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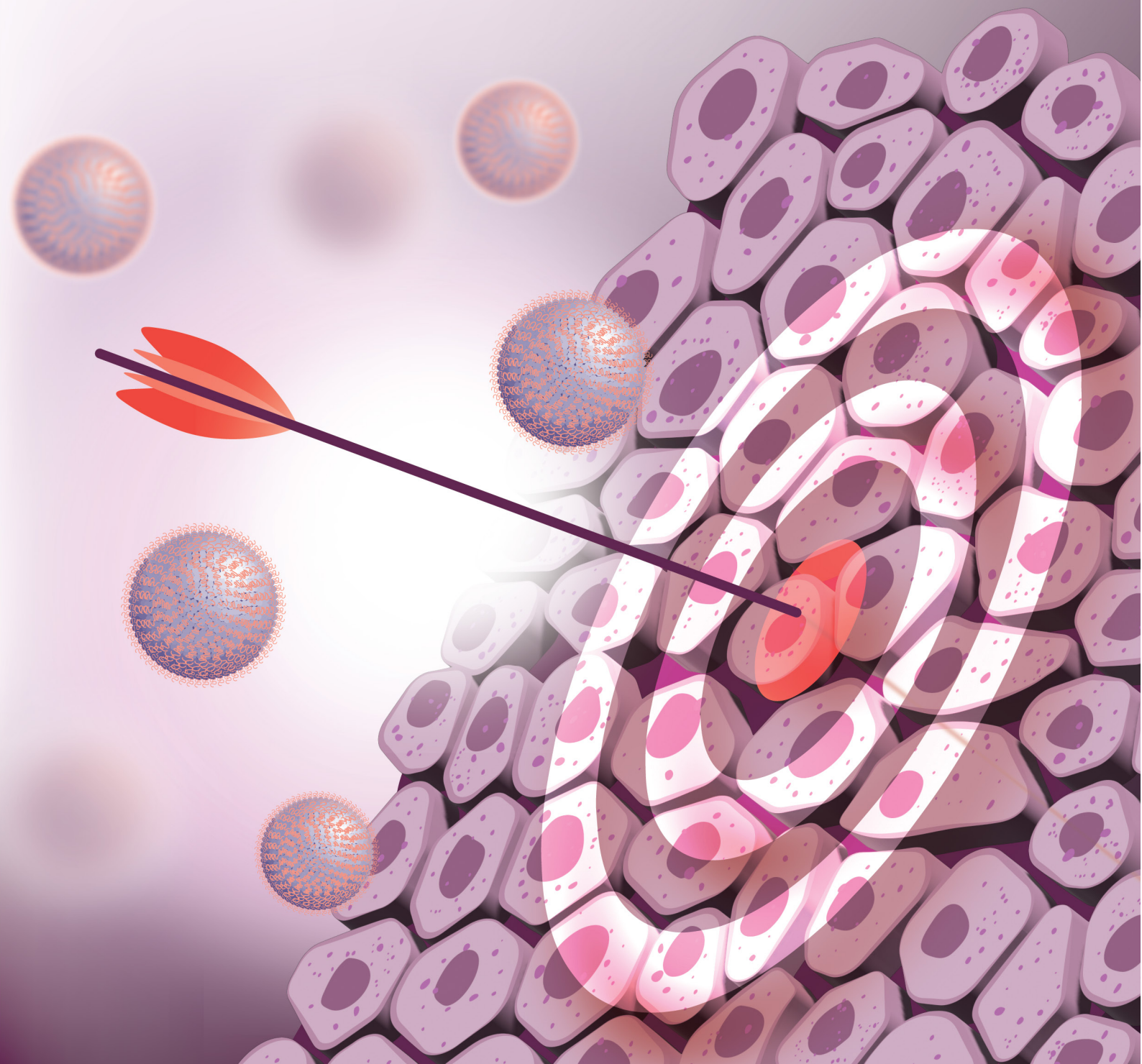
NUCLEIC ACID INSIGHTS

SPOTLIGHT ON
Delivery and formulation



NUCLEIC ACID INSIGHTS

Volume 1, Issue 2

CONTENTS

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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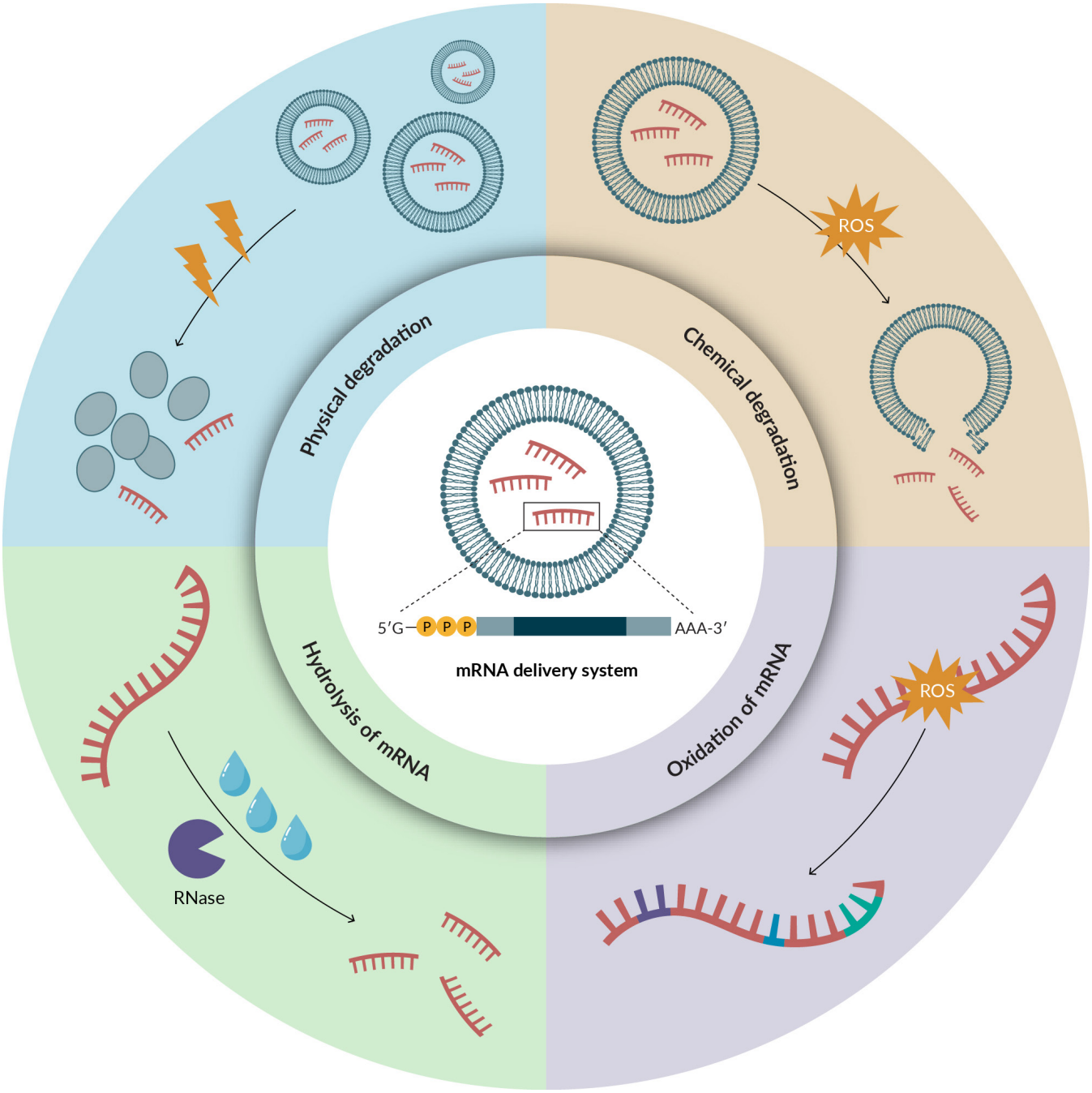
GRAPHICAL ABSTRACT



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].

