

Emerging Nucleic Acid Cargos for Next-Generation RNA Vaccines and Therapeutics

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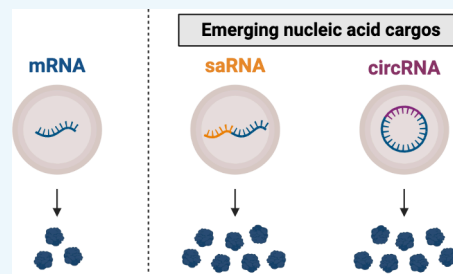
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ABSTRACT: Messenger RNA (mRNA) lipid nanoparticles (LNPs) have emerged as a transformative technology with broad applications in vaccines, protein replacement therapy, and gene editing. However, the transient nature of mRNA expression often necessitates high or repeated dosing regimens, limiting its therapeutic potential. Thus, there is a critical need for innovation at the interface of RNA biology and drug delivery that prolong the duration of RNA translation. In this Viewpoint, we provide an overview of emerging nucleic acid cargos that address these challenges, specifically self-amplifying RNA (saRNA) and circular RNA (circRNA), and provide a framework for how these nucleic acid cargos can enable the next generation of vaccines and therapeutics for diverse clinical applications.



INTRODUCTION

Between 2020 and 2024, greater than 13 billion doses of COVID-19 vaccines were administered, preventing millions of deaths worldwide.¹ Both the Pfizer-BioNTech and Moderna COVID-19 vaccines are composed of lipid nanoparticles (LNPs) encapsulating messenger RNA (mRNA) specific for the SARS-CoV-2 spike protein, providing immunological memory against viral infection. The safety and efficacy of these products has ushered in a new era of RNA nanomedicine.^{2,3} Ongoing trials in patients include mRNA-LNP vaccines for several infectious diseases and mRNA-LNP therapeutics to introduce or edit genes implicated in pediatric disorders, hereditary diseases, and cancer.^{4,5} The modularity of mRNA-LNP platforms enables this breadth of clinical application. Interchangeable lipid-based building blocks (ionizable lipid, helper lipid, cholesterol, polyethylene glycol) modulate organ and cellular biodistribution of LNPs, while customizable mRNA payloads allow for tailored biological activity and function.^{6,7}

Despite this, conventional mRNA-LNP platforms have several limitations. High mRNA doses and/or repeat immunizations may be required to elicit a sufficient immune response after vaccination. The transient nature of mRNA expression also limits the utility of mRNA-based therapeutics for diseases with chronic pathologies.^{7–9} Thus, there is a critical need for advancements at the interface of RNA biology and drug delivery that extend the duration of RNA cargo translation. In this Viewpoint, we provide an overview of novel nucleic acid cargos that address these challenges, including self-amplifying RNA (saRNA) and circular RNA (circRNA) (Figure 1A). We further discuss engineering strategies for the biochemical modification of these RNA cargos and their encapsulation in optimized nanoparticle formulations. As the

toolbox for RNA-based platforms continues to grow, implementation of these emerging technologies will facilitate the next generation of vaccines and therapeutics for diverse clinical applications.

SELF-AMPLIFYING RNA

saRNA is a class of nucleic acids that has been engineered to self-replicate inside cells to enhance protein production relative to standard mRNA. saRNA shares structural similarities to mRNA, including a 5' cap, 5' and 3' untranslated regions (UTRs), and a polyadenylated (polyA) tail, and is synthesized in a manner similar to mRNA using *in vitro* transcription.¹⁰ However, unlike mRNA, saRNA encodes replicase proteins which enables self-replication of the RNA within cells, amplifying protein expression over an extended period.^{10,11}

The replication element of saRNA is typically derived from self-replicating, single-stranded RNA viruses, and the majority of saRNA constructs are based on alphaviruses. These replication elements include regions encoding non-structural proteins, such as RNA-dependent RNA polymerase (RdRp), which is vital for RNA self-replication.^{10,12,13} Once translated in the cytoplasm, these nonstructural proteins form a replicase complex that produces complementary negative-strand RNA, which can be used to repeatedly synthesize new positive-strand saRNAs and subgenomic RNAs, amplifying both the saRNA

