Toxicologic Pathology
2024, Vol. 52(8) 545–552

DOI: 10.1177/01926233241298572
journals.sagepub.com/home/tpx


Session 4: mRNA and Self-Amplifying RNA (saRNA): Opportunities for Disease Prevention and Therapy

Rani S. Sellers[1], Lila Ramaiah[2], Sue-Jean Hong[3], Prashant Nambiar[4], Eric Jacquinet[5], and Shan Naidu[6]

Abstract

The unprecedented speed of developing vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the COVID-19 pandemic, has propelled mRNA technologies into the public eye. The versatility of mRNA technology, often referred to as "plug and play," offers immense promise for rapidly updating vaccines to address newer variants of respiratory diseases and combat emerging infectious diseases and lethal pathogens, such as the Ebolavirus. However, the potential applications of mRNA technology extend well beyond prophylactic vaccines. This session explored the two primary mRNA platforms: nonreplicating mRNA and self-amplifying mRNA (variably referred to as saRNA, samRNA, or SAM). Presentation topics were on current research efforts aimed at broadening the applications of mRNA modalities beyond vaccines. Topics included opportunities for delivering mRNA via intra-tumoral and inhalational routes, immunological and systemic inflammatory responses elicited by these modalities, and regulatory considerations involved in the development and licensing of these technologies.

Keywords

mRNA, self-amplifying mRNA, saRNA, samRNA, regulatory, safety, nonclinical, pathology, vaccines, RNA therapeutics

Introduction

This manuscript is a synopsis of Session 4 of the 2024 Symposium of the Society of Toxicologic Pathology: mRNA and Self-Amplifying RNA (saRNA): Opportunities for Disease Prevention and Therapy. Drs. Shan Naidu from Moderna and Rani Sellers from the University of North Carolina co-chaired the session. Dr. Sellers opened the session by noting that the power of mRNA therapies came to the forefront as a result of COVID-19 vaccine development, but in fact had been in vaccine and therapeutic investigative and clinical studies for decades. Its importance in scientific progress was acknowledged by the presentation of the 2023 Nobel Prize in Physiology or Medicine to Katalin Karikó and Drew Weissman for their discoveries related to nucleoside base modifications, which directly contributed to the development of an effective mRNA vaccine against COVID-19. This session reviewed the nature of mRNA and self-amplifying mRNA (saRNA) modalities and nonclinical safety concerns, potential uses for treating chronic diseases such as cystic fibrosis, its use for expressing therapeutic molecules in tumors, assessing reactogenicity using biomarkers in nonclinical studies, and regulatory guidelines relevant for mRNA modalities.

Safety and Immunogenicity of Self-Amplifying RNA Vaccines

Dr. Sue-Jean Hong, mRNA Platform Lead, Pfizer Vaccines Research and Development, led off the session by defining the fundamental concepts around mRNA-based vaccines and explaining the differences between modified mRNA and self-amplifying RNA (saRNA) platforms, specifically for prophylactic vaccines. Her presentation was entitled "Safety and immunogenicity of self-amplifying RNA vaccines."

[1] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA
[2] Johnson and Johnson, New Brunswick, New Jersey, USA
[3] Pfizer, Inc., Pearl River, New York, USA
[4] Strand Therapeutics, Inc., Boston, Massachusetts, USA
[5] SalioGen Therapeutics, Lexington, Massachusetts, USA
[6] Moderna, Inc., Cambridge, Massachusetts, USA

Corresponding Author:
Rani S. Sellers, University of North Carolina at Chapel Hill, 160 Medical Dr, Chapel Hill, NC 27599, USA.
Email: rsselle@unc.edu

In vaccine development, there are currently two primary RNA platforms: nonreplicating mRNA and self-amplifying RNA (saRNA). The mRNA-lipid nanoparticle (LNP) COVID-19 vaccines utilized nonreplicating mRNA; these mRNA had all the required components for generating the antigen. In addition to having the sequences for the antigen of interest, the mRNA vaccine had 5′ and 3′ untranslated regions, a 5′cap, and a polyadenylated tail. However, in order to help reduce reactogenicity and improve stability and translatability, the mRNA contained modified nucleosides (e.g. N1-methylpseudouridine) as well as other sequence modifications such as codon optimization, increased GC content, etc. Once in the cell, the mRNA is released into the cytoplasm and translated by the host machinery, and within days, degraded.[28,36] In order to get adequate antigen expression to develop a protective immune response, relatively high doses of the mRNA-LNP are required, which likely contributed to the reactogenicity identified during clinical trials and in the post-emergency use phase.