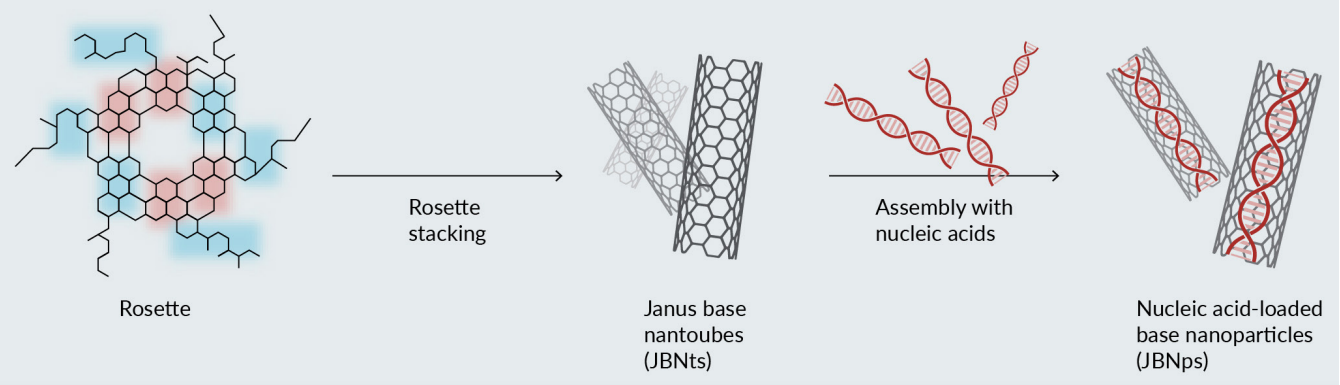
Addressing chemical degradation is also of paramount importance. This involves incorporating tailored chemical modifications into the delivery system, specifically designed to withstand enzymatic actions and environmental factors that could otherwise compromise stability. For instance, varying the cholesterol 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

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NUCLEIC ACID INSIGHTS

DELIVERY AND FORMULATION

SPOTLIGHT

COMMENTARY

Comparative analysis of nucleic acid delivery systems for gene therapy: assessing viral and non-viral approaches with emphasis on extracellular vesicles

Nizar Y Saad

In the landscape of modern medicine, nucleic acid therapeutics have emerged as groundbreaking agents, holding the promise to revolutionize disease treatment at its genetic roots. These therapeutics encompass a spectrum of approaches, including gene, oligonucleotide drug, and mRNA therapies, each designed to modulate gene expression or function. The potential of nucleic acid therapeutics lies in their ability to address genetic disorders by directly targeting and manipulating the underlying genetic material. However, the translation of nucleic acid therapeutic concepts into effective clinical interventions faces challenges, spanning delivery hurdles, immunogenicity concerns, and the need for sustained efficacy. This Commentary article navigates the complexities associated with nucleic acid therapeutics, with a particular focus on gene therapy, and explores the recent emergence of extracellular vesicles as a potential solution to overcome the hurdles in nucleic acid delivery.

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INTRODUCTION

Gene therapy stands at the forefront of medical innovation, offering a promising avenue for treating genetic disorders by introducing therapeutic nucleic acids (RNA, DNA and their modified derivatives) into target cells. Multiple nucleic acid delivery systems were developed with some have been only tested in pre-clinical setups and others have reached clinical trials. Nucleic acid delivery systems are composed of either re-purposed natural carriers such as viruses, bacteriophages, and extracellular vesicles (EVs), or synthetic carriers such as lipid nanoparticles (LNP), synthetic nucleic acids, synthetic peptide conjugates, or tissue-specific antibodies [1–6]. These systems transport DNA or RNA to target specific cells or tissues within the body, depending on whether they were designed to achieve broad delivery across multiple cell types or to specifically target particular cells based on the intended therapeutic goal. These delivery systems are essential in the field of gene therapy, where the goal is to introduce therapeutic genes, replace defective ones or modulate gene expression for the treatment of various genetic diseases.

The nucleic acid delivery systems are divided into viral and non-viral delivery vehicles. The viral vectors encompass adenoviruses (AdV), adeno-associated viruses (AAV), lentiviruses (LV), or LV vectors (LVV), retroviruses, herpes simplex viruses (HSV), baculoviruses, bacteriophages, sendai virus (SeV) and vesicular stomatitis viruses (VSV). The non-viral vectors comprise LNPs, EVs, and virus-like particles (VLPs) [7]. The non-viral conjugates include peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) and antibodies. Nucleic acids can be modified to be self-deliverable, and these comprise modified messenger RNAs (mRNAs), RNA aptamers and phosphorodiamidate morpholino oligomer (PMO)-based antisense or splice-switching oligonucleotides (ASOs or SSOs).

Several gene therapies have already been approved by the US FDA, marking significant milestones in the evolution of medical treatment paradigms. These groundbreaking approvals underscore the potential of gene therapy to address previously incurable genetic disorders and offer novel therapeutic options. As of 2023, twelve gene therapies using viral vectors (AAV, LVV, retroviral vector [RVV] or HSV) for *in vivo* or *ex vivo* delivery were approved by the FDA (Table 1). In addition, eighteen non-viral based nucleic acid therapeutics (oligonucleotide drugs not gene encoded) were also approved by the FDA (Table 2). Both viral and non-viral delivery systems have several advantages and limitations when delivering nucleic acids for gene therapy. Viral delivery systems may offer high efficiency, long-term expression, and relatively good tissue specificity, making them suitable for one-time targeted gene delivery *in vivo*. However, they carry risks of immunogenicity and insertional mutagenesis. Non-viral delivery systems, on the other hand, may provide safety, ease of manufacturing, and flexibility in design, with relatively lower immunogenicity and potential for repeat administration. Their versatility allows for diverse delivery strategies, although some non-viral delivery systems may not be suitable for scaled-up production and may have lower transduction efficiency compared to viral vectors.

Despite these approvals and the high growth in the number of gene therapy clinical trials over the past years, viral-based gene therapy still faces many challenges regarding tissue-specificity, immunogenicity, and off-target toxicity [8]. Achieving precise targeting of therapeutic genes to specific tissues remains a challenge. Tissue-specific promoters have been used in several pre-clinical setups to enhance tissue-specific gene expression but is not enough to achieve complete tissue specificity [9]. To achieve this high level of tissue specificity, directed evolution steps and complex engineering of tissue-specific viral serotypes is also necessary. An example is the myotropic AAVs (MYOAAVs) that showed increased targeting to striated muscles in mice

TABLE 1

List of FDA-approved viral-based gene therapies.

Therapy name	Delivery vehicle	Transgene	Disease	Affected gene	Age	Year of approval	Company
CASGEVY™ (exagamglogene autotemcel [exa-cel])	Hematopoietic stem cell CRISPR-based gene therapy	CRISPR–Cas9 to disrupt *BCL11A* expression	SCD	HBB	>12 years	2023	Vertex and CRISPR Therapeutics
LYFGENIA™ (lovotibeglogene autotemcel [lovo-cel])	Hematopoietic stem cell-LVV based gene therapy	Modified β-globin gene ($\beta^{A\text{-}T87Q}$) to produce anti-sickling hemoglobin (HbA^{T87Q})	SCD	HBB	>12 years	2023	Bluebird bio
ELEVIDYS (delandistrogene moxeparvovec-rokl)	AAV	*Microdystrophin*	DMD	DMD	4–5 years boys	2023	Sarepta therapeutics
ROCTAVIAN™ (valoctocogene roxaparvovec-rvox)	AAV	B-domain deleted SQ form of *human coagulation factor VIII* (*hFVIII-SQ*)	Hemophilia A (congenital factor VIII deficiency)	Factor VIII	>18 years	2023	BIOMARIN
VYJUVEK® (beremagene geperpavec)	HSV	*COL7A1*	Dystrophic epidermolysis bullosa	COL7A1	>6 months	2023	Krystal Biotech
HEMGENIX® (etranacogene dezaparvovec-drlb)	AAV	*Factor IX*	Hemophilia B (congenital Factor IX deficiency)	Factor IX	>18 years	2022	CSL Behring
SKYSONA™ (Elivaldogene autotemcel)	Genetically modified autologous cells	*ABCD1*	CALD	ABCD1	4–17 years	2022	Bluebird bio
ZYNTEGLO™ (betibeglogene autotemcel)	Hematopoietic stem cell LVV-based gene therapy	Modified beta-globin gene (*βA-T87Q-globin* gene)	β-thalassemia	HBB		2022	Bluebird bio
ZOLGENSMA® (onasemnogene abeparvovec-xioi)	AAV	*SMN1*	SMA	SMN1	<2 years	2019	Novartis Gene Therapies
LUXTURNA (voretigene neparvovec-rzyl)	AAV	*RPE65*	RPE65 mutation-associated retinal dystrophy	RPE65	>8 years	2017	Spark Therapeutics
KYMRIAH® (tisagenlecleucel)	CAR-T cell- LVV based gene therapy	*CD19*	ALL	t(9;22), t(4;11) or t(8;14)	<25 years	2017	Novartis
YESCARTA® (axicabtagene ciloleucel)	CAR-T cell- RVV based gene therapy	*CD19*	Relapsed or refractory large B-cell lymphoma	*BCR*, *MYD88*, *CD79A/B*, *CARD11* and *TNFAIP3*, *BCL2* and *MYC* gene re-arrangements, *EZH2* and *PTEN*	Adult patients	2017	Kite, a Gilead Company

ABCD1: human adenosine triphosphate binding cassette, sub family D, member 1 gene; ALL: acute lymphoblastic leukemia; BCL2: B-cell lymphoma 2; BCR: B-cell receptor; CALD: cerebral adrenoleukodystrohy; CARD11: caspase recruitment domain family, member 11; CD79A/B: Cluster of Differentiation 79; *COL7A1*: collagen type VII alpha 1 chain gene; *DMD*: Dystrophin gene; DMD: Duchenne muscular dystrophy; *EZH2*: Enhancer of zeste homolog 2; *Factor VIII*: factor 8 gene; *Factor IX*: factor 9 gene; *HBB*: Beta-globin gene; HSV: herpes simplex virus; LVV: lentiviral vector; *MYC*: MYC proto-oncogene; MYD88: Myeloid differentiation primary response 88; *PTEN*: phosphatase and tensin homolog; RPE65: retinoid isomerohydrolase; SCD: sickle cell disease; SMA: spinal muscular atrophy; SMN1: survival of motor neuron 1 gene; TNFAIP3: tumor necrosis factor, alpha-induced protein 3; t(9;22), t(4;11) or t(8;14): chromosomal translocations between chromosome 9 and 22, 4 and 11 or 8 and 14.

