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From bench to bedside: advancing mRNA therapies for cardiovascular diseases

The mRNA therapeutics field is heavily engaged in extending its reach to new cells, targets, and patient populations, and the opportunities don't come any bigger than those in the cardiovascular diseases area. **David McCall**, Senior Editor, BioInsights, talks to **Ajit Magadum**, Assistant Professor, Department of Cardiovascular Sciences and Aging and Cardiovascular Discovery Center (ACDC), Lewis Katz School of Medicine at Temple University, about his work in identifying novel targets and addressing mRNA targeting specificity to improve patient outcomes.

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Q What are you working on right now?

AM: As a molecular and cellular biologist specializing in cardiovascular biology and diseases, my research primarily focuses on leveraging mRNA technology to address cardiovascular diseases (CVD). CVDs are the leading cause of death globally, with an estimated 19.9 million deaths in 2022 projected to rise to over 23 million by 2030, and there are 607 million people worldwide who are susceptible to them. My work aims to address this significant health challenge.

Over the past decade, I have been exploring various molecular and cellular approaches to identify novel therapeutic targets for CVD using mouse models. By utilizing modified mRNA (modRNA) or mRNA as a gene therapy tool, we aim to enhance cardiac outcomes for patients with cardiovascular conditions. A significant focus of my research is the targeted delivery of mRNA to specific cell types within the heart. A notable development in this area is the Specific Modified mRNA Translation System (SMRTS), which we developed a few years ago. This system allows for precise targeting of both cardiomyocytes and non-cardiomyocytes, and we are currently expanding its application to other cell types within the heart. Furthermore, our research is investigating the potential for systemic delivery of mRNA to target the heart. While we are still in the early stages of this technology, its potential to revolutionize the treatment of CVD is immense.

Q How would you frame both the current state-of the-art and the nature and scale of the opportunity for mRNA therapeutics in the CVD area?

AM: This is an exciting era for CVD therapeutics, especially with the advent of mRNA technology. Despite advances in diagnosis and treatment, outcomes, survival rates, and prognosis for ischemic heart disease and heart failure (HF) patients remain suboptimal. Over the past two decades, a variety of approaches-including proteins, viruses, and small molecules have been tested in preclinical models and clinical trials (Phases 1 and 2) to treat CVDs. However, these methods face significant limitations, such as short half-lives, complicated administration protocols, lack of cell specificity, limitations in combinatorial gene expression, and challenges in delivering intracellular genes like transcription factors. While adeno-associated virus (AAV) has been widely used in cardiovascular research and therapy development, it comes with its own set of challenges, including chromosomal integration, limited gene insertion capacity (4.5 kb), a lengthy period of transgene expression (of more than a year), and potential adverse effects such as cardiac hypertrophy, edema, and arrhythmia. Furthermore, over 60% of the population has AAV neutralizing antibodies, which can impede its effectiveness. Chemical or small molecule-based cardiac repair holds promise, but precise delivery to the heart or specific cells at the desired concentration and timing remains a significant challenge. While transgenic mouse models are invaluable for research, they are not practical for therapeutic applications. Heart transplants, although an option, are severely limited by the scarcity of donor organs. Therefore, there is a substantial unmet need for novel, clinically appropriate therapeutics to repair injured cardiac tissue and reverse pathological remodeling.

modRNA or mRNA gene therapy offers a promising solution, capable of prompt protein translation, controlled and robust yet transient expression patterns, enhanced RNase resistance, and cell-specific mRNA expression in the heart (via SMRTs) without the risk of genomic integration or activation of innate immunity. The VEGF mRNA program (AZD8601), which reached Phase 2 clinical trials sponsored by AstraZeneca and Moderna Therapeutics, was

"The integration of mRNA therapeutics in cardiovascular treatment strategies holds immense promise..."

recently discontinued. However, Moderna Therapeutics' Relaxin (mRNA-0184) program continues in Phase 1 trials. Beyond these clinical efforts, mRNA technology is primarily being utilized in preclinical studies for CVDs in animal models.

The market potential for targeting CVDs is vast. In 2023, the cardiovascular drugs market was valued at approximately US$146.02 billion. The global cost of CVDs was US$863 billion in 2010, projected to exceed US$1 trillion by 2030. The integration of mRNA therapeutics in cardiovascular treatment strategies holds immense promise, offering new avenues for effectively addressing and managing CVDs.