Reductions in RNA vaccine dose can potentially be achieved using a self-amplifying RNA (saRNA) platform. SaRNAs are synthetic RNA molecules derived from an alphavirus-derived replicon. Like the mRNA vaccine, saRNA vaccines can also be formulated with a lipid nanoparticle (LNP) delivery system that is designed to enhance vaccine stability, delivery, and immunogenicity.[16] saRNA is constructed by retaining the viral replication machinery (nonstructural proteins: nsp1-4) but replacing viral structural proteins with target antigen sequences under key regulatory elements. The nsp1-4 proteins come together to form a replicase, which replicates the saRNA, allowing for high antigen expression without the formation of infectious viral particles that can spread from cell to cell. Unlike conventional mRNA vaccines, which only encode the antigen of interest and are translated directly from the incoming mRNA molecules, saRNA can generate many copies of the antigen-encoding transcripts in the target cell, leading to high and prolonged expression of the antigen and additional self-adjuvanting of innate immune responses. As a result, saRNA vaccines can elicit protective humoral and cellular immune responses at lower doses, potentiating this platform to be particularly useful for the rapid global response to a pandemic outbreak or emerging infectious pathogens.

Nonclinical toxicology studies have a favorable safety profile. Findings in rats administered saRNA vaccines delivered using a cationic nanoemulsion rather than LNP have been the result of an inflammatory/immune response, which is an expected finding after prophylactic vaccine administration.[7,26,35] Clinically, rats may develop transiently higher body temperatures compared to saline controls. In these studies, transiently higher fibrinogen, alpha-1-acid glycoprotein [A1AGP], and alpha-2 macroglobulin [A2M] as compared to saline controls are present and expected. Similarly, white blood cells, mostly neutrophils and large unstained cells, are transiently higher compared to PBS controls. Additionally, in saRNA nonclinical studies, transiently higher AST and ALT are observed compared to controls. SaRNA-administered rats have enlarged draining lymph nodes at the end of the dosing phase. Microscopically, the injection site has minimal to moderate inflammation and myodegeneration/necrosis. Draining lymph nodes had mild to moderately greater cellularity compared to saline control animals. Despite increases in AST and ALT in these studies, there is no associated microscopic change in the liver, which suggests that AST and ALT activity increases may not be of hepatic origin. All findings, except increases AST and ALT are commonly evident in nonclinical repeat-dose prophylactic vaccine studies. Findings resolved or were resolving at the end of the recovery phase.

Currently, there are several saRNA vaccines in clinical trials which have thus far proved to be safe and potent in clinical studies against SARS-CoV-2 and rabies, as well as in oncology programs. These vaccines have demonstrated balanced and durable immune responses for optimal protective immunity.[1] These findings have led to the recent approval of two COVID-19 vaccines by the national drug agencies of India and Japan, underscoring the promising potential of this technology.[18,31] In addition, the ongoing clinical trials for rabies and influenza are an exciting opportunity for the field of saRNA vaccines and will no doubt be informative as to the characteristics of the immune response, required dose, duration of immunity, and required regimen. The field is also starting to consider methods to modulate the innate response to saRNA, which will no doubt be imperative to the clinical success of these vaccines.[2,27]

samRNA/mRNA Opportunities in Oncology

Dr. Prashant Nambiar, Senior Vice President of R&D at Strand Therapeutics, spoke on the use of RNA modalities for treating cancer in his presentation entitled *"SamRNA/mRNA opportunities in Oncology"* elaborating on advancements in cancer immunotherapy through synthetic self-replicating mRNA technology.

The evolution of mRNA technology has transformed therapeutics, particularly in the development of vaccines and treatments. From its inception in the 1990s to the present day, mRNA has revolutionized the field, playing a critical role in the success of vaccines developed by Pfizer/BioNTech and Moderna. The therapeutic potential of mRNA is particularly notable in combating infectious diseases and cancer. mRNA stands out due to its high scalability, transfection efficiency, systemic delivery capabilities, and robust gene expression without genome integration, making it advantageous over other therapeutic modalities like plasmid DNA (pDNA) and viral vectors such as HSV, AAV, AdV, and Lentivirus. mRNA modalities are currently being evaluated to address various therapeutic needs:

- modRNA: Modified mRNA is useful for vaccines due to its shorter-expression and lower innate immune activation.

- repRNA: Replicating mRNA (aka self-amplifying mRNA) is well suited for immuno-oncology, offering longer expression and higher immunogenicity.
- circRNA: Circular RNA is valuable for cell therapy editing and protein replacement, providing extended expression with lower immunogenicity.

The specific applications of these technologies in cancer therapy were elaborated upon, using a Strand Therapeutic molecule STX-001. STX-001 is a self-replicating mRNA expressing the cytokine IL-12. IL-12 is a cytokine with pro-inflammatory properties and has potential as an immunotherapy for the treatment of "immune-cold" cancers (i.e., low anti-tumor immune responses). STX-001 is intended for the treatment of solid tumors (such as melanoma and triple-negative breast cancer [TNBC]) through intra-tumoral injection. By injecting self-replicating mRNA encapsulated in lipid nanoparticles (LNPs) directly into tumors, STX-001 mRNA encoded IL-12 is expressed, turning "cold" tumors "hot" (i.e., high anti-tumor immune responses) and eliciting a robust anti-tumor response.