TABLE 2

List of FDA-approved ONDs

Therapy name	Delivery vehicle/method	Route of administration	Oligonucleotide	Target gene	Disease	Affected gene	Age	Year of approval	Company
VITRAVENE (formivirsen)	ASO	Intravitreal	ASO	*CMV IE-2*	CMV retinitis	*CMV IE-2*	>18 years	1998	Isis Pharmaceuticals
KYNANRO (mipomersen)	Gapmer ASO with 2′-O-MOE/2′-H modification	Subcutaneous	ASO	*APOB*	HoFH	*APOB*	>18 years	2013	Genzyme Corporation
SPINRAZA (nusinersen)	Fully phosphorothioate- and 2′-MOE-modified ribonucleotide ASO	Intrathecal	ASO	*SMN2*	SMA	*SMN1*	>1 month	2016	Ionis/Biogen
EXONDYS 51 (eteplirsen)	PMO	Intravenous	ASO	*DMD*	DMD	*DMD*	4–19 years	2016	Sarepta therapeutics
TEGSEDI (inotersen)	Gapmer ASO with 5′ and 3′ 2′-MOE-modified wings	Subcutaneous	ASO	*TTR*	hATTR	*TTR*	>18 years	2018	Ionis pharmaceuticals
WAYLIVRA (volanesorsen)	Gapmer ASO with 2′-O-MOE/2′-H modification	Subcutaneous	ASO	*APOC3*	FCS	*APOC3*	>18 years	2019	Ionis pharmaceuticals
VYONDYS 53 (golodirsen)	PMO	Intravenous	SSO	*DMD*	DMD	*DMD*	4–19 years	2019	Sarepta therapeutics
VILTEPSO (viltolarsen)	PMO	Intravenous	SSO	*DMD*	DMD	*DMD*	5–18 years	2020	Nippon Shinyaku
AMONDYS 45 (casimersen)	PMO	Intravenous	SSO	*DMD*	DMD	*DMD*	7–20 years	2021	Sarepta therapeutics
MACUGEN (pegaptanib)	Pegylated aptamer	Intravitreal	Aptamer	*VEGF*	nAMD	*VEGF*	>50 years	2004	Eyetech pharmaceuticals
ONPATTRO (patisiran)	siRNA with 2′-OH/2′-OMe/2′-H modification	Intravenous	siRNA	*TTR*	hATTR	*TTR*	>18 years	2018	Alnylam Pharmaceuticals
GIVLAARI (givosiran)	Ligand containing three N-acetyl-galactosamine (GalNAc) residue	Subcutaneous	siRNA	*ALAS1*	AHP	*ALAS1*	19–65 years	2019	Alnylam pharmaceuticals
OXLUMO (lumasiran)	N-acetyl-D-galactosamine (GalNAc) ligand	Subcutaneous	siRNA	*HAO1*	PH1	*HAO1*	>4 months	2020	Alnylam pharmaceuticals
LEQVIO (inclisiran)	Triantennary N-Acetylgalactosamine (GalNAc)	Subcutaneous	siRNA	*PCSK9*	ASCVD	*PCSK9*	>18 years	2021	Novartis
AMVUTTRA (vutrisiran)	N-acetyl-D-galactosamine (GalNAc) ligand	Subcutaneous	siRNA	*TTR*	hATTR	*TTR*	>18 years	2022	Alnylam pharmaceuticals
HEPLISAV-B	CpG-containing	Intramuscularly	DNA	..	Hepatitis B	..	>18 years	2017	Dynavax Technologies Corporation
COMIRNATY BNT162b2 (tozinameran)	LNPs	Intramuscularly	mRNA (1N-Me pseudouridine with 2′-OMe in 5′-cap)	..	SARS-CoV-2	..	>12 years	2021	BioNTech Manufacturing
SPIKEVAX (elasomeran)	LNPs	Intramuscularly	mRNA (1N-Me pseudouridine with 2′-OMe in 5′-cap)	..	SARS-CoV-2	..	>12 years	2022	Moderna Tx

AHP: acute hepatic porphyria; *ALAS1*: 5′-aminolevulinate synthase 1 gene; APOB: apolipoprotein B; APOC3: apolipoprotein C3; ASCVD: primary hypercholesterolemia or clinical atherosclerotic cardiovascular disease (who require additional lowering of low-density lipoprotein cholesterol); ASO: anti-sense oligonucleotide; DMD: Duchenne muscular dystrophy; *DMD*: Dystrophin gene; FCS: familial chylomicronemia syndrome; *HAO1*: glycolate oxidase gene; hATTR: hereditary transthyretin-mediated amyloidosis; HoFH: homozygous familial hypercholesterolemia; *IE-2*: immediate-early; nAMD: neovascular age-related macular degeneration; OND: oligonucleotide drugs; *PCSK9*: proprotein convertase subtilisin/kexin type 9 encoding gene; PH1: primary hyperoxaluria type 1; SMA: spinal muscular atrophy; *SMN1*: survival of motor neuron 1 gene; *SMN2*: survival of motor neuron 2 gene; SSO: splice-switching oligonucleotides; *TTR*: transthyretin gene; *VEGF*: vascular endothelial growth factor.

and non-human primates when compared to other AAV serotypes such as AAV9 [10,11]. It remains to be seen whether these MYOAAVs will show similar targeting efficacy in clinical trials. Immunogenicity poses a hurdle as the immune system may recognize therapeutic genes or viral vectors as foreign, prompting the exploration of immune-evading vectors or the use of immunosuppressant drugs. Other strategies to circumvent viral (e.g., AAV) immunogenicity were used such as specific routes of administration, enzymatically degrading IgGs [12], empty capsids decoys [13], and plasmapheresis [14,15]. The success of the latter was hampered by high-titer antibodies and by the need for repeated sessions to eliminate anti-AAV antibodies.

Despite the FDA-approval of several oligonucleotide drugs (Table 2), the application of most of them remains limited to certain easy to target organs or tissues and with diseases not requiring systemic drug delivery. However, oligonucleotide drugs that were injected intravenously such as the PMO-conjugated ASO drugs for Duchenne muscular dystrophy (DMD) showed poor and uneven efficacy among patients and affected striated muscles (Table 2) [16,17]. The non-viral delivery methods used for the FDA-approved ASOs face many challenges such as achieving effective delivery of ASOs to target tissues, improving cell penetration, increasing target engagement, and decreasing toxicity. The intricacies of tissue-specific targeting demand innovative strategies to enhance ASO penetration and cellular uptake, ensuring optimal therapeutic efficacy. Additionally, the stability of ASOs in the biological milieu poses a challenge, necessitating the integration of chemical modifications to withstand nuclease degradation and maintain the integrity of the oligonucleotide during transit. Another critical challenge in the development of ASOs is the potential induction of immune responses by ASOs, adding an additional layer of complexity. For instance, delivered oligonucleotide drugs (ONDs) are known for their activation of the pattern recognition receptors (PRR), leading to the induction of the innate immune response and the secretion of inflammatory cytokines and type I interferons (IFNs) [18–20]. In another instance, when compared to viral delivery systems, ONDs were shown to trigger less the immune system prior to their entry into target cells [21–23]. Strategies to minimize immunostimulatory effects are crucial to enhance safety and tolerability. Addressing these challenges collectively will contribute to the advancement of ASOs as a robust and precise therapeutic modality with broader clinical applications.