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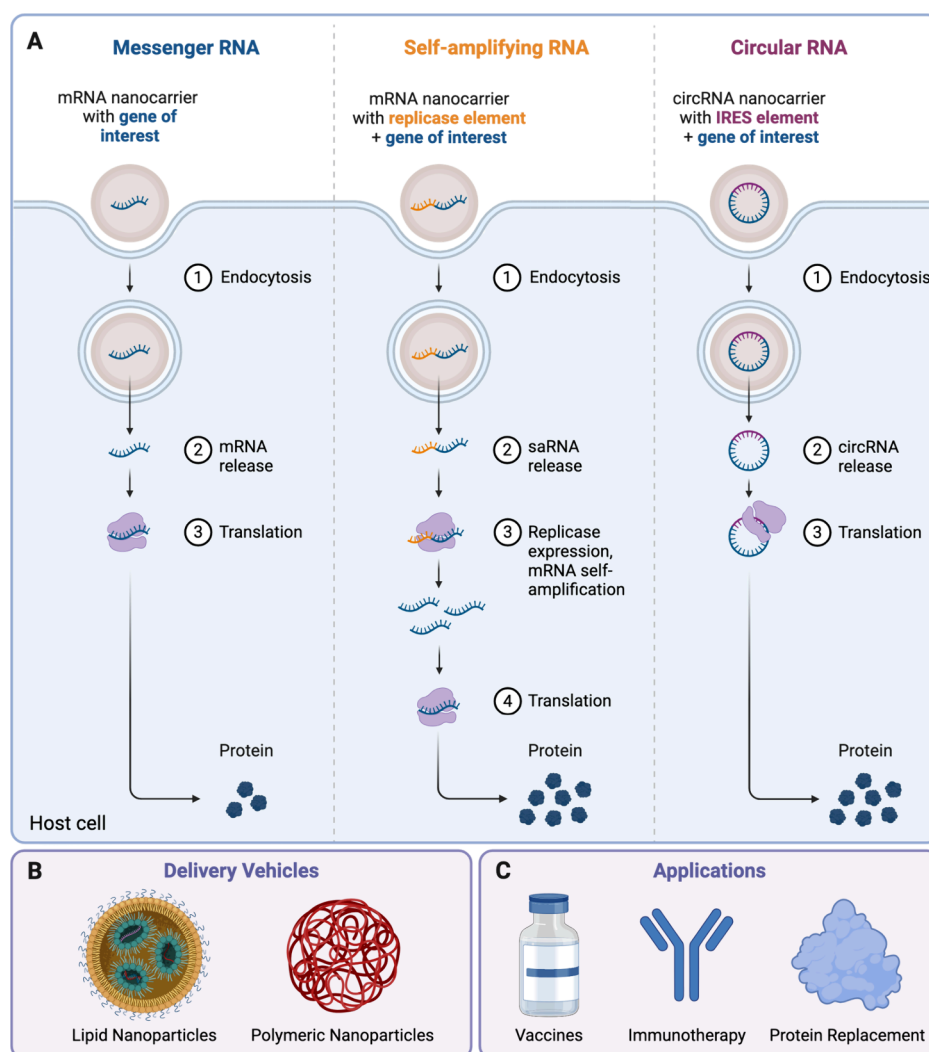


Figure 1. Schematic overview of emerging RNA cargos highlighting (A) the mechanisms of translation of self-amplifying RNA (saRNA) and circular RNA (circRNA) compared to messenger RNA (mRNA), (B) encapsulation strategies to enhance the delivery of translation of saRNA and circRNA and (C) potential therapeutic applications of saRNA and circRNA.

itself and the encoded transgene for sustained intracellular protein expression.^{13,14} The performance of non-structural proteins can vary across different cell types, suggesting that amplification efficiency may be improved by optimizing the non-structural protein region of the saRNA sequence.^{11,15}

Despite amplifying protein expression, saRNA is highly immunogenic, which may limit its potential as a therapeutic modality. During replication, saRNA generates double-stranded RNA (dsRNA) intermediates that activate the innate immune system and trigger an inflammatory response.^{11,16} Specifically, these intermediates are sensed by pattern recognition receptors (PRRs) which recognize exogenous RNA and initiate the secretion of pro-inflammatory cytokines and type I interferons (IFNs).^{13,17} This immune activation can inhibit the translation of saRNA and limit the potential of saRNA therapeutics.

Even with elevated immunogenicity, saRNA therapies offer several advantages over traditional mRNA approaches. By amplifying protein production, saRNA may reduce the required dosage and administration frequency of a therapy compared to mRNA. This reduction may in turn lower the risk of adverse side effects and reduce manufacturing burdens and

costs. While saRNA has shown strong potential for vaccine development, its application in protein-replacement therapies is limited unless the innate immunogenicity of saRNA can be mitigated.

