or degradation of the encapsulated mRNA. Furthermore, lipid oxidation products may be recognized by the immune system, thus altering the immunogenic profile of the LNP formulation [27]. Such alterations are not merely structural but can have profound functional implications. Specifically, destabilization of the lipid carrier due to oxidation compromises the efficacy of mRNA delivery, impeding the mRNA's capacity to reach its intended target and undergo successful translation into the requisite protein [28]. Moreover, the constituent lipids in LNPs are susceptible to hydrolytic reactions,

particularly at ester or amide bonds [29]. Such hydrolysis leads to the disintegration of lipid molecules into glycerol, fatty acids, and other by-products. This process can critically undermine the structural integrity of the nanoparticles, thereby impairing their capacity for effective mRNA delivery. These considerations underscore the essentiality of maintaining the stability of lipid components within LNPs to ensure the effective delivery of mRNA-based therapeutics.

Numerous polymers utilized in mRNA delivery, such as polylactic-co-glycolic acid, are similarly prone to hydrolytic degradation [30]. This degradation, characterized by the cleavage of ester bonds within the polymer's backbone, is catalyzed by water molecules. The rate of hydrolysis, influenced by factors like the polymer's composition, molecular weight, and the presence of catalytic agents, can sometimes lead to premature degradation. Such premature hydrolytic degradation of the polymer matrix can result in the untimely release of the encapsulated mRNA, potentially compromising the efficacy of the therapeutic delivery. Furthermore, certain polymers, while engineered to respond to specific environmental conditions like pH or temperature, may degrade unexpectedly under non-ideal conditions [31]. This can be particularly problematic for polymers designed to degrade in acidic environments, such as endosomes, as uncontrolled degradation can occur before the polymer reaches the targeted cellular compartment. Additionally, susceptibility to oxidative degradation in the presence of ROS can further destabilize these polymers [32, 33]. Oxidative stress can lead to the breaking of polymer chains, thereby diminishing their structural integrity and reducing their ability to effectively encapsulate and deliver mRNA. These negative aspects highlight the challenges in ensuring the stability and controlled degradability of polymer-based delivery systems for effective mRNA therapy.

The stability and delivery efficacy of protein-based mRNA delivery systems are significantly influenced by chemical degradation processes, including proteolysis, denaturation, deamidation, and oxidation [34]. Proteolysis, which involves the fragmentation of proteins by proteases, compromises the structural integrity of the delivery systems. Environmental shifts induce denaturation, altering the three-dimensional configurations of proteins and impacting mRNA interaction and encapsulation. Deamidation, on the other hand, changes the protein's structure and charge, thereby affecting mRNA stability and interaction. Additionally, oxidation, triggered by ROS, leads to structural changes in proteins, influencing their capacity to protect and deliver mRNA. These mechanisms highlight the challenges associated with maintaining the functional stability of protein-based delivery systems for effective mRNA therapy.

Hydrolysis of mRNA phosphodiester backbone

It is widely accepted that mRNA as a molecule is inherently more unstable than DNA due to the ribose 2′ OH group that can cleave its neighboring phosphodiester bond by in-line nucleophilic attack, a mechanism that is typically favored at alkaline pH and can be catalyzed by amines that are present in some LNPs and other delivery systems [35]. In this way, hydrolysis is a key degradation process for mRNA, predominantly targeting its phosphodiester bonds that interconnect nucleotides [36]. This reaction, catalyzed by RNases, fragments the mRNA into smaller nucleotide sequences, thereby compromising its functional integrity. Notably, the rate of hydrolysis is accelerated in aqueous environments and is further modulated by factors such as pH and the ionic composition of the surrounding environment. This susceptibility to hydrolytic degradation presents a formidable challenge in mRNA delivery as the molecule may undergo premature degradation en route to target cells. The stability of the mRNA's phosphodiester backbone is, therefore, pivotal in maintaining its structural integrity and ensuring its therapeutic viability.

Oxidation of mRNA ribose nucleobases

Oxidative degradation is a crucial factor impacting mRNA stability and its delivery efficiency. ROS target mRNA, leading to structural damage by attacking the ribose sugar and nucleobases [36]. This oxidative stress can cause strand breaks or base alterations, potentially hindering the translation process or resulting in aberrant protein synthesis. Such modifications to mRNA's nucleobases or ribose backbone, induced by oxidation, lead to structural changes that can significantly impede its translational accuracy. These oxidative effects not only compromise the integrity of mRNA but also alter the effectiveness of mRNA-based therapeutic applications, underscoring the importance of safeguarding mRNA from oxidative damage in delivery systems.