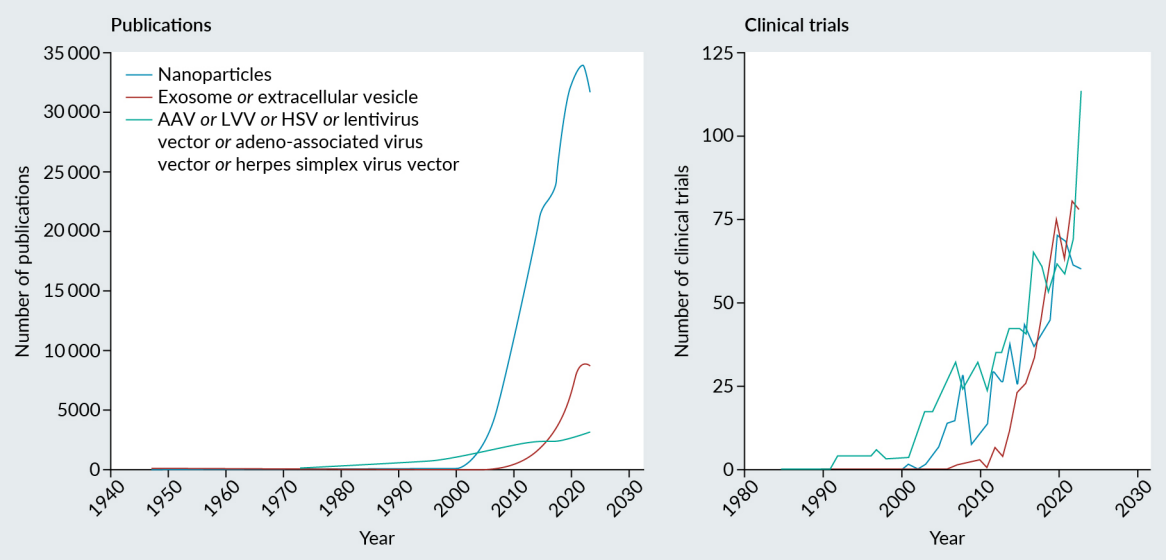
LNPs underwent clinical validation for their use in the delivery of the SARS-CoV-2 mRNA vaccine. In this context, the primary objective was to stimulate the immune system by administering doses of LNP-mRNAs that are conventionally deemed sub-optimal for achieving therapeutic efficacy in the treatment of genetic diseases. Nevertheless, certain LNPs must address concerns regarding biocompatibility, stability, and potential immunogenicity to ensure safe and effective clinical use [24–26]. Achieving scale-up production and tissue-specific targeting further complicate their widespread implementation. Overcoming these challenges demands comprehensive approaches, including optimized formulations, modifications for stability, and strategies to enhance tissue specificity [17,27,28]. Ongoing research efforts are dedicated to refining these delivery systems and oligonucleotides, aiming to unlock their full potential for precision medicine applications [24–26].

EXTRACELLULAR VESICLES: PROMISING CARRIERS TO RESHAPE GENE THERAPY'S LANDSCAPE

EVs are non-replicative, lipid-bilayer delimited particles naturally released from tissues and organs into biofluids, carrying organ- and tissue-specific molecules (e.g., nucleic acids, peptides, proteins, lipids and metabolites)

FIGURE 1

Graph representation of the number of publications (left graph) and clinical trials (right graph) involving nanoparticles, exosomes, or EVs and viral-based therapies.



We obtained the number of publications by searching in the PubMed database using the terms "nanoparticles", "exosome OR extracellular vesicle" and "AAV OR LVV OR HSV OR lentivirus vector OR adeno-associated virus vector OR herpes simplex virus vector". We obtained the number of clinical trials by searching in the ClinicalTrials.gov website using the same terms. In 2023, 26 interventional and 13 observational clinical trials using EVs were shown to be active and/or recruiting participants. EV: extracellular vesicle; HSV: herpes simplex viruses; LVV: LV vector.

[29–32]. In recent years, EVs have emerged as novel delivery vehicles with potential to overcome some of the obstacles associated with viral- and non-viral synthetic-based nucleic acids delivery systems. The number of publications and clinical trials involving EVs has consistently grown since their initial description in 1956, indicating their rising significance in both research and clinical applications **(Figure 1)** [33–36]. This surge in interest underscores their potential for various biomedical purposes, such as diagnostics, therapeutics (as stem cell-derived native EVs), and as delivery vehicles for therapeutic nucleic acids. **Figure 1** shows that publications related to EVs have seen tremendous exponential growth that started 12 years ago. It also shows a disparity wherein the greater volume of publications pertaining to nanoparticles (i.e., membrane-delimited particles), in contrast to those focused on EVs and viral-based therapies, does not frequently translate into clinical trials. This could be due to many factors such as the complexity and diversity of nanoparticles, their intended use, safety concerns, regulatory hurdles, and clinical trial design challenges. In addition, nanoparticles research and basic applications could be more accessible to broader labs and institutes with limited funding, leading to higher publication rates that do not necessarily translate into costly clinical trials requiring the establishment of intricate infrastructure and interdisciplinary teams.

In this commentary we will delve into the potential benefits and drawbacks of harnessing EVs for the delivery of nucleic acids.

Overcoming the challenges of cellular uptake and intracellular trafficking is pivotal for success of any gene therapy. Viral delivery systems rely on specific receptors and co-receptors to enter the cell, governing their tissue tropism (e.g., most AAV serotypes use AAV receptor (AAVR) and GPR108 or TM9SF2 co-receptors to enter the target cell [37–40]). Consequently, the efficacy of viral vectors in gene therapy is intricately linked to the expression of these receptors on target cells,

especially when their expression is affected in a disease state. In this context, the variability in receptor expression across different tissues and cell types poses a challenge, influencing the vectors' ability to achieve precise and consistent targeting. The entry of viral vectors into target tissues can be also affected by the presence of naturally occurring single nucleotide polymorphisms (SNPs) in their receptors, preventing efficient viral vector transduction, and hampering therapeutic efficacy. Moreover, AAV tropism is influenced by variations in capsid proteins dictating the interaction of each AAV serotype with specific cell surface receptors and co-receptors. Similarly, the lipid composition of LNPs influence their tissue specificity [41,42]. Despite the wide use of AAVs and LNPs, in nucleic acid delivery, the multifactorial nature—needed for an effective delivery system—of these delivery systems is limited to certain aspects. Alternatively, many more interconnected factors, in addition to the abovementioned ones, contribute to the success of native or engineered EVs to deliver nucleic acids. For examples, EVs possess complex lipid architecture and composition, making them highly suitable carriers for absorption by recipient cells. In addition, the presence of cell adhesion molecules (CAMs) (e.g., integrins, cadherins and immunoglobulins) on the surface of EVs influence their biodistribution in vivo and enhance their interaction with and uptake by recipient cells [43–45]. EVs collected from allogeneic or autologous donor cells use various mechanisms and receptors to enter the target cell. When compared to LNPs, EVs showed higher uptake and cargo delivery, indicating higher endosomal escape capacity [46,47].

Based on their size, EVs can be divided into different sub-populations such as exosomes (30–150 nm in diameter), microvesicles (150 nm–1 μm) or apoptotic bodies (1–5 μm). EV molecular payload or cargo varies based on the type and state of their producer cells. EVs transmit their molecular cargo to a variety of target cells to maintain homeostasis in normal physiological conditions or to drive pathogenesis during disease [48–52]. Therefore, EVs can be exploited for disease biomarker discovery and therapeutic delivery [3,32,53–55]. EVs have been already used in multiple therapeutic treatment and regenerative strategies to deliver their molecular payload to specific target cells. These strategies can either rely on native EVs, carrying natural molecular payload that is intrinsically therapeutic or EVs supplemented with exogenous natural or artificially designed therapeutic agents [6,49,50,56–61].

As the field of gene therapy advances, addressing challenges associated with viral and non-viral vector tropism, immunogenicity becomes paramount for enhancing therapeutic efficacy and ensuring the safety of genetic interventions. EVs have great potential to become widely used as therapeutic delivery vehicles not just because of their low immunogenicity but also due to their wide tissue biodistribution, low toxicity and modulability [62]. Compared to viral or non-viral synthetic delivery systems, EVs exhibit reduced immunogenicity because they can be derived from autologous cell sources or from allogeneic cells (i.e., red blood, mesenchymal stem cells or HEK293 cells), some of which can be engineered to eliminate the expression of immunogenic surface markers, further enhancing their biocompatibility and potential for therapeutic applications. This intrinsic characteristic diminishes the risk of immune recognition and subsequent clearance of therapeutic cargo, reinforcing the potential efficacy of EV-delivered gene therapy. When delivered systemically, EVs accumulate predominantly in the liver, spleen, kidney and lungs, and to a lower degree in the heart, GI tract, bone, bladder, pancreas, and skeletal muscles [48]. The fast clearance of EVs from the bloodstream may reduce their exposure to humoral immunity, but may compromise their delivery efficiency to the target organ [48]. Because of their modulability, EV tropism to a determined set of tissues can be achieved by decorating EVs with more than

one tissue-specific moieties (i.e., peptides and fusogens). This customization minimizes off-target effects, elevating the overall precision of EV-based gene therapy interventions.

A bottleneck for certain delivery systems is the payload capacity limitations. For example, AAV has a packaging capacity of approximately 4.7 kb when it contains a single-stranded DNA genome and about 2.3 kb when it adopts a self-complementary DNA genome [63,64]. Alternatively, EVs have relatively larger cargo capacity [65]. This versatility transforms EVs into carriers capable of accommodating a diverse range of nucleic acids, expanding the scope of gene therapy applications to include various genetic elements, from small interfering RNAs (siRNAs) to large messenger RNAs and CRISPR-based genome, base and prime editors [51,66–70]. One aspect of sustaining high therapeutic efficacy of gene therapy, is ensuring long-term expression of therapeutic genes. EVs have the potential to envelop minicircle expression DNA cassettes (MCs), that become 'DNA episomes' when delivered into the host proliferating or differentiated cell [71–73]. These DNA episomes would prolong gene expression, contributing to the durability of gene therapy effects. MCs are circular DNA vectors only encompassing an eukaryotic therapeutic DNA sequence derived from a parental DNA (classical plasmid DNA), and lacking the prokaryotic DNA backbone found in the parental DNA [74]. MCs can accommodate large inserts compared to plasmids containing inverted terminal repeats (ITRs), which form the genome of AAV vectors. MCs are expected to be safer than classical viral genomes, as they are less likely to cross-package bacterial backbone sequences, which can occur with traditional viral vectors [75]. Another aspect in which EVs can enhance the efficacy and longevity of the therapeutic intervention is their ability to deliver therapeutic nucleic acids to stem cells, a characteristic that most viral and non-viral synthetic delivery systems are not efficient at achieving [76–79]. Targeting stem cells is beneficial as it allows for the transmission of the therapeutic benefits to differentiated daughter cells. So far, there is limited evidence to suggest that the cellular origin of EVs plays a significant role in determining their ability to target stem cells.