■ CIRCULAR RNA

CircRNA is a nucleic acid cargo characterized by its unique, single-stranded, covalently closed structure without 5' and 3' ends. The structure of circRNA confers enhanced stability and resistance to exonuclease degradation, resulting in a longer half-life and prolonged protein expression compared to linear mRNA.^{11,18,19}

Because circRNA lacks a 5' cap, it cannot initiate translation through traditional cap-dependent pathways. Instead, circRNA constructs contain an internal ribosomal entry site (IRES) which recruits ribosomes to initiate translation in a cap-independent manner.^{18,19} However, cap-independent translation via IRES is generally slower and less efficient than the cap-dependent translation mechanism utilized by mRNA.^{11,20} Additionally, these IRES elements are typically derived from viral sequences and different IRES motifs can exhibit tissue-specific translation. This has led to screening of IRES motifs

Table 1. Nucleoside Modifications for saRNA and circRNA and Their Effects on Innate Immunity and Translation/Replication^a

Nucleoside modification	Effects on innate immunity		Effects on translation/replication		refs
	saRNA	circRNA	saRNA	circRNA	
Pseudouridine (Ψ)	Reduces RIG-I/TLR7/8 activation, lowers IFN responses	Ψ helps reduce immunogenicity	Incompatible with saRNA replication, full Ψ substitution prevents replication/translation	Ψ substitution has been used to prolong translation <i>in vivo</i> and has shown circRNA translation for weeks with minimal immune clearance	20, 24, 29–31
N1-Methylpseudouridine (m1 Ψ)	m1 Ψ leads to poor recognition by some viral polymerases and can block replication	Strongly immunoevasive, m1 Ψ -modified RNA does not efficiently bind TLR7 and protects circRNA from TLR7/8 sensing	m1 Ψ leads to poor recognition by some viral polymerases and can block replication due to replicase incompatibility	Hypothesized to improve translation and RNA half-life by preventing stress response, improves cumulative protein expression	51
5-Methylcytidine (m5C)	m5C-modified saRNA triggers little to no interferon response, likely avoids RIG-I/MDAS activation and reduces IFIT binding by mimicking host mRNA 2'-O-methylation patterns	Reduces any residual immune sensing and if bacterial sequences or double-stranded regions are present, m5C will dampen OAS/RIG-I activation, generally, lowers IFN induction	Preserves high protein expression and replication competency, a fully m5C-substituted saRNA demonstrated sustained translation in cells and animals and without early shutoff	m5C would prolong RNA half-life and could improve translation consistency, especially in long-term applications	27, 29
5-Methyluridine (m5U)	Uridine methylation suppresses TLR endosomal sensors and reduced TLR7/8 recognition, often used in combination with other modifications	Makes circRNA less visible to innate immune cells and useful for repeat dosing applications	Improves translation efficiency when combined with m5C to give high protein yields <i>in vivo</i> , <i>in vitro</i> , complete m5U substitution increased the fraction of cells expressing protein by about 8-fold compared to m1 Ψ -modified replicon, generally compatible with replicase function and does not stall RNA polymerase	Functions analogously to pseudouridine in promoting translation, by decreasing innate immune responses, m5U-modified circRNA avoids shutdown of protein synthesis, m5U may also slightly increase thermal stability of RNA duplex regions, potentially aiding certain IRES or structural elements	27, 29, 31
2-Thiouridine (s2U)	Not yet widely applied in saRNA, but known from mRNA studies, s2U attenuates TLR7/8 and TLR3 activation, it also reduces RIG-I binding to 5'-triphosphate RNAs	s2U in circRNA would be expected to dampen TLR3 and TLR7/8 responses as it does in other RNAs, if any double-stranded byproducts form, s2U also interferes with OAS activation and PKR binding.	Effect of s2U during replication is under exploration, high s2U content could potentially interfere with base-pairing and partial substitution might be preferable, if compatible, it would be expected to prolong saRNA translation by preventing immune-mediated shutdown	In circRNA, s2U would be expected to prolong its half-life further but s2U may slightly reduce base-pairing fidelity, if an IRES contains many uridines, complete substitution might affect its functionality	31, 52, 53
N6-Methyladenosine (m6A)	m6A-modification confers a potent immune-evasion signal	m6A prevents RIG-I recognition of circRNA, YTHDF2 binding blocks immune activation	Generally neutral for saRNA replication/translation, not routinely used in saRNA	Acts as a cap-independent translation enhancer when strategically placed, m6A sites in circRNA recruit initiation factors via m6A readers, enabling efficient translation without a 5' cap, extensive m6A can also recruit decay pathways, potentially reducing protein output	21, 54