APPROACHES TO INCREASE STABILITY

To enhance the stability of mRNA delivery systems, it is useful to focus on two primary aspects: preventing physical and chemical degradation. The application of stabilizing agents or protective coatings plays an essential role in shielding these systems from mechanical and thermal stresses. These preventative measures are carefully engineered to preserve the system's integrity under varying physical conditions, effectively preventing premature degradation. Notably, it has been shown that the buffering species chosen for the formulation is of key importance and has the potential to improve the stability of RNA drug products, especially in the case of LNP/RNA drug products [37].

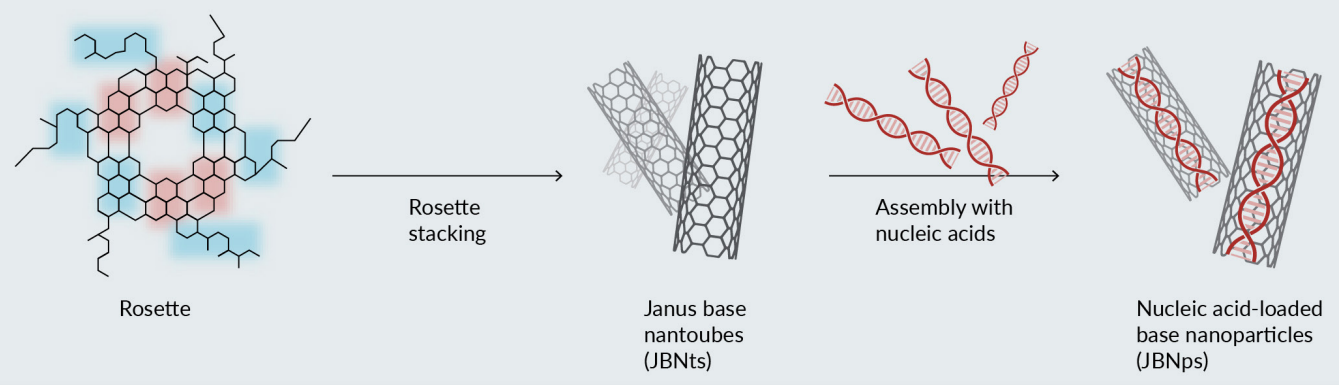
Addressing chemical degradation is also of paramount importance. This involves incorporating tailored chemical modifications into the delivery system, specifically designed to withstand enzymatic actions and environmental factors that could otherwise compromise stability. For instance, varying the cholesterol composition of some systems can stabilize lipid layers, which promotes the cohesion and liquid-ordered phases of lipids [38].

At present, freezing and lyophilization (freeze-drying) are the most common approaches to addressing and overcoming the above forms of degradation in the case of long-term storage [39]. Freeze-drying has been proven to increase the shelf life of pharmaceutical products by removing water. Studies have shown that the efficacy of LNPs, particularly during freeze-thaw cycles, can be maintained by adding cooling agents and cryoprotectants such as trehalose and sucrose [40]. This aspect is crucial as it addresses the challenge of maintaining stability in LNPs under conditions such as freezing and thawing, which are common for pharmaceutical transport. However, relying on keeping these products frozen to maintain stability is undesirable due to the high cost, as well as barriers to transport and accessibility of the therapeutics when they are required to be kept below the temperature of a standard refrigerator.

Additionally, strategies to counteract the hydrolysis of the mRNA phosphodiester backbone and oxidation of the mRNA ribose nucleobases are critical. Chemically modified nucleotides can be used to reduce susceptibility to hydrolysis, thus enhancing the mRNA's stability within the delivery system. The resistance to hydrolysis may be further increased by chemical modification of the mRNA, such as the addition of pseudouridine which can similarly enhance stability of the mRNA [41, 42]. In these cases, the pseudouridine is essentially stopping the innate immune system from recognizing that the mRNA molecule is exogenous, therefore preventing degradation caused by the immune system itself. Another effective strategy to mitigate mRNA hydrolysis involves the redesign of RNA molecules to form double-stranded regions [43]. This structural alteration provides protection against in-line cleavage and enzymatic degradation while maintaining the capability to code for the intended proteins. Moreover, integrating

FIGURE 1

Diagram of the delivery system of Janus base nanotube.



antioxidants like ascorbic acid and glutathione into the formulation provides a protective barrier against oxidative stress, ensuring the preservation of the structural integrity of mRNA [44]. These approaches collectively contribute to the development of robust mRNA delivery systems, capable of maintaining their functional efficacy in therapeutic applications.