Preclinical studies have shown promising results for STX-001, demonstrating its ability to induce significant IL-12 expression and maintain tumor control over longer periods compared to nonreplicating mRNA expressing IL-12. These studies also revealed the synergy between STX-001 and anti-PD-1 antibodies, which enhanced the therapeutic response in mouse models with tumor cells that are typically resistant to PD-1 treatment alone.

The importance of understanding the drug product, developing reliable bioanalytical methods, and implementing effective biomarker strategies is essential. Addressing systemic exposure and off-target effects is also crucial to maximizing the therapeutic index. As STX-001 moves into clinical trials, the primary goal is to establish its safety while also seeking early efficacy signals. Early engagement with regulatory agencies and a science-driven development strategy have been key elements in the drug development pathway for STX-001.

In summary, the presentation showcased the immense potential of synthetic self-replicating mRNA technology in advancing cancer immunotherapy. These types of innovative therapies have the potential to make significant contributions to the fight against cancer.

Inhaled mRNA Therapy for Cystic Fibrosis

Dr. Eric Jacquinet, Vice President of Non Clinical Development at SalioGen Therapeutics, presented on the opportunities for respiratory tract-specific delivery of mRNA expressing normal cystic fibrosis transmembrane conductance regulator (CFTR) proteins may have in the treatment of cystic fibrosis. Cystic fibrosis (CF) is an autosomal recessive disease related to mutations in the CF Transmembrane Conductance Regulator (CFTR) gene (more than 2000 different mutations have been reported). The CFTR is a chloride channel regulating the transportation of chloride ions and controls epithelial ion and fluid absorption and secretion. CF affects the respiratory, gastrointestinal, and reproductive tracts. Over the past 2 decades, drug modulators have been developed to target some specific CFTR mutations. Despite providing great improvement, they are accompanied by significant side effects, and about 10% of the patient population carries mutations that are unresponsive to this class of medicines. Improvements in therapies for CF are essential, as there is still an unmet need and a desire for fewer side effects. Because of this, genetic and mRNA-based therapies are being developed.

During the last decade and, particularly, within the last 5 years, lipid nanoparticles (LNP)-encapsulated mRNAs have proven to be a new class of viable medicines for the treatment of CF. Beside the intramuscular delivery for vaccines, LNP-RNA therapeutics have been delivered by other routes, including intravenous, intra-tumoral, sub-cutaneous, and intra-nodal for various conditions.

The delivery of mRNA therapeutics through the respiratory route, in this case, to treat CF, has unique issues. Here, we present the challenges and the results of delivering CFTR mRNA encapsulated in LNP by inhalation in different preclinical models.

Clinical Pathology Findings With mRNA Vaccine Compared to Relevant Vaccine Adjuvants

Dr. Lila Ramaiah, Global Head of Clinical Pathology & Safety Biomarkers in the Department of Predictive Sciences & Translational Safety at Johnson & Johnson presented on the expected findings for mRNA-LNP vaccines as compared to traditional adjuvanted vaccines.

Messenger ribonucleic acid-lipid nanoparticle (mRNA-LNP) has emerged as a transformative technology in drug development and enabled fast-track vaccine development during the COVID-19 pandemic.[30] mRNA-LNPs are elegant as a vaccine platform because they co-deliver antigen and adjuvant in a single unified self-adjuvanting formulation. Interestingly, mRNA-LNPs induce adjuvant effects on the innate immune system that differ from conventional (conjugated or subunit adjuvanted) vaccines. These effects can be recognized on routine clinical pathology assessments and provide insights into origins and mechanisms of adjuvanticity.

Nonclinical repeat dose toxicology studies for vaccines are unusual, as dosing is intermittent—usually administered every 2-4 weeks.[32] As the goal of vaccination is to develop antigen-specific immune response, clinical pathology changes are reflective of a transient inflammation and immune activation. Such changes most commonly include transiently higher acute phase proteins and increased white blood cell counts. The intramuscular administration of vaccines may cause transient elevations in CK and AST because of muscle injury (e.g., mechanical or inflammatory).

In order to evaluate the magnitude of the acute phase response, blood collection is usually 1 to 3 days after the first and last dose administrations and at the recovery-phase necropsy. In rats, acute phase protein increases generally peak 24-72 hours after vaccine administration (in rats: alpha-1-acid glycoprotein [A1AGP], alpha-2 macroglobulin [A2M]; fibrinogen).[29] Other markers of an acute phase response include increased serum alpha- and beta-globulins and decreased albumin (and a decreased albumin-to-globulin ratio). However, the magnitude of these responses may depend on the antigen or the adjuvant used.[29] Increased white blood cell counts after vaccine administration are generally the result of higher numbers of circulating neutrophils and large unstained cells (LUC), although increases in eosinophils, monocytes and lymphocytes may also be evident.[32] Enzymes such as CK and AST may be increased due to intramuscular injection-related local tissue injury and/or inflammation at injection site. Control groups administered saline or buffer help to distinguish changes attributed to the injection procedure from vaccine reactogenicity.