^aLegend: Retinoic acid-inducible gene 1/melanoma differentiation-associated gene 5 (RIG-I/MDAS), interferon-induced proteins with tetratricopeptide repeats (IFIT), toll-like receptors (TLR), pattern recognition receptors (PRRs), 2',5'-oligoadenylate synthetase (OAS), YTHDF2 binding blocks

from different viral sequences to optimize for cell-specific IRES translation.^{11,19–21}

The most widely used technique for *in vitro* circRNA synthesis is the permuted intron exon (PIE) approach. In this splicing technique, fused partial exons are flanked by half intron sequences and during autocatalysis, the introns are excised and the circularized RNA product is formed.^{11,19,22} The PIE technique is useful in generating large RNA sequences.¹⁷ However, the PIE method has low efficiency, and the introduction of exogenous sequences during splicing may result in a final product that differs from the original linear RNA precursor.^{19,23} Alternative circularization methods include chemical and enzymatic ligation strategies, both of which can produce high-purity circRNA. However, these approaches are generally limited to the synthesis of shorter RNA sequences.^{17,19}

The use of circRNA instead of mRNA has many advantages. Primarily, the extended half-life of circRNA and the resultant enhanced durability of protein expression enable dose-sparing effects, which may be beneficial for both for vaccine and protein replacement therapies. Additionally, purified circRNA, without nucleoside modifications, induces a lower innate immune response compared to mRNA as it lacks the 5'-triphosphate motif that commonly triggers retinoic acid-inducible gene I (RIG-I) activation.^{19,24} The lack of a polyA tail, reduced need for costly nucleoside modifications, and enhanced stability may simplify manufacturing requirements for circRNA, potentially decreasing production costs.^{17,18} Despite the promise of circRNA, the therapeutic efficacy of circRNA ultimately depends on its successful encapsulation into a delivery vehicle to ensure efficient cellular uptake and translation.

■ NUCLEIC ACID MODIFICATIONS IN SARNAs AND CIRC RNAs

Nucleic acid modifications may overcome some of the current challenges of saRNA and circRNA platforms. Standard saRNA platforms have their translation impeded by innate immune sensing during replication via RIG-I and toll-like receptor (TLR) 7 and 8 activation that lead to interferon and protein kinase R (PKR) mediated silencing. To overcome this, saRNA transcripts are produced with modified immune-evasive nucleosides while maintaining compatibility with the viral replicase. Key nucleic acid modifications in saRNA have been compiled (Table 1). Notably, the widely used mRNA modification N1-methylpseudouridine (m1Ψ) was found to be ineffective in saRNA, as this substitution severely hampered replication and protein yield. Recent studies found that alternative substitutions like 5-methylcytidine (m5C), 5-hydroxymethylcytidine, and 5-methyluridine can be incorporated without disrupting the alphavirus RNA-dependent RNA polymerase.²⁵ These modifications mitigate innate responses by dampening TLR and dendritic cell interferon induction to prevent translational cessation. Consequently, base-modified saRNAs drive higher antigen expression at low doses. For example, a fully m5C-modified saRNA COVID-19 vaccine achieved protective immunity at ~100-fold lower dose than an m1Ψ-modified mRNA COVID-19 vaccine.^{26,27} The net effect of nucleoside modifications on saRNA platforms is enhanced translation and potency with reduced reactogenicity.

circRNA platforms must overcome the lack of a 5' cap for translation and the risk of host immune recognition of foreign circRNAs. To address this challenge, circRNAs are engineered

with cap-independent translation elements such as an IRES or N⁶-methyladenosine (m6A) motifs that recruit initiation factors known as the m6A-induced ribosome engagement site (MIREs).²⁸ A single m6A site can drive efficient translation initiation in circRNAs by recruiting eukaryotic initiation factor 4 gamma 2 (eIF4G2), enabling protein yields comparable to capped mRNAs. At the same time, innate immune sensing of circRNAs is minimized by incorporating modified nucleotides during *in vitro* transcription. Chemically modified bases such as m5C, 5-methyluridine (m5U), 2-thiouridine, and pseudouridine have been substituted into circRNA to avoid activation of RIG-I and endosomal TLR3/7/8 (Table 1).^{20,24,29–31} These modifications have been shown to mimic natural epitranscriptomic markers. For example, m6A residues on circRNA not only enhance translation but also signal the RNA as self, suppressing interferon responses to exogenous circRNA.¹⁹ Importantly, utilizing such modifications does not impede RNA circularization processes, such as ligation or splicing. High-purity circRNAs with optimal modifications have demonstrated prolonged stability, high protein expression, and low immunogenicity in preclinical models.¹⁹