Q Can you go into more depth on your current work in CVD, and specifically, the approaches (both LNP- and non-LNP-specific) that you are taking to enhance cell-specific targeting of mRNA therapeutics?

AM: Our research predominantly employs mouse models of ischemic heart diseases, including acute MI, ischemia/reperfusion, transverse aortic constriction (TAC), and the HF. Currently, we administer modified RNA (modRNA) directly into the myocardium using a sucrose-citrate buffer. However, we are actively exploring LNP-based delivery methods to achieve stable mRNA delivery to the heart.

One of the significant challenges in cardiac gene therapy is achieving targeted delivery of mRNA to specific cell types within the heart. The heart is composed of four to five major cell types essential for cardiovascular function, development, homeostasis, and disease response, making precise targeting crucial. Cardiomyocytes, the primary cell type, suffer extensive death following ischemic injury, leading to HF. Unlike lower organisms like zebrafish and newts, or 1-day-old mice, which can regenerate their hearts through cardiomyocyte proliferation, adult mammalian cardiomyocytes do not proliferate. Therefore, identifying novel genes or molecular pathways to induce cardiomyocyte proliferation is a key focus of our laboratory. Additionally, inducing proliferation of non-cardiomyocytes, such as fibroblasts and immune cells, can be detrimental to cardiac repair. Over the last decade, we have identified approximately four novel genes/molecular pathways capable of inducing cardiomyocyte proliferation and cardiac repair using mRNA approaches. Cardiomyocytes can also be targeted to inhibit apoptosis and oxidative stress, and cardiac hypertrophy post-MI, while fibroblasts can be targeted to inhibit fibrosis, and endothelial cells to induce angiogenesis.

Previously, there was no mRNA-based system capable of delivering genes in a cell-specific manner within the heart. The mRNA does not differentiate between the cells and they are

translated by the cell's translational machinery. This non-selectivity poses challenges, especially in the context of CVD, where targeted cellular processes are crucial for effective treatment. Developing cell-specific mRNA therapeutics could significantly enhance the precision and efficacy of interventions for various cardiac conditions. To address this, we developed the first cardiomyocyte-specific modRNA system. This system uses two distinct modRNA constructs: one encoding the suppressor/repressor protein L7AE regulated by cardiomyocyte-specific microRNA ($_{CMS}$miR) binding sites (miR1 and miR208), and another construct carrying a gene-of-interest modRNA regulated by a kink-turn motif (k motif), a specific binding site for L7AE. This 'suppress the suppressor' approach ensures that the gene-of-interest modRNA is expressed exclusively in cardiomyocytes, sparing other heart cells. SMRTs provide additional benefits by inhibiting detrimental cardiomyocyte-specific microRNAs like miR1 and miR208 following CVDs. We have also expanded the SMRTs approach to specifically target non-cardiomyocytes in the heart. Using this SMRTs approach, we expressed two genes, *Pkm2* and *Lin28a*, exclusively in cardiomyocytes and demonstrated that they induce cardiomyocyte proliferation without affecting other cells. This approach highlights the potential of cell-specific mRNA therapies to precisely target and treat CVD, paving the way for advanced clinical applications.

LNPs are the most widely used delivery system for mRNA therapeutics, protecting mRNA from degradation and facilitating its entry into target cells or organs. While LNPs have been established for cell-specific mRNA delivery in some organs, their application in the cardiovascular system remains limited. Ionizable lipids are known to target specific cells by altering their charge and size and incorporating targeting ligands. We are exploring promising approaches, including functional LNPs with ligands, aptamers, or antibodies that bind to cell-specific receptors on heart cells, facilitating the entry of LNP-mRNA complexes into the cell.

Q As someone with a foot in both camps, so to speak, what are your concerns and expectations regarding the future of LNP-mediated delivery in this particular field?

AM: As a researcher deeply involved in both CVD and mRNA therapeutics, my perspective on the future of LNP-mediated delivery in the cardiovascular system is shaped by both its immense potential and the challenges it presents. Although LNPs are designed to be biocompatible, there remains a risk of immune reactions that can lead to inflammation or adverse effects. This is particularly concerning for chronic and ischemic conditions like CVDs, where repeated dosing might be necessary. Some lipid components can be toxic at higher doses or with prolonged exposure. Therefore, screening and developing non-immunogenic and non-toxic LNPs to target mRNA specifically to the heart is crucial, and AI-driven approaches could play a significant role in achieving this.