The effects of mRNA-LNP COVID-19 vaccine candidates on clinical pathology were assessed as part of nonclinical GLP-compliant studies in Wistar Han rats and have been published.[30] The clinical pathology findings were consistent with inflammation/immune responses routinely identified in nonclinical vaccine studies, but with some unique features. As with routine nonclinical vaccine studies, most of the clinical pathology changes identified during the dosing phase returned to control levels at the end of the recovery phase.

The magnitude of many mRNA-LNP-related changes was dose-related (A2M, A1AGP, albumin, AG ratio, globulins, and most leukocytes). Reported increases in A2M in female Wistar Han rats 72 hours after administered 30 μg mRNA-LNP COVID-19 vaccine IM was estimated, on average, to be ~ 33x higher than in Wistar Han rats dosed with the full dose of the tetanus toxoid, reduced diphtheria toxoid and acellular pertussis (TDaP) vaccine (Estimated from publications by Reagan et al[29] and Rohde et al[30]). This is consistent with strong immune response to the mRNA-LNP vaccine candidates.[30]

Increased WBC after mRNA-LNP administration in rats was the result of increased neutrophils, monocytes and LUC, and was correlated with increased cellularity in the bone marrow. Similar to acute phase proteins, the magnitude of neutrophil increases (up to 7.8x controls) was greater than generally observed for conventional vaccines. An unexpected finding was the presence of hypersegmented neutrophils on peripheral blood smears of vaccinated animals, reflective of prolonged retention in the circulation.[40] Under physiologic conditions, neutrophils stay in circulation for fewer than 24 hours. Neutrophils that are not consumed at the site of inflammation by macrophages may be cleared by the bone marrow.[24] Therefore, hypersegmented neutrophils may be observed in circulation when there is an excess of neutrophils remaining (and aging) in circulation relative to the number of neutrophils transmigrating to tissues or bone marrow to be cleared.

There were two other unexpected hematology findings: a transiently lower reticulocyte count (to 0.27x, Day 4 only) that was followed by a slight decrease in red blood cell mass (to 0.86x, Day 17 only) and a decrease in platelets (to 0.66x, Day 17) which was only evident at the highest dose 100 μg mRNA-LNP. The reduction in reticulocytes likely reflects suppression of erythropoiesis in favor of myelopoiesis, which is mediated in part by TNFα, interferon-γ (IFNγ), and IL-1β.[41] These cytokines may directly and/or indirectly impact erythropoiesis and erythrocyte clearance. It is also possible that reticulocyte decreases were due to TLR-mediated downregulation, degradation, and endocytosis of ferroportin, limiting iron availability for hemoglobin synthesis resulting in suppression of erythropoiesis.[22] Reticulocyte decreases secondary to inflammation have been reported after administration of endotoxin or heat-killed Brucella abortus.[3,23]

Platelet counts were decreased in animals administered the highest dose of mRNA-LNP vaccine when compared with concurrent controls but were generally still within historical reference intervals and were not associated with changes in coagulation times, evidence of bleeding, or microscopic bone marrow megakaryocyte changes. Decreased platelet counts have been reported in humans after infusion of LPS producing low-grade endotoxemia.[34] Platelets have TLR receptors, and it is hypothesized that activation of these receptors causes platelet decreases.[5]

In conclusion, mRNA-LNP-based COVID-19 vaccines induce an inflammatory/immune response consistent with other non-mRNA-based vaccines when administered to rats. However, the magnitude of the inflammatory response appears to be higher with other more classical vaccine modalities.

Regulatory Considerations With mRNA Modalities—Working Without Guidelines

Dr. Rani Sellers from the Department of Pathology and Laboratory Medicine in the School of Medicine at the University of North Caroline at Chapel Hill closed out the session with a review of the relevant nonclinical guidelines for mRNA modalities and where gaps exist in these guidance documents.

The use of mRNA in prophylactic vaccines against infectious agents has significant potential for rapidly developing vaccines against emerging viruses and seasonal viruses that have antigenic shifts. Similarly, the treatment of virus-associated diseases such as hepatitis B, HIV, and human papillomavirus using therapeutic vaccines holds great promise. Finally, the value of delivering RNAs expressing therapeutic proteins cannot be understated for the treatment of genetic diseases and cancer. The use of mRNA modalities in treating and preventing disease is becoming a reality.

Regulatory guidelines around nonclinical toxicology studies inevitably lag behind emerging technologies—and mRNA modalities (e.g., modified mRNA and self-amplifying mRNA) are no exception. Recently, regulatory authorities have sought to include mRNA vaccines and therapeutics through the generation

Table 1. Nonclinical Regulatory Guidelines/Documents Relevant to mRNA Modalities.