Recent advancements in the realm of mRNA delivery have heralded the advent of innovative vehicles such as DNA-inspired nanoparticles and hybrid nanoparticles, each exhibiting remarkable potential for enhanced stability and sustained efficacy [45–48]. One example of DNA-inspired materials is Janus-based nanotubes (JBNts), deriving their nomenclature from the dual-faced Roman deity and exhibiting an architecturally distinct bifunctional design [49–51]. JBNts (Figure 1) represent a cutting-edge class of biomimetic nanotubes, distinguished by their unique ability to self-assemble into elongated bundles featuring hollow channels, adept for the encapsulation of therapeutic agents [52,53]. The structural foundation of JBNts is rooted in rosette nanotubes, which are composed of guanine and cytosine DNA base pairs [54, 55]. Augmenting this structure are the six-amino-acid fusions of adenine and thymine DNA base pairs, which confer enhanced biocompatibility and biodegradability to the JBNts [56]. Central to the architecture of JBNts is the DNA base analogue, specifically the adenine–thymine motif, whose building blocks spontaneously orchestrate into stable nanotubes upon exposure to aqueous environments [57]. This self-assembly is driven by a confluence of hydrogen bond formation, π-stacking interactions, and hydrophobic effects, culminating in a structurally robust and functionally versatile nanotube [58]. This unique configuration facilitates concurrent targeting and release modulation, thereby ensuring precise and protected delivery of the mRNA payload [59,60]. Their asymmetric composition is strategically crafted to bolster resistance against enzymatic degradation and environmental adversities.

Concurrently, hybrid nanoparticles have emerged at the forefront, amalgamating the virtues of organic and inorganic materials into a singular platform. These nanoparticles are typically characterized by a core-shell architecture, where the inorganic core imparts structural resilience and controlled release dynamics, while the organic shell amplifies biocompatibility and augments targeted delivery capabilities [61]. Moreover, core–shell structured lipopolyplex nanoparticles and nanostructured lipid carriers, integral components of certain mRNA COVID-19 vaccines, have received licensure for human use across various global regions. This includes the SW-BIC-213® vaccine from Stemirna Therapeutics which is currently in a Phase 3

clinical trial, as well as the Gemcovac®-19 vaccine from Gennova Biopharma which was approved for use in 2022 [62–64]. These delivery systems have claimed to remain stable and bioactive at refrigerated temperatures or in a lyophilized powder form for more than several months [65]. The interplay between organic and inorganic components in these hybrid structures not only accentuates stability but also enables the customization of release profiles—a pivotal attribute for extending the therapeutic impact of mRNA treatments [66]. Collectively, these cutting-edge vehicles represent a significant paradigm shift in mRNA delivery methodologies, offering robust and versatile alternatives to traditional systems and heralding a new era in mRNA-based therapeutic interventions.

TRANSLATIONAL INSIGHT

Despite significant advances in mRNA delivery systems, limitations persist. One primary challenge lies in the intricate balance between stability and efficiency of delivery platforms. For instance, the structural modifications necessary for stability can sometimes impede efficient cellular uptake or release of mRNA. Additionally, the diverse physiological environments encountered by these systems en route to their target cells introduce complexities in maintaining functional integrity. Furthermore, potential immunogenic responses and off-target effects remain a concern, especially in lipid-based and inorganic nanoparticle systems. Addressing these limitations requires ongoing research and innovative design strategies.

In conclusion, the field of mRNA delivery systems stands at a promising juncture, with substantial advancements already achieved and numerous possibilities on the horizon. Future research should focus on developing delivery platforms with enhanced stability, targeted delivery capabilities, and minimal immunogenicity. Exploration into novel materials and structural designs is crucial, as is the refinement of existing systems for specific therapeutic applications. Continued interdisciplinary efforts in bioengineering, material science, and molecular biology are essential to overcome current limitations and fully realize the potential of mRNA therapeutics in diverse clinical settings.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

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