The natural biocompatibility of EVs, stemming from their endogenous origin, reduces the risk of adverse reactions, making them an attractive option for delivering therapeutic nucleic acids when compared to viral-based delivery systems. For example, high-dose vascular AAV or LVV delivery has been linked to adverse events [80,81]. In the case of AAVs, which is more amenable for *in vivo* therapeutic settings, adverse events like complement activation, thrombocytopenia, and liver toxicity have been seen. Thus, AAV safety and immunogenicity are now primary concerns [82,83]. The lipid bilayer enveloping EVs provides a protective shield against extracellular nucleases, enhancing the stability of encapsulated nucleic acids [84,85]. It also provides a shield from pre-existing neutralizing antibodies developed against EV molecular cargos and may prevent the immune system from developing immunity against these cargos. Hence, the idea of enveloping AAVs with EVs was developed and has been successfully tested in few animal models to increase the transduction efficiency of hard to transduce tissues [66,86–92]. These examples show the versatility of EVs in cargo encapsulation.

Addressing the practical challenge of large-scale production, EVs harvested from various cell types, along with advancements in isolation and purification technologies, contribute to the scalability of production. This scalability is pivotal to standardize EV-based therapies and to meet the demands of widespread gene therapy applications. One aspect in EV scaled production that still need development is the reproducibility in generating EV batches with similar therapeutic efficacy [93,94]. This is particularly the case for EVs derived from stem cells, where alterations in their culture conditions may lead to changes in the packaging of their inherent molecular

payload [95,96]. The potential variability in the composition of EVs becomes particularly critical when the maintenance of a specific ratio among multiple molecular payloads is crucial for ensuring consistent therapeutic efficacy across different batches of EVs. In addition, the role of EVs in inter-cellular communication adds another layer of complexity to their therapeutic potential. In this context, cells can be genetically edited *in vivo* or *ex vivo* to produce therapeutic EVs that can target neighboring cells, thereby benefiting the entire tissue or organ. Leveraging this natural messenger system opens the door to diverse therapeutic interventions.

The potential for personalized medicine, afforded by isolating EVs from a patient's own cells and engineering them for specific applications, aligns with the evolving landscape of individualized medicine. However, this must be accompanied with securing regulatory approval, which is a pivotal step in the clinical translation of gene therapies. This regulatory approval is nuanced for EVs. Being naturally occurring vesicles, EVs present a potentially favorable safety profile. However, the path to regulatory approval demands comprehensive preclinical and clinical data to establish their safety and efficacy, necessitating a thorough navigation of the evolving regulatory landscape to meet stringent criteria. This should be accompanied, by increasing the levels of standardization in the field of EVs, ensuring reproducibility of EV-related benefits [29,97–99]. The amalgamation of these advancements positions EVs as a promising delivery vehicle that addresses the challenges of gene therapy and may contribute to reshape the landscape of future medical interventions. However, questions linger regarding the fate of EVs post-administration, including their clearance from the bloodstream and biodistribution to target tissues. Comprehensive understanding of these pharmacokinetic aspects is vital for optimizing the therapeutic efficacy of EV-based gene therapies.

CONCLUSION

In navigating the complexities of nucleic acid delivery for gene therapy, EVs emerge as one of the delivery vehicles with high promise. Their natural biocompatibility, versatile packaging ability, and cell-specific targeting capabilities address longstanding challenges, paving the way for more precise and efficacious gene therapies. As researchers continue to unravel the intricacies of EV-based approaches, collaborative efforts are essential to overcome remaining challenges and usher in a new era of personalized and effective gene therapies. The potential benefits are vast, but the journey requires a concerted commitment to advancing the science and technology of EV-mediated nucleic acid delivery.

Looking ahead, the advancement of EV-mediated gene therapy hinges on a strategic and collaborative approach that addresses the challenges outlined in this commentary. The optimization of EV isolation and engineering protocols demands focused research to streamline processes and establish standardized methodologies, ensuring scalability and reproducibility. Standardizing EV preparations becomes imperative, urging researchers to delve into the intricacies of heterogeneity and establish protocols that guarantee consistency across diverse applications. A deeper understanding of EV pharmacokinetics, including clearance mechanisms and biodistribution patterns, will be pivotal in optimizing dosing regimens and addressing safety concerns. Rigorous safety evaluations and collaborative efforts with regulatory bodies will lay the foundation for the seamless translation of EV-based gene therapies from laboratories to clinics.

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NUCLEIC ACID INSIGHTS

DELIVERY & FORMULATION

SPOTLIGHT

Glimpsing the future of siRNA delivery strategies and the broader therapeutic application of oligonucleotides

Naim Nazef
Head of Oligonucleotide Chemistry and Delivery Platforms, Takeda

"The aim is to target a spectrum of diseases, including both rare conditions and those more prevalent in the human population."

VIEWPOINT

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On December 19, 2023, **David McCall**, Senior Editor, *Nucleic Acid Insights*, spoke to **Naim Nazef**, Head of Oligonucleotide Chemistry and Development Platforms, Takeda, about the challenges and advances in siRNA delivery, and the burgeoning promise of

oligonucleotide therapeutics for targeting a wide array of diseases. Naim Nazef's research focuses on direct conjugates. He successfully developed a GalNAc-siRNA conjugate platform (GalXC®) for targeted delivery to hepatocytes that has led to major partnerships with big pharma companies.

RNAi therapeutics—and siRNA therapeutics in particular—pose challenges to drug developers due to their poor physiochemical properties. These include high molecular weight, strong negative charge, poor cellular permeability, rapid renal excretion, and poor tissue uptake and biodistribution. Additionally, naked siRNA is metabolically unstable and susceptible to rapidly nuclease degradation, making its delivery for therapeutic purposes a difficult and persistent challenge.

In 2018, Alnylam Pharmaceuticals achieved a major milestone for the field with Patisiran, the first FDA-approved siRNA drug, using lipid nanoparticles (LNP) as a delivery vehicle. LNPs effectively protect siRNA from nuclease-mediated degradation, facilitate rapid liver uptake, and ensure endosomal escape for efficient target engagement. Despite the success of LNPs in this context, drawbacks include limited therapeutic potential via repeated IV administration, complex manufacturing process, immunogenicity, and a relatively low therapeutic index.

In the early 2010s, the industry shifted focus from LNPs to direct conjugate approaches, particularly GalNAc-siRNA conjugates, addressing challenges in understanding the rules to chemically modify siRNA to increase metabolic stability while simultaneously maintaining intrinsic RNAi potency. GalNAc-siRNA conjugates leverage the asialoglycoprotein receptor (ASGPR), which is specifically and highly expressed by hepatocytes, ensuring efficient targeted delivery resulting in a wide clinical therapeutic index. The pairing of GalNAc and the ASGPR receptor advanced the oligonucleotide (and particularly the siRNA) field into the clinic, resulting in more than six marketed GalNAc-siRNA products. The success of this approach sparked interest in exploring other cell-surface receptors and ligands, leading to Takeda's 2016 publication [1] on the use of antibodies as targeting ligands to the CD71 transferrin receptor for muscle delivery. This paved the way for antibody-based oligonucleotide conjugates, with subsequent developments by companies utilizing the transferrin receptor, such as Dyne Therapeutics using a Fab antibody antisense oligonucleotide (ASO) conjugate and Avidity Therapeutics utilizing a full length mAb siRNA conjugate for muscle delivery. Takeda, among others, are exploring targeting the transferrin receptor not only for muscle delivery but to cross the blood–brain barrier into CNS deep brain regions via systemic delivery versus the more invasive intrathecal or intracerebroventricular local delivery. The challenge now lies in discovering additional cell-surface receptors for different tissues or cell types, necessitating screening approaches and workflows to identify ideal receptors with desirable characteristics for productive siRNA delivery. However, in practice, the discovery process of novel receptors and the receptor properties required for efficient delivery and translatability from *in vitro* internalization or mRNA knockdown data to *in vivo* is not well understood. While other receptors have been explored such as the integrin family of receptors, there is currently still a lack of validated receptors for efficient oligonucleotide delivery. The main culprit for inefficient delivery is the lack of endosomal escape, a barrier that has plagued the industry for decades and limited the therapeutic potential of oligonucleotides.

The requirement for endosomal escape agents depends on the type of receptor being targeted, but it is widely recognized that improving endosomal escape would enhance the success and potential reach of oligonucleotide therapeutics considerably. Takeda and others are actively exploring this

area, but success to date has been limited due to the often cytotoxic nature of endosomal escape agents. Patience and ongoing research effort will be required to discover a safe endosomal escape agent and while this work continues, other receptors are being explored.