Prophylactic vaccines	
WHO	Evaluation of the quality, safety, and efficacy of messenger RNA vaccines for the prevention of infectious diseases: regulatory considerations, Annex 3, 2022[37]
	Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines, 2013[38]
	Guidelines on the nonclinical evaluation of vaccines, 2005[39]
EMA	Guideline on Vaccine Adjuvants for Human Use (dormant and no longer being updated), 2005[10]
FDA	Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications, 2006[11]
ICH	ICH S5(R3): Detection of Reproductive and Developmental toxicity for Human Pharmaceuticals, 2020[20](does not include prophylactic vaccine exception for male fertility and F1 post-weaning studies [previous versions had a prophylactic vaccine exception])
Therapeutic vaccines	
FDA	Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products 2013[12]
	Considerations for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications, 2006[11]
Gene therapy	
FDA	Human Gene Therapy Products Incorporating Human Genome Editing, Guidance for Industry, 2024[14]
	Human Gene Therapy for Rare Diseases, Guidance for Industry, 2020[13]
	Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products 2013[12]
EMA	Draft Guideline on quality, nonclinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials, 2024[8]
	Guideline on the quality, nonclinical and clinical aspects of gene therapy medicinal products, 2018[9]
ICH	ICH S12: Guideline on nonclinical biodistribution considerations for gene therapy products, Step 5, 2024[21]

Abbreviations: EMA, European Medicines Authority; FDA, Food and Drug Administration; ICH, International Council for Harmonization; WHO, World Health Organization.

of new and revision of old nonclinical guidance documents. However, contradictions between guidelines and between global regulatory authorities exist, leading to challenges in nonclinical safety studies. Regardless of these issues, the basic foundation for nonclinical studies can generally be defined within existing and recently added guidance documents (Table 1).

The regulatory branch of the Food and Drug Administration (FDA) handling mRNA-based modalities is the Center for Biologics Evaluation and Research (CBER). This contrasts with small RNA therapies, such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), which are submitted through the Center of Drug Evaluation and Research (CDER). There are two divisions in CBER: the first is Office of Vaccine Research and Review (OVRR) and the second is the Office of Therapeutic Products (OTP). OVRR evaluates prophylactic vaccines, regardless of modality. OTP evaluates all other biological products such as cell therapies, therapeutic vaccines and proteins, and gene therapies. Nucleic acid-based therapeutic vaccines, including mRNA and self-amplifying mRNA, are not currently considered gene therapy products by the FDA. Therapeutic vaccines incorporate a wide array of targets including against cancer, infectious agents, and endogenous proteins. Interestingly, if you submit a therapeutic vaccine against an infectious agent, it will go to OTP even if it is identical to a prophylactic vaccine submitted to OVRR. These offices may have different expectations on the nonclinical safety studies required for IND submissions. European Medicines Agency (EMA) guidelines related to therapeutic vaccines are not currently available, and it is uncertain if they are under the gene therapy umbrella. RNA-based products that express therapeutic proteins, however, would fall under the gene therapy umbrella for both the FDA and EMA.

Regulatory guidance documents for prophylactic vaccine nonclinical toxicology studies are the most amenable to mRNA modalities, and additional guidelines have been recently published. Most regulatory authorities defer to the World Health Organization (WHO) expectations for nonclinical safety studies for prophylactic vaccine development. These guidelines have effectively replaced FDA and EMA guidelines (see Table 1). However, the 2005 guideline from EMA on adjuvanted vaccines has not been officially replaced by the WHO guideline on adjuvanted vaccines. mRNA vaccines are not currently considered adjuvanted vaccines, thus, this guideline may not be relevant for mRNA-based prophylactic vaccines. In 2022, the WHO has published a guideline specifically on mRNA vaccines and the EMA has also developed a concept paper on quality aspects on mRNA vaccines. These documents are roughly similar in their expectations for nonclinical studies. Thus far, no specific comments on other RNAs (such as self-amplifying or circular RNAs) are included in these documents.

The 2022 WHO Guideline *Evaluation of the Quality, Safety and Efficacy of Messenger RNA Vaccines for the Prevention of Infectious Diseases* clarified some of the regulatory expectations around mRNA vaccine development. Specifically, they reiterated the expectation for nonclinical studies to determine mRNA biodistribution and mRNA expressed protein biodistribution with clearance as well as distribution and elimination

kinetics for carrier materials (e.g., lipids, polymers, etc). The guideline also stated the importance for identifying vaccine components with potential risk in humans (e.g., PEG) and projections on the reactogenicity and clinical SAEs based on the formulation. mRNA products may contain modified nucleosides to reduce activation of pathogen-associated molecular patterns; to date these have been naturally occurring nucleosides. The use of non-naturally occurring nucleoside analogs in mRNA vaccines would require a risk assessment plan. Additionally, any novel materials/lipids included in the formulation must be assessed for systemic toxicity and, on a case-by-case basis, genotoxicity (see ICH S2(R1)).[19]

Importantly, the WHO also clarified that toxicity studies *may not be required* if there are only minor sequence changes to the expressed protein (in response to antigenic variation) when using an existing mRNA-LNP platform (with demonstrated comparability). In these cases, the WHO suggested that some safety endpoints be included in pharmacology studies. However, novel antigens using an existing mRNA-LNP platform technology do require GLP toxicity studies. Safety data from clinically approved traditional vaccine antigens may be leveraged to support the safety of equivalent antigens expressed using an mRNA vaccine platform.