Achieving precise targeting of LNPs to the heart, or even specific cardiac cells, while avoiding off-target effects in other tissues is complex. Further research is needed to design novel LNPs that can minimize off-target effects in other organs and cells. Integrating LNP

approaches with SMRTs platform could offer a more specific and versatile method. Continued innovation is expected to yield more sophisticated LNPs with enhanced targeting capabilities. This includes developing LNPs that can effectively target specific cell types within the heart, such as cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells, and immune cells, each with unique receptors and uptake mechanisms. Customizing LNP formulations based on individual patient profiles and disease characteristics can significantly enhance therapeutic outcomes. Personalized approaches will be critical in targeting the diverse cell types in the heart.

There are significant challenges associated with LNPs, such as scaling up production while maintaining consistency, quality, and stability. The cost of producing high-quality LNPs is another hurdle, potentially limiting accessibility and widespread adoption. However, the unmet need for CVD treatments justifies the use of these expensive gene delivery products. As more mRNA-based therapies gain regulatory approval, there will be a clearer path for new cardiovascular applications, including streamlined processes and guidelines from regulatory bodies. Successful clinical trials will validate the safety and efficacy of LNP-mediated mRNA therapies for CVD. While we are in the early stages of clinical implementation for mRNA therapeutics in CVD, a single success will likely spur further investment and innovation in this promising area.

Q How is the nonclinical toolbox evolving to help provide the answers around delivery that the field requires?

AM: We are leveraging a diverse array of tools and technologies across *in vivo*, *in vitro*, and *in silico* platforms to advance the development of mRNA therapeutics for CVD. These tools address critical aspects such as target identification, delivery, efficacy, safety, and targeting specificity. Here is an overview of the key tools and the evolving nonclinical toolbox supporting this field:

For initial proof-of-concept studies, we use mice and rats to understand the biodistribution, efficacy, and safety of mRNA therapeutics and their effects on cardiac function. Models mimicking human myocardial infarction (MI), hypertrophic cardiomyopathy, cardiac fibrosis, ischemic injury, and HF are particularly valuable. Larger animal models, such as pigs and sheep, provide a closer approximation to human cardiovascular physiology and are essential for replicating positive outcomes observed in rodent models. These models are also crucial for testing different delivery approaches for mRNA before transitioning to human clinical trials. We are using many *in vitro* cell culture models, including rodent primary cardiomyocytes, fibroblasts, endothelial cells, and human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes, to study cellular uptake, expression, and the functional impact of mRNA therapeutics. These models optimize mRNA delivery systems and assess cytotoxicity. Additionally, we are looking in 3D culture systems such as cardiac spheroids and organoids, which provide a more physiologically relevant environment compared to traditional 2D cultures.

"An exciting area of research involves developing LNPs with additional target structures and surface modifications..."

Magnetic resonance imaging (MRI) and echocardiography are employed to non-invasively monitor cardiac function and structure in animal models, assessing the therapeutic effects on CVD. Fluorescence and bioluminescence imaging tools are used to study cardiovascular biology and track mRNA distribution and expression *in vivo* with fluorescent reporters and tagged nanoparticles. Various microscopes, PCRs, and histological tools are utilized to analyze the impact of mRNA therapies on cardiovascular function, molecular pathways, gene expression, and cellular processes in cardiac tissues. We integrate genomics, proteomics, and transcriptomics to gain a comprehensive understanding of the molecular and cellular effects of mRNA therapeutics. Multiomics data guide the optimization of therapeutic strategies, assessing efficacy and safety. Different bioinformatics tools are employed for sequence optimization and secondary structure prediction of mRNA to enhance stability and translational efficiency.

Collaborations are underway to screen and identify novel lipids, polymers, and hybrid materials to improve mRNA delivery efficiency and specificity to the heart. Surface modifications of nanoparticles with targeting ligands, such as antibodies or peptides, are being explored to enhance cell-specific delivery and reduce off-target effects. Despite significant advancements, challenges remain in achieving systemic mRNA delivery to the heart, such as via intravenous (IV) or intraperitoneal (IP) routes. Continued innovation in targeting ligands, nanoparticle surface modifications, and the development of non-immunogenic and non-toxic LNPs will be crucial.