Leveraging the targeting agent's selectivity ensures delivery to specific cell types. This involves 2D selectivity, considering the receptor's tissue expression profile, such as with hepatocytes and the ASGPR receptor. Combining this with siRNA targeting a specific gene ensures significant drug selectivity, reducing off-target effects and thereby addressing safety concerns. However, this approach introduces significant complexity (and with it, cost) with some molecules reaching a molecular weight >150 kDa. The repercussions of this, together with the novelty of the approach, call for access to specific development, regulatory, and manufacturing expertise. Additionally, with interest in oligonucleotides in general and siRNA in particular continuing to grow, the industry's oligonucleotide manufacturing capacity is currently insufficient, impacting timelines for clinical material production.

Short-term opportunities for siRNA lie in leveraging the transferring receptor for efficient siRNA delivery, especially to muscles and for rare disease indications. Additionally, crossing the blood–brain barrier is a short-term focus of research. Although both siRNA and antisense oligonucleotides (ASOs) have approved drugs for CNS indications, as previously noted, current local delivery methods such as intrathecal delivery are invasive. Developing approaches such as using antibody-oligonucleotide conjugates to overcome this barrier could significantly improve the patient experience.

Looking further ahead, the primary focus will remain on advancing delivery approaches for siRNA. Discovering new methods is an ongoing challenge, but with better understanding and improved *in vitro*/*in vivo* correlation, which is currently lacking for oligonucleotides, there is hope for progress in leveraging siRNA for diverse therapeutic indications.

Long-term opportunities chiefly depend on the wider field recognizing oligonucleotides as another valuable option in the pharmaceutical modality toolbox for exploring difficult-to-treat targets. What sets oligonucleotides apart from small molecules and antibodies is their ability to drug targets whether they are intracellularly located or expressed extracellularly, and with exquisite selectivity and precise therapeutic action, when combined with a targeting ligand. Unlike antibodies that are designed for extracellular targets and small molecules with their broad distribution, siRNAs exhibit greater selectivity and in principle, can drug any target in the human genome, and address targets once considered undruggable. When antibody therapeutics were introduced in the 1990s, they initially faced skepticism as a viable class of drug due to their substantial size and the challenges associated with manufacturing and engineering. Subsequently, siRNAs and oligonucleotides in general were met with similar skepticism, largely relating to the challenges described previously. However, with GalNAc-siRNA and ASOs now approved as drugs, oligonucleotides are emerging as a more widely accepted modality, akin to small molecules and antibodies.

Oligonucleotides have the potential to extend beyond the liver, and this has prompted an ongoing collective effort to leverage this modality for diseases in other organs. Takeda is exploring how to harness the strengths of oligonucleotides, focusing on gastroenterolgy, oncology and neuroscience, including disorders such as Alzheimer's disease and Parkinson's disease. The aim is to target a spectrum of diseases, including both rare conditions and those more prevalent in the human population. The long-term objective is to realize the potential of oligonucleotides across diverse therapeutic areas.

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BIOGRAPHY

NAIM NAZEF is the Head of Oligonucleotide Chemistry and Delivery Platforms at Takeda Pharmaceuticals, with over 25 years of experience in drug discovery and development. With a focus on RNAi and nucleic acid-based therapeutics, Naim has contributed significantly to the field for over 15 years. Notably, he guided the discovery of the GalXC® GalNAc-siRNA delivery platform at Dicerna Pharmaceuticals, resulting in multiple clinical stage programs. One of these programs, Rivfloza™, recently received FDA approval for the treatment of primary hyperoxaluria, a rare genetic disease that can lead to end-stage kidney failure. At Takeda, Naim is dedicated to expanding the therapeutic potential of RNAi and actively involved in discovering novel targeted delivery approaches to expand its application beyond liver-related diseases. Naim Nazef holds a BS in Biochemistry from the University of Manchester, an MS in Chemistry from Northeastern University, and a PhD in Natural Product Synthetic Chemistry from the University of Edinburgh.

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NUCLEIC ACID INSIGHTS

DELIVERY & FORMULATION

SPOTLIGHT

INTERVIEW

Focusing on the fundamentals: what do and don't we know about mRNA delivery?

As excitement around the evolving field of mRNA continues to grow, there remain some fundamental questions to be answered. **David McCall**, Senior Editor, *Nucleic Acid Insights*, spoke with Monash University's **Colin Pouton**, Professor of Pharmaceutical Biology and **Angus Johnston**, Associate Professor, ARC Future Fellow about the key limitations and unknowns of current mRNA delivery approaches, and critical areas for further research and innovation to propel the field forward.

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Q What are you working on right now?

CP: I am a pharmaceutical scientist by training. What fascinates me most in the mRNA delivery space is the success of lipid nanoparticles (LNPs) and the questions around how they

work, what makes them unique, what makes them particularly amenable to manufacturing, and what alternatives there are. We are still at the very early stages of this technology, and—in the public domain, at least—a lot of what I'd consider the core pharmaceutical aspects are still not well known. This includes things like the fundamentals of delivery and biodistribution, and even the quality control assays you use in manufacturing.

A lot of our work involves looking at how we can better understand these aspects to move the field forward, and writing up papers so that we can present information to the public domain. We also have a number of collaborations set up to do proof-of-concept studies for a variety of therapeutic applications and novel vaccines.

AJ: I was originally a chemist, before moving more into materials science. More recently, cell and molecular biology became my main areas of interest. On a fundamental level, I am interested in trying to be more accurate and specific with our delivery of mRNA. This means being precise and delivering to the right type of cells, but then also to the right place within those cells to maximize activity.

I also want to leverage those fundamentals of biodistribution to enhance understanding of where are we able to deliver mRNA in the body—it's still an emerging area of research and we don't really know what the limitations are.

In terms of disease focus, my interest falls into the area of immune disorders, and in particular, autoimmune diseases. We want to see what we can do in that space to get more control over how immune cells are differentiating, and achieve better outcomes in a range of diseases.

Q What for you is the current state of the art in terms of what we can do with mRNA?

CP: It goes without saying that vaccines are the most immediate use of mRNA. The vaccines that were developed for COVID-19 were efficient, but although COVID-19 gave us all sorts of problems globally, it was quite an easy target for vaccination. Although people may think we will be able to readily apply mRNA technology to other vaccines, it won't necessarily be easy. Some of the difficult infectious diseases that we have been battling for a long time will still be a problem for mRNA technology. There is a lot of activity going on in that space and I am sure vaccines for other infectious diseases will come through, but others will remain intractable.

People are also looking at a whole variety of therapeutic applications. The most obvious approach in that area is targeting diseases where you can replace a missing or defective protein. One fairly straightforward option we have is to express a protein in the liver. We know that we can deliver LNPs effectively to the liver, and if you can use the liver as a factory for making proteins with systemic activity, you could use mRNA to deliver the protein rather than use a therapeutic protein itself.

"...I think the next cutting edge is going to involve specificity and targeting. There are some companies in the US that are starting to explore precision delivery in earnest, and there is a lot of scope for that approach to be broadened."

Another area that is particularly interesting and is seeing a lot of activity is using mRNA to achieve gene editing in genetic disease. Here, the real advantage of mRNA is that it is potentially safer to deliver a gene editing construct using mRNA versus DNA.

AJ: The tumor neoantigen area is very interesting as well—in Australia, Georgina Long and Richard Scolyer have just received the 2024 Australian of the Year award for the tumor neoantigen therapy they developed, which was taken by Richard to treat his own brain tumor.

Turning to gene editing, as Colin was saying, I think there's a lot of scope. Perhaps because it is my own area of research area, I think the next cutting edge is going to involve specificity and targeting. There are some companies in the US that are starting to explore precision delivery in earnest, and there is a lot of scope for that approach to be broadened. It is not going to be the answer in every instance because that additional level of complexity is going to add significant costs, but equally, there will be cases where that enhanced specificity is required and gives you a huge advantage.

Q Can you expand on some of the key limitations in extending the reach of mRNA technology?

CP: The most obvious is the duration of action, particularly when you compare mRNA strategies with, say, successful gene therapies based on AAV vectors. AAV-based gene therapies have been approved for human use and are delivering DNA—and once the DNA gets into the nucleus, it tends to hang around, so you get much longer expression of proteins than with RNA.

With mRNA you avoid the immunogenicity issue associated with viral vectors, and you don't have the possible problem of insertional mutagenesis of the DNA inside the AAV vector. But on the other hand, duration of action is measured in days. This means you are talking about giving injections of a therapy on a weekly basis, or perhaps even more frequently, which is impractical. There is a lot of activity going on around mechanisms that could be used to extend the duration of action. Those will be pivotal studies for the field: if we can extend the duration of action from days to weeks, then mRNA will really take off as a therapeutic modality.

The other limitation is one we have alluded to already: the biodistribution of formulated mRNA in LNPs. Because they are particulates, they are subject to the typical distribution of particulate materials when they get into the circulation. This means limited access to certain tissues, and strong delivery to clearance tissues, i.e., the liver, spleen, and to some extent the bone marrow.

This is where Angus's work to understand how and to what extent we can change that biodistribution really comes in. It is currently limited, so we have to look at what can be done to exploit those delivery systems that we know go to the liver, spleen, and so on. There is a lot of interest in applications that are tangible.