Therapeutic vaccine guidance documents are also relatively adaptable to the mRNA modality, although guidelines have only been published by the FDA. The goal of therapeutic vaccines is to generate an immune response against a specific protein target to treat a disease. These target proteins may be self or non-self. In the realm of anti-cancer vaccines, there are both "one size fits all" and "patient-specific" anti-tumor antigen-based vaccines. A number of therapeutic vaccines are also being developed against infectious agents, such as human papilloma virus, herpes B virus, and HIV, to name a few.[33] Additionally, there are therapeutic vaccines that target self-antigens. These can include therapeutic vaccines to treat asthma (e.g., targeting cytokines and IgE), Alzheimer's disease (e.g. alpha and beta amyloid and tau protein), hypercholesterolemia (e.g., anti-PCSK9), and even nicotine addiction.[25,4,17,15,6]

Therapeutic nucleic acid-based vaccines, at least by the FDA, don't fall under the category of gene therapy, as the administered material has no direct effect on the target (they fall under the category of Cellular Therapies; https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products). The 2013 guidance document, Preclinical Assessment of Investigational Cellular and Gene Therapy Products,[12] covers therapeutic vaccines, and is adaptable to mRNA modalities as there is language that includes non-viral vectored modalities. Biodistribution studies are expected, and genome integrations studies have *generally* not been expected. The EMA has no specific guidelines on therapeutic vaccines. The recent EMA Draft Guideline on Quality, Non-Clinical and Clinical Requirements for Investigational Advanced Therapy Medicinal Products in Clinical Trials[8] is not helpful for understanding EMA expectations for mRNA-based therapeutic vaccines. This guidance defines what is considered a gene therapy product, and specifically excludes "vaccines against infectious organisms" as gene therapy medicinal products. However, there is no reference to "cancer vaccines" or other therapeutic vaccines. Because such vaccines have no direct effect on cell function or gene expression related to a target, it seems like it might not fall under the category of gene therapy. However, wording in the EMA document on what is considered gene therapy may indicate mRNA-based therapeutic vaccines fall into this category. Therefore, specific interactions should be sought with the EMA to understand their expectations for mRNA-based therapeutic vaccines.

mRNA-based therapeutics are categorized as gene therapy by both the FDA and EMA. However, regulatory expectations are not fully aligned between these two regulatory authorities, although updated documents have helped to narrow this gap. Interactions with regulatory authorities early in development are important for defining nonclinical safety study expectations for mRNA-based gene therapy submissions. For mRNA therapeutic products, the FDA Guidance for Industry Preclinical Assessment of Investigational Cellular and Gene Therapy Products includes wording on non-viral vectors, so is relevant to mRNA therapies. This guideline also includes expectations on assessing toxicities due to the components of the final formulation (e.g., liposomes and various excipients/contaminants). While the FDA released a Guidance for Industry in 2020: Human Gene Therapy for Rare Diseases,[13] Expectations for Nonclinical Studies referred the reader back to the 2013 Guidance to Industry noted above. Additionally, in 2024 the EMA released a Draft Guideline on Quality, Nonclinical and Clinical Requirements for Investigational Advanced Therapy Medicinal Products in Clinical Trials—Second version.[8] This document is relevant to mRNA therapeutics, as they mention *nucleic acid sequences for transgene expression*, but do not specifically mention mRNA. This document does acknowledge that if the product persists for a short time in the body without long-lasting effects, safety evaluation can be adapted accordingly. Expectations for biodistribution for gene therapy products were outlined in an update of ICH S12: Guideline on Nonclinical Biodistribution Considerations for Gene Therapy Products, Step 5, and is relevant to RNA products.[21] While not stated in the guidance document, it is presumed that shedding studies need not be included, and that genomic alteration and germline transmission studies are *likely* unnecessary except for genome editing mRNA molecules.

An important specific guidance on gene editing is highly relevant to mRNA modalities was published by the FDA in 2024: Human Gene Therapy Products Incorporating Human Genome Editing, Guidance for Industry.[14] The primary concern with this modality is around on and off-target gene editing, specifically the risk of mutagenicity, carcinogenicity, and alterations in cell maturation/proliferation. For gene-editing products, it is expected that there is "*Verification of off-target sites should be conducted using methods with adequate sensitivity to detect low frequency events."* However, there are no suggestions for what studies are acceptable for this endpoint. These types of

products should include early discussions with regulatory authorities to define nonclinical expectations.

In summary, existing nonclinical regulatory guidelines and documents relevant to mRNA modalities are sufficient for prophylactic vaccines. For therapeutic vaccines, regulatory guidelines from the FDA are sufficient. Submissions of mRNA-based therapeutic vaccines outside of the United States will require regulatory authority consultation, as currently only the FDA has relevant guidance documents. Because gene therapy encompasses many divergent technologies all with different risks, clarity from regulators on expectations for mRNA-based nonclinical safety packages may be needed on a case-by-case basis.