■ DELIVERY STRATEGIES FOR SARNAs AND CIRC RNAs

To overcome the biological barriers associated with intracellular nucleic acid delivery, delivery vehicles, such as LNPs or polymeric nanoparticles, are essential for the encapsulation of saRNA or circRNA (Figure 1B). Many of the LNP formulations originally developed for mRNA or small interfering RNA (siRNA) delivery, including the FDA-approved Moderna and Pfizer LNP formulations, have been repurposed for the delivery of saRNA and circRNA.^{27,32–34} Researchers have also explored new strategies to optimize the encapsulation and delivery of saRNA and circRNA.^{35–39} For example, Xu et al. screened a library of 96 ionizable lipids and identified an LNP capable of delivering circRNA to lung cancer cells.³⁹ Blakney et al. compared LNPs and polymer nanoparticles for saRNA delivery and found that polymer nanoparticles enabled greater protein expression *in vivo*, whereas LNPs were more immunogenic and provided better protection against influenza infection.⁴⁰ Finally, Barbieri et al. demonstrated that incorporating DSPC, a high melting point and cylindrical phospholipid, into LNP formulations improved both the stability and potency of saRNA delivery compared to alternative phospholipids.⁴¹

■ APPLICATIONS OF SARNAs AND CIRC RNAs

Both saRNA and circRNA have primarily been explored for vaccine applications, while circRNA has also shown promise for sustained protein delivery (Figure 1C). The largest thrust of saRNA vaccine research has been for immunity against SARS-CoV-2, leading to the approval of ARCT-154 in Japan, a saRNA SARS-CoV-2 vaccine developed by Arcturus Therapeutics.^{42,43} A phase III clinical trial demonstrated that ARCT-154 elicited a stronger immune response than the Pfizer mRNA vaccine at a reduced dose of total RNA.^{42,44} Several other clinical trials are currently ongoing investigating saRNA vaccines against SARS-CoV-2 and influenza, while preclinical studies have explored saRNA vaccines against the rabies virus, zika virus, and human immunodeficiency virus.^{16,37,45,46} Similarly, early circRNA research has focused on developing

SARS-CoV-2 vaccines.^{47–49} However, circRNA has also been explored for cancer immunotherapy, wound healing, and protein replacement therapies.^{38,39,50} Notably, Liu et al. reported that a single dose of VEGF circRNA, delivered via LNPs, significantly accelerated wound healing in mice compared to a VEGF mRNA control.³⁸

As saRNA and circRNA platforms advance through preclinical development and expand into new disease indications, the immunogenicity of each RNA product must be considered. For vaccines, which often require fewer repeat doses and rely on activation of the immune system, the inherent immunostimulatory properties of saRNA may be beneficial. In contrast, for applications such as protein replacement therapy, where prolonged protein expression and minimal immune activation are desired, circRNA may offer a better solution. Careful selection of these RNA cargos based on their immunogenic profiles will be essential to maximize their therapeutic efficacy across many clinical applications.

OUTLOOK

saRNA and circRNA hold significant promise to revolutionize RNA-based medicine, offering the potential for sustained protein expression, reduced dosing frequency, and simplified manufacturing practices. However, despite the rapid development of the COVID-19 mRNA vaccines, driven by decades of foundational research on mRNA, further optimization of saRNA and circRNA constructs is required before their successful implementation in clinical products. Research efforts should continue to focus on reducing the innate immunogenicity of these RNA cargos, potentially through nucleic acid modifications or alternative design strategies. Additionally, the translation efficiency of these cargos is highly dependent on replicase and IRES elements, which have demonstrated varied performance across different cell types and animal models. A deeper understanding of the mechanisms behind these discrepancies in translation will be needed prior to clinical translation.

Nanoparticle engineering and design will also play a critical role in the success of saRNA and circRNA platforms. Continued advancements in lipid and polymeric nanoparticle design are necessary to enable cell-specific targeted delivery, potent endosomal escape, and controlled modulation of an immune response. As both RNA construct design and delivery technologies continue to evolve, the therapeutic potential of saRNA and circRNA will expand, enabling the development of next-generation RNA therapeutics and vaccines with enhanced efficacy, durability and safety.

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Notes

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