AJ: A related consideration is the efficiency of delivery. We estimate that even if you get the mRNA to the right cell, only about 2–5% of that mRNA will end up getting into the cytosol where it is active. There is huge potential for an efficiency gain there. Part of the challenge is our limited understanding of how anything is able to get into the cytosol. We have some ideas for certain viruses, but we don't have a generalizable understanding of how those materials naturally escape. If we understood this more, we would have a better chance of engineering our materials to escape more efficiently.

Q Looking more closely at the question of targeting, what can and can't we target with mRNA, and can you expand on the specific applications where this technology will work?

AJ: Some of this relates to quantity, as we are not delivering huge amounts of mRNA. For example, if you have an application with a protein replacement therapy that requires very large amounts of protein, then there is a limitation in that the mRNA is just not giving you enough of that protein. But for things like vaccines, growth factors, or other types of protein that are active at much lower concentrations, mRNA is having a major impact already.

When it comes to which tissues you can target and how, to firstly lend some context, we are trying to take the standard LNPs that have been used for vaccines and modify them. These existing LNPs are designed to essentially interact with any cell. You can change the way they interact a little by changing the composition of the lipids, but in general, you are relying on non-specific interactions to facilitate that LNP delivering to a cell.

What we are trying to do is firstly to lower those non-specific interactions, and then secondly, to control that interaction by adding an antibody or antibody fragment. The antibody fragments we use are called nanobodies—they are the same concept as an antibody, and are comprised of the small variable domain from a single domain antibody. We are then trying to program the interaction with the cells based on that nanobody interaction. What we hope to achieve there is specificity from the nanobody, so it will recognize a particular receptor on the surface of particular cells and only deliver to them. Higher levels of delivery to those cells should also lower off-target delivery, but that is yet to be properly demonstrated.

"...you have made a product, it is in a vial, but can you actually show what you have made? Can you show what the concentration of the lipids in an LNP is, and what concentration of mRNA you have in the vial?"

A potential limitation of relying on non-specific interactions is that current LNPs rely on proteins adsorbing to the LNP to achieve delivery. Companies are starting to see that there is significant variability in how that adsorption occurs, both from species to species and from person to person. It therefore becomes hard to predict exactly how the LNP is going to perform from one organism to another.

Our contention is that if you are no longer relying on that non-specific adsorption and instead relying on an interaction that you have pre-programmed, then you will have better translation from one organism to the next, and potentially better translation from patient to patient.

In terms of a more direct answer to the question of what we can target, certain organs are much easier to target, i.e., liver, spleen, lung, and things that are circulating in the blood. Depending on the route of administration, you can potentially start to localize your delivery into certain areas—for example, into a lymph node. The first specific applications will likely be those organs and cell types where you get good distribution of the nanoparticles. An example of a major challenge would be LNP delivery into somewhere like the brain due to obstacles like the blood–brain barrier.

Q You specialize in part on honing analytical techniques that can help identify potential application areas for mRNA technology. Can you tell us more about the tools and methods you utilize for this purpose?

CP: This is worth considering in two sections. Firstly, there's the quality control (QC) of the product and the pharmaceutical side: you have made a product, it is in a vial, but can you actually show what you have made? Can you show what the concentration of the lipids in an LNP is, and what concentration of mRNA you have in the vial? Companies with approved products will have had to deal with these QC aspects, and they are quite tricky.

In laboratories, we tend to take some of those aspects for granted. We make LNPs with a certain amount of mRNA, and we assume that a lot of the mRNA is present—but in fact, establishing that it really is present and is in an active state is difficult with a macromolecule of that complexity. So is separating mRNA from LNPs, which are completely different in terms of their solute properties. Lipids dissolve in organic solvent and mRNA is really a polyanionic aqueous solute, which is quite difficult to deal with, so there is no single solvent you can use to

> "It is clear that in order to understand the mechanisms of action and the applications of mRNA in the long run, we need a lot more information...There are some challenges with mRNA that we are not used to facing."

dissolve the materials and analyze them. An mRNA isn't something you can analyze in the way we analyze drugs, with HPLC and liquid chromatography-mass spectrometry (LC-MS), for example, where you can get a single peak and show that it is exactly what you think it is. With mRNA, you have all sorts of complexities around small changes in the molecule that might affect its biological activity, which you cannot necessarily detect using something simple like HPLC. There are aspects of the QC of the product that are important but not well understood in the public domain, and we need to do more work to make those assays easier to do. We also need to show that we have validated them correctly.

The other aspect is what happens when you administer the material *in vivo*? How do you go about analyzing the distribution of the drug? mRNA is a much more complicated molecule to analyze than a small molecule drug. We are particularly interested in techniques that we can use to explore biodistribution. Can we use molecular biological techniques such as quantitative (q) PCR or even sequencing of mRNA to analyze distribution *in vivo*? Can we use techniques to identify which cells have taken up the mRNA, and which cells have intact and active mRNA in them? Then, within tissues, is that distribution even, or is mRNA delivered specifically to certain cell types?

It is clear that in order to understand the mechanisms of action and the applications of mRNA in the long run, we need a lot more information. Angus has been leading an investigation into whether we can use nanopore sequencing *in vivo*, for example. It looks very promising in the sense that we can pick up and quantitate delivery of mRNA to particular tissues (and maybe even particular cells) going forward. There are some challenges with mRNA that we are not used to facing. Academics can play an important role in teasing out the best methods.

AJ: Next-generation sequencing (NGS) techniques like nanopore, single-cell sequencing, and spatial genomics could be the way forward. The standard tools that you would use for a small molecule are less applicable. It makes it a much harder challenge, but because there are molecular biology tools that we can potentially start applying, we may be able to get a lot more information about the distribution of mRNA than we would about a small molecule drug, for example. We can start to see interactions that the mRNA might be having, things that it might be doing to the cells, and potentially home in on individual cells to understand what is happening in them.

The complexity of mRNA is both a blessing and a curse. There is a lot more that you have to do to gain a full understanding, but it also means that there is actually a range of cutting-edge techniques that you can start applying. You get a set of data that is almost too rich. Trying to

get your head around it is a challenge, because you get a lot of information from some of the NGS experiments.

Q Can you highlight some of your specific learnings in terms of monitoring mRNA biodistribution and activity in vivo, and how these might be harnessed to advance the mRNA vaccine and therapeutic fields?

CP: One of the paradoxes of this field is that often, measuring activity is easier than measuring mechanism of action. Tracking the fate of mRNA and working out exactly how it gets to its site of action, and its exact quantity and how efficient it is, is often much more difficult to analyze than just measuring a biological endpoint. There could even be a biological endpoint in terms of a physiological effect.

It might also be a biological endpoint like an expression of a protein. We often use mRNA encoding for what we call reporter genes. For example, we use nanoluciferase, which is incredibly sensitive. We can inject mRNA and measure nanoluciferase in tissues easily. What we can't do is work out why is it not being delivered to certain tissues and what the limitations are—particularly the barriers to improved intracellular delivery.

One of the key learnings is that you are delivering a particle to the cells, and it often gets taken up into the endocytic system, but the endocytic system in a cell is largely designed to take up material, destroy it, and use it as a nutrient. When molecules like RNA get into endosomes they are often largely broken down when endosomes fuse with lysosomes.

People often talk about endosomal escape, which means trying to get the RNA into the cytoplasm. We know this is a very inefficient process, and we can't control it at the moment. It is also difficult to quantitate it. So, the challenge is not so much measuring biological activity, but measuring mechanisms of action and efficiencies of delivery at various stages. There are no well-established assays for these different processes, and in particular intracellular trafficking processes. But once we have got good assays in place, it will hopefully allow us to improve the delivery systems.

AJ: The amount of lipid delivered to a particular organ doesn't necessarily correlate to the amount of mRNA that was delivered actively to those cells. The amount of mRNA that was delivered also doesn't necessarily correlate well to the amount of protein expression that you get. There are so many additional factors to go from LNP delivery to mRNA release inside the cell, to translation, and even to correlating protein expression in the cell, because cells will have different efficiencies of translation. Just because you have a cell that is giving you a huge amount of therapeutic protein doesn't necessarily mean it had a huge amount of mRNA delivered to it, or any more mRNA delivered than another cell that might be giving significantly less output. The key to developing better delivery systems will be pulling apart the complexity of these multiple stages between initial delivery and outcome.

Q What do your findings to date regarding mRNA delivery tell us about future research and innovation priorities in this field?

AJ: There are a couple of levels here. When measuring how much mRNA is being delivered to the cells, you can do the more traditional RT-qPCR, or you can start using some of the sequencing techniques. We are using long-read sequencing, but you could also use conventional sequencing.