Nonclinical Animal Studies

Nonclinical animal studies were performed under protocols approved by respective individuals' Institutional Care and Use Committee, in accordance with current guidelines for animal welfare.

Acknowledgments

RSS would like to thank Cynthia Rohde, Claudia Lindemann, Doug Donahue, Prashant Nambiar, and Eric Jacquinet for their input on the presentation and review of the write-up of the regulatory expectations for mRNA modalities.

Author Contributions

Rani S. Sellers organized, reviewed, and edited the manuscript and was responsible for the content in Regulatory Considerations with mRNA Modalities section; Lila Ramaiah was responsible for the content in the Clinical Pathology Findings with mRNA Vacine Compared to Relevant Vaccine Adjuvants; Sue-Jean Hong was responsible for the content in the Safety and Immunogenicity of Self-Amplifying RNA Viruses section; Prashant Nabiar was responsible for the content in the samRNA/mRNA Opportunities in Oncology section; Eric Jacquinet was responsible for the content in the Inhaled mRNA Therapy for Cystic Fibrosis section; Shan Naidu reviewed and edited the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Blakney AK, Ip S, Geall AJ. An update on self-amplifying mRNA vaccine development. *Vaccines (Basel)*. 2021;9(2):97.
2. Blakney AK, McKay PF, Bouton CR, Hu K, Samnuan K, Shattock RJ. Innate inhibiting proteins enhance expression and immunogenicity of self-amplifying RNA. *Mol Ther*. 2021;29(3):1174-1185.
3. Brooks MB, Turk JR, Guerrero A, et al. Non-lethal endotoxin injection: a rat model of hypercoagulability. *PLoS ONE*. 2017;12(1):e0169976.
4. Chackerian B, Remaley AT. PCSK9 vaccines: a promising new strategy for the treatment of hypercholesterolemia? *J Lipid Res*. 2024;65(3):100524.
5. Cognasse F, Nguyen KA, Damien P, et al. The inflammatory role of platelets via their TLRs and Siglec receptors. *Front Immunol*. 2015;6:83.
6. Damase TR, Sukhovershin R, Boada C, Taraballi F, Pettigrew RI, Cooke JP. The limitless future of RNA therapeutics. *Front Bioeng Biotechnol*. 2021;9:628137.
7. Donahue DA, Ballesteros C, Maruggi G, et al. Nonclinical safety assessment of lipid nanoparticle-and emulsion-based self-amplifying mRNA vaccines in rats. *Int J Toxicol*. 2023;42(1):37-49.
8. European Medicines Agency. Draft guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials. Published 2024. https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-quality-non-clinical-clinical-requirements-investigational-advanced-therapy-medicinal-products-clinical-trials-second-version_en.pdf
9. European Medicines Agency. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products. Published 2018. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-and-clinical-aspects-gene-therapy-medicinal-products_en.pdf
10. European Medicines Agency. Guideline on vaccine adjuvants for human use. Published 2005. https://www.ema.europa.eu/en/adjuvants-vaccines-human-use-scientific-guideline
11. Food and Drug Administration. Guidance for industry: considerations for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications. Published 2006. https://www.fda.gov/files/vaccines,%20blood%20&%20biologics/published/Guidance-for-Industry--Considerations-for-Developmental-Toxicity-Studies-for-Preventive-and-Therapeutic-Vaccines-for-Infectious-Disease-Indications.pdf
12. Food and Drug Administration. Guidance for industry: preclinical assessment of investigational cellular and gene therapy products. Published 2013. Accessed November 1, 2024. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm
13. Food and Drug Administration. Guidance to industry: human gene therapy for rare diseases. Published 2024. Accessed November 1, 2024. https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics/biologics-guidances
14. Food and Drug Administration. Guidance to industry: human gene therapy products incorporating human genome editing. Published 2024. Accessed November 1, 2024. https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics
15. Fraleigh NL, Lewicky JD, Martel AL, Diaz-Mitoma F, Le HT. Assessing neutralized nicotine distribution using mice vaccinated with the mucosal conjugate nicotine vaccine. *Vaccines (Basel)*. 2021;9(2):118.
16. Geall AJ, Verma A, Otten GR, et al. Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci U S A*. 2012;109(36):14604-14609.
17. Gharailoo Z, Plattner K, Augusto G, Engeroff P, Vogel M, Bachmann MF. Generation of a virus-like particles based vaccine against IgE. *Allergy*. 2024;79(8):2207-2221.
18. Hồ NT, Hughes SG, Ta VT, et al. Safety, immunogenicity and efficacy of the self-amplifying mRNA ARCT-154 COVID-19 vaccine: pooled phase 1, 2, 3a and 3b randomized, controlled trials. *Nat Commun*. 2024;15(1):4081.
19. International Council for Harmonization. Guidance for industry S2(R1) genotoxicity testing and data interpretation for pharmaceuticals intended for human use. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Published 2012. Accessed November 1, 2024. https://www.ema.europa.eu/en/ich-s2-r1-genotoxicity-testing-data-interpretation-pharmaceuticals-intended-human-use-scientific-guideline
20. International Council for Harmonization. ICH S5 (R3) Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals Step 5. International council for harmonisation of technical requirements for pharmaceuticals for human use (ICH). Published 2020. https://www.ema.europa.eu/en/ich-s5-r3-guideline-detection-reproductive-developmental-toxicity-human-pharmaceuticals-scientific-guideline