By integrating these sophisticated tools, we are enhancing our understanding of delivery mechanisms, optimizing formulations, and predicting therapeutic outcomes. These efforts are primarily focused on rodent models but aim to accelerate the translation of mRNA therapies from bench to bedside, ultimately improving treatment options for CVD.

Q ...And where should further innovations in this regard be targeted, for you?

AM: In order to address existing challenges and maximize the therapeutic potential of mRNA therapeutics and delivery for CVD, there are several key areas for future innovation. One major opportunity lies in achieving cell and organ-specific mRNA expression. An exciting area of research involves developing LNPs with additional target structures and surface modifications, such as ligands, antibodies, or peptides targeting specific receptors on cardiac cells. Leveraging AI-driven approaches to screen and optimize these LNPs can significantly enhance targeting specificity. Additionally, further developing SMRTs for *in situ* regulation of cell-specific mRNA expression, and combining these with LNPs, can maximize therapeutic effects

while minimizing systemic side effects. Enhancing the stability of mRNA, improving cellular uptake, and ensuring efficient endosomal escape through advanced LNP formulations are also critical objectives.

Another essential focus is identifying novel therapeutic targets that can significantly improve cardiac function in CVD. This involves exploring and validating new targets such as functional genes, inducers of cardiomyocyte proliferation and angiogenesis, and inhibitors of cardiomyocyte apoptosis, oxidative stress, cardiac hypertrophy, and fibrosis. Identifying growth factors and reprogramming factors and delivering them in the form of mRNA for CVD, focusing on tissue regeneration and functional repair, are also promising areas of investigation. To identify these novel candidates, we study cardiovascular biology across various conditions, employing molecular and cellular approaches. Techniques include reverse engineering from CVD patient datasets, gene expression analysis, proteomics, extracellular vesicle biology, and multiomics datasets. Overall, our efforts aim to find novel targets and enhance the specificity and efficacy of mRNA therapies for CVD. By fostering the development of innovative treatments that target specific cardiac cells and pathways, we aim to improve patient outcomes while minimizing adverse effects.

Q Can you sum up your vision for the role that mRNA therapeutics will play in the CVD space in future—and what will be some key next steps?

AM: mRNA therapeutics is set to revolutionize the treatment landscape for CVD by providing highly specific, flexible, and efficient methods to target a broad spectrum of pathological processes. In the coming years, it is anticipated that mRNA technology will be extensively employed to overexpress functional genes in the heart, thereby enhancing cardiac function. Identifying novel genes that promote cardiac repair and gaining a deeper understanding of the molecular and cellular mechanisms underlying CVD and its repair are of paramount importance. Moreover, mRNA technology holds the potential to reprogram resident cells into functional cardiomyocytes, aiding in the restoration of heart function in degenerative conditions. This approach can also facilitate the delivery of gene-editing tools like CRISPR/Cas9, enabling precise correction of genetic mutations responsible for hereditary cardiovascular disorders. This capability is particularly significant for personalized medicine, as mRNA therapeutics can be tailored to individual genetic profiles, maximizing efficacy and minimizing adverse effects.

Enhancing targeting mechanisms such as using LNP's or SMRTs-like approaches to ensure mRNA reaches the desired cells and tissues without off-target effects remains a crucial challenge. Success in this area would greatly expand the possibilities for mRNA therapeutic delivery to treat CVD. Exploring systemic delivery of mRNA to target the heart represents a particularly promising avenue, potentially enabling non-invasive treatments for CVD patients. Advancements in these areas in the next few years could significantly transform the field of cardiovascular medicine.

Q Finally, can you highlight one or two of your key goals for your work over the foreseeable future?

AM: As a researcher deeply engaged in the development of mRNA therapeutics for CVD, I have three primary goals for the foreseeable future:

My first goal is to discover and validate novel therapeutic targets that can significantly enhance cardiac function and tissue regeneration, using both mouse and larger animal models. The aim is to find targets that promote cardiomyocyte proliferation, angiogenesis, and tissue repair while mitigating detrimental processes such as apoptosis, oxidative stress, hypertrophy, and fibrosis.

Building on the success of the Specific Modified mRNA Translation Systems (SMRTs) approach for targeting cardiomyocytes (CMs) and non-cardiomyocytes (non-CMs), my second goal is to extend this strategy to other heart cell types such as fibroblasts, endothelial cells, smooth muscle cells, and immune cells. By utilizing mRNA and lipid nanoparticles (LNPs), we can achieve cell-specific modulation, thereby enhancing therapeutic outcomes in CVD. The potential applications of this platform extend beyond CVD, offering possibilities for targeting other organs and diseases with cell-specific mRNA therapies.

Collaborating with other laboratories, my third goal is to design and validate LNPs with various formulations and surface modifications, such as ligands or antibodies, to selectively target the heart without affecting other organs. Additionally, we are developing LNP formulations that enhance mRNA stability, improve cellular uptake, and facilitate endosomal escape. Preclinical studies using rodent and large animal models will assess the biodistribution, targeting efficiency, and therapeutic efficacy of these modified mRNA-LNP complexes.

These goals aim to advance the field of mRNA therapeutics for CVD by enhancing targeting specificity, therapeutic efficacy, and the potential for tissue regeneration, ultimately improving patient outcomes.

BIOGRAPHY

AJIT MAGADUM is an Assistant Professor in the Department of Cardiovascular Sciences and ACDC at the Lewis Katz School of Medicine, Temple University in Philadelphia, PA, USA. He earned his PhD from the Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany in 2014, focusing on the molecular and cellular aspects of cardiovascular diseases (CVD) and repair mechanisms. During his postdoctoral tenure at Mount Sinai, he made significant advancements in developing mRNA delivery systems tailored for the cardiovascular system, utilizing various carriers to ensure robust and sustained mRNA expression within cardiac tissues. In 2016, he developed the first-of-its-kind cell-specific mRNA delivery platform known as Specific Modified mRNA Translation System (SMRTs). This innovation allowed for precise targeting of mRNA expression in either cardiomyocytes or non-cardiomyocytes within the heart, paving the way for cell-specific mRNA therapeutics for CVD. He joined Temple University in 2020 as an Associate Scientist. His groundbreaking research has identified a series of novel genes (five targets) delivered as mRNA to the heart, promoting cardiomyocyte proliferation, cardiac regeneration, angiogenesis, and inhibition of cardiac hypertrophy, oxidative stress, and fibrosis. He has published over 25 research papers

n peer-reviewed journals and has successfully filed three patents, which have been licensed to leading biotechnology companies. In recognition of his contributions, he received the Outstanding Research Innovation Award from Mount Sinai Hospital, New York, in 2017 for his work on mRNA therapeutics for CVD. In 2022, he was honored with the ISHR-NAS Young Investigator Award (runner-up) and the Melvin L Marcus Early Career Investigator Award from the American Heart Association (AHA; finalist) in 2022. Currently, his research focuses on identifying novel targets and leveraging mRNA and cell-specific mRNA as innovative therapeutic modalities to target CVD.

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NUCLEIC ACID INSIGHTS

mRNA: PRECLINICAL AND TRANSLATIONAL TOOLS

SPOTLIGHT

COMMENTARY

Impact of pharmacometrics in advancing mRNA therapeutics and vaccines

Husain Attarwala

The article discusses the role of pharmacometrics in the development of mRNA therapeutics, emphasizing its significance in informing dose decisions and guiding clinical study designs through mathematical modeling and simulations. It highlights how pharmacometric modeling aids in predicting doses for first-in-human clinical studies based on preclinical data, with case studies illustrating the predictive power of these models. Additionally, the application of pharmacometric modeling in vaccine development is discussed, notably with immunostimulatory/immunodynamic models, showcasing how these models help in successfully predicting clinical responses, thereby guiding dose decisions during clinical development. As the field of mRNA medicines continues to evolve and expand, pharmacometric methodologies are poised to play an increasingly influential role in their clinical development, leveraging data-driven, model-informed strategies.

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Pharmacometrics is a vital field that integrates mathematical modeling and simulations to explore and predict drug behavior in biological systems. This interdisciplinary approach focuses on understanding pharmacokinetics