Then, as we touched upon earlier, one of the real challenges is just because the RNA is delivered, it doesn't mean it is in the right place. This has been a big focus of what Colin and I have been working on, and we have developed two complementary molecular sensors. In each case, the sensor molecule gives no signal if it is inside an LNP or inside the endosome. But if the mRNA escapes into the cytosol, we have a complementary protein present that will come in and snap a little bit of the sensor molecule off. This generates light, either as fluorescence or luminescence, which can then be measured by one of the two sensors. The fluorescence sensor is good for single-cell analysis and allows us to quantitatively measure the percentage of mRNA that is being delivered to the cytosol—that is where we get the 2–5% efficiency result. We can do this in quite a high-throughput manner, using tools like flow cytometry to analyze tens of thousands of cells. What was very nice to see in terms of that 2–5% range was that there is some work by Marino Zerial of the Max Planck Institute from about a decade ago that used cryo-electron microscopy to meticulously count individual LNPs using high-resolution imaging techniques, and they reached approximately the same result. The luminescent version of the sensor doesn't work at a single cell level, but can work on an organ or multiple cell level. Is has a high level of sensitivity, and again, we see very good correlation between the numbers from these two sensors.

This range of 2–5% seems to be the natural limit of delivery that you get to the cytosol. You have to start doing quite special things to get it higher than that. However, you don't have to do much to get it below 2%—there are many things that you can do to drop your efficiency. With the engineering of any new delivery system, you want to benchmark it by saying that you are achieving at least 2% delivery, otherwise, you know that you have gone backwards. We are yet to come across the next leap forward that takes this number higher.

CP: Viruses are often held up as the ultimate in terms of the efficiency of their delivery to cells. In the virology literature, there is a lot of information that suggests that some viruses have evolved mechanisms to get out of the endosome and deliver to different sites within the cell. Those mechanisms have quite often been teased out at the molecular level, but strangely, we don't actually know how efficient they are. For example, adenoviruses have a well-documented mechanism to get out of the endosome. But if you ask a virologist how efficient it is, they don't really know, because as a virus is an infective agent you don't need a hugely efficient system. It would be very interesting to use the sensors that Angus has been describing to tease out how efficient the viral systems really are—then, perhaps, we could simulate those with a synthetic approach.

"The ability to re-engineer your lipid nanoparticles so it doesn't stimulate the immune system as much is going to be an important leap forward."

Q Looking to the future, where do you see advances coming in terms of innovations that can address the targeted delivery limitations of current LNP and other delivery platforms?

CP: We have to make sure that in trying to explore different mechanisms of targeting and distribution, we don't over-engineer the product.

As a pharmaceutical scientist, I come back to this often because LNPs have obviously evolved from many years of study and have been optimized for delivering nucleic acids. Now that all that work has been done and we have a successful delivery system, they are remarkably easy to manufacture. When we make modifications, we must ensure that we don't create a lot of difficulties with manufacturing. What I mean is not just making the product, but characterizing it. You have got to be able to characterize the surface of the particle you are targeting and the orientation of the molecules, and that is something we are focusing on. It is important to think about how you are going to characterize the product and demonstrate it is working in the way that you think it is.

AJ: I would add that when it comes to putting targeting molecules like antibodies or nanobodies onto the LNP, they are directional molecules. You cannot stick them on in just any orientation; they have a particular region that recognizes the antigen on the surface of the cell that you are delivering to. The ability to control orientation of a protein on an LNP is critical. What people usually do is to stick a few hundred antibodies on, and rely on enough of them pointing in a good enough direction that you will get the required delivery. That is obviously far from ideal not just from an efficiency point of view, but also in terms of manufacturability. You might find that you start getting batch-to-batch or antibody-to-antibody variation if they naturally have a propensity to orientate in a slightly different way. We want all of the antibodies we put on the surface to be in the same, optimal orientation, so you have a defined, manufacturable product.

Another area for innovation, particularly as we move beyond vaccines, is that it is known that LNPs have a level of reactogenicity that is favorable for vaccines but is not favorable for other applications. The ability to re-engineer your LNP so it doesn't stimulate the immune system as much is going to be an important leap forward. Again, this combines our areas of interest—if you are relying on non-specific interactions, you are more likely to have an LNP that is more reactogenic. We hope if we can take away that non-specific interaction and we are governing the interactions based on an antibody, then we can start changing the LNP a little bit more and reducing that reactogenicity.

CP: We all know from having received mRNA vaccines that we are treading a thin line between a good outcome and poorly tolerated products. A lot of people have experienced some reactogenicity from the mRNA vaccines, and the dose is right up against the limit of tolerability for widespread use. Then you have to think about other aspects, such as to what extent is my target indication a life-threatening disease? When you are talking about vaccination of a large population in a non-pandemic scenario, you are treating perfectly healthy people and don't want to do damage. What can be considered as well tolerated in different circumstances is an important ethical dilemma for the field to address.

Q Finally, can you each sum up one or two key goals or priorities for your work over the foreseeable future?

CP: One of the things that interests me is that for straightforward therapeutic applications where you want to replace a protein, we don't really know how much protein we need for particular diseases. If you are comparing it with administering a pharmaceutical protein, it isn't quite as easy as it sounds. Take Pompe disease—we have a protein pharmaceutical, Lumizyme®, and we give it at a certain dose. However, the cell doesn't really have an active mechanism for taking up a protein under normal circumstances, it's just a passive mechanism. That makes it hard to work out how much of the pharmaceutical protein is being delivered intracellularly and match that up with how much protein you can make intracellularly with mRNA. These are some basic things we need to find out because depending on the purpose of the protein, mRNA may prove to be a much more potent alternative. For example, if it is an activity that only involves a few molecules of protein, like enzymatic activity or a transcription factor, those applications may be very amenable to mRNA therapy. For others where you need quite a lot of systemic protein, it may be a different story.

Personally, I am also very interested in using gene editing in different disease settings. That comes in a number of different guises, and it's not one answer fits all. I'm particularly interested in whether we can use knock-in approaches, such as an integration of copy DNA (cDNA) encoding a protein to replace a protein. That gets around the problem of the duration of action of mRNA. If you can use gene editing to put in an entire cDNA, then you can have longstanding expression.

AJ: My fundamental interest has always been in understanding what is going on inside the cell, how can we control delivery, and how we can improve efficiency of delivery. The problem of how we get material from the endosome into the cytosol has been the fundamental question I have been interested in for the last 10 years.

With RNA there is so much potential for new therapies that at the moment, it's a little like being a child in a sweet shop—you start thinking of all the things that the mRNA could possibly do, but you have got to control yourself and see where you can apply it first. There are so

many different diseases and interesting biological things that you could do. I am now interested in trying to do a lot of translational work that I probably wouldn't have even considered just a couple of years ago.

BIOGRAPHIES

COLIN POUTON is Professor of Pharmaceutical Biology at Monash Institute of Pharmaceutical Sciences (MIPS), Melbourne, Australia. Pouton is a pharmaceutical scientist with broad experience in drug discovery, delivery, and product development. He began his postdoctoral career at the University of Bath, UK, where he remained before moving to Monash University in 2001. Pouton is best known internationally for his program of research on delivery systems to enhance the oral bioavailability of poorly absorbed small molecule drugs, which began during his PhD at the University of London. Since the mid-1990s he has directed two other academic research programs in parallel, on nucleic acid therapeutics and pharmaceutical applications of stem cell technology. He has published over 180 peer-reviewed papers and supervised over 75 PhD students. In 2017, his nucleic acid research group moved away from attempting to enhance nuclear delivery of DNA to focus on *in vivo* delivery of mRNA. His current focus is a collaborative program to foster early proof-of-concept studies to study the potential of mRNA for vaccination, therapeutic use in chronic diseases, and treatment of genetic diseases by genome editing. In response to COVID-19, Pouton's research group developed mRNA vaccines encoding membrane-anchored variant RBDs (RBD-TM) derived from the SARS-CoV-2 spike protein. Preclinical studies were conducted in collaboration with virologists and immunologists at the Doherty Institute. A Phase 1 study using the RBD-TM mRNA vaccine platform was carried out in parallel with an RBD-Fc protein (+ adjuvant) vaccine developed by colleagues at the Doherty Institute. This joint Phase 1 study using beta variant vaccines was completed in 2022. The RBD-TM platform is currently being evaluated at MIPS as a potential basis for broad spectrum multivalent COVID vaccines.

ANGUS JOHNSTON is an Associate Professor and leads the NanoMaterials for Biology group at the Monash Institute of Pharmaceutical Sciences (MIPS), Melbourne, Australia. He completed his PhD in DNA sequencing at the University of Queensland in 2006, and following this he was a postdoctoral research fellow at the University of Melbourne. In 2013, he established his own research group at Monash University. Johnston's research focuses on developing targeted drug delivery systems and understanding the trafficking of nucleic acids, proteins, and nanoparticles in cells. He has published over 130 papers and in leading journals including *Nature Nanotechnology*, *Nature Communications* and *Nature Protocols*. Johnston has received a number of awards for his research, including the Grimwade Prize for Industrial Chemistry, Young Tall Poppy Award, and was a finalist for the Eureka Prize for Outstanding Young Researcher and Eureka Prize for Innovative Use of Technology.

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AUTHORSHIP & CONFLICT OF INTEREST

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In the version of this FastFacts poster initially published, Adam Armengol was listed as the sole author. However, the poster was in fact co-authored by Angel Lorenzo. This error has been corrected in the HTML and PDF versions of this article as of March 6, 2024. The amended FastFacts poster can be accessed here.

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