21. International Council for Harmonization. S12 Guideline on nonclinical biodistribution considerations for gene therapy products. International council for harmonisation of technical requirements for pharmaceuticals for human use (ICH). Published 2023.
22. Kim A, Fung E, Parikh SG, et al. A mouse model of anemia of inflammation: complex pathogenesis with partial dependence on hepcidin. *Blood*. 2014;123(8):1129-1136.
23. Kim A, Fung E, Parikh SG, Gabayan V, Nemeth E, Ganz T. Isocitrate treatment of acute anemia of inflammation in a mouse model. *Blood Cells Mol Dis*. 2016;56(1):31-36.
24. Kraus RF, Gruber MA. Neutrophils-from bone marrow to first-line defense of the innate immune system. *Front Immunol*. 2021;12:767175.
25. Marciani DJ. A retrospective analysis of the Alzheimer's disease vaccine progress—the critical need for new development strategies. *J Neurochem*. 2016;137(5):687-700.
26. Maruggi G, Mallett CP, Westerbeck JW, et al. A self-amplifying mRNA SARS-CoV-2 vaccine candidate induces safe and robust protective immunity in preclinical models. *Mol Ther*. 2022;30(5):1897-1912.
27. McGee JE, Kirsch JR, Kenney D, et al. Complete substitution with modified nucleotides in self-amplifying RNA suppresses the interferon response and increases potency *Nat Biotechnol*. 2024. doi: 10.1038/s41587-024-02306-z.
28. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines—a new era in vaccinology. *Nat Rev Drug Discov*. 2018;17(4):261-279.
29. Reagan WJ, Shoieb AM, Schomaker SJ, Markiewicz VR, Clarke DW, Sellers RS. Evaluation of rat acute phase proteins as inflammatory biomarkers for vaccine nonclinical safety studies. *Toxicol Pathol*. 2020;48(7):845-856.
30. Rohde CM, Lindemann C, Giovanelli M, et al. Toxicological assessments of a pandemic COVID-19 vaccine-demonstrating the suitability of a platform approach for mRNA vaccines. *Vaccines (Basel)*. 2023;11(2):417.
31. Saraf A, Gurjar R, Kaviraj S, et al. An omicron-specific, self-amplifying mRNA booster vaccine for COVID-19: a phase 2/3 randomized trial. *Nat Med*. 2024;30(5):1363-1372.
32. Sellers RS, Nelson K, Bennet B, et al. Scientific and regulatory policy committee points to consider: approaches to the conduct and interpretation of vaccine safety studies for clinical and anatomic pathologists. *Toxicol Pathol*. 2020;48(2):257-276.
33. Son S, Lee K. Development of mRNA vaccines/therapeutics and their delivery system. *Mol Cells*. 2023;46(1):41-47.
34. Stohlawetz P, Folman CC, von dem Borne AE, et al. Effects of endotoxemia on thrombopoiesis in men. *Thromb Haemost*. 1999;81(4):613-617.
35. Stokes A, Pion J, Binazon O, et al. Nonclinical safety assessment of repeated administration and biodistribution of a novel rabies self-amplifying mRNA vaccine in rats. *Regul Toxicol Pharmacol*. 2020;113:104648.
36. Tombácz I, Weissman D, Pardi N. Vaccination with messenger RNA: a promising alternative to DNA vaccination. *Methods Mol Biol*. 2021;2197:13-31.
37. World Health Organization. Evaluation of the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases: regulatory considerations. Vol annex 3, TRS no 1039. Published 2022. Accessed November 1, 2024. https://www.who.int/publications/m/item/annex-3-mRNA-vaccines-trs-no-1039
38. World Health Organization. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines. Vol technical report series no 987, annex 2. Published 2013. Accessed November 1, 2024. https://cdn.who.int/media/docs/default-source/biologicals/vaccine-standardization/trs_987_annex982.pdf?sfvrsn=ea991caca_983&download=true
39. World Health Organization. WHO guidelines on nonclinical evaluation of vaccines. Vol technical report series, no. 927, 2005 annex 1. Published 2005. Accessed November 1, 2024. https://www.who.int/publications/m/item/nonclinical-evaluation-of-vaccines-annex-1-trs-no-927
40. Zabolotzky SM, Walker DB. Peripheral blood smears. *Cowell and Tyler's Diagnostic Cytology and Hematology of the Dog and Cat*. 5th ed. Amsterdam, The Netherlands: Elsevier Mosby; 2020.
41. Zarychanski R, Houston DS. Anemia of chronic disease: a harmful disorder or an adaptive, beneficial response? *CMAJ*. 2008;179(4):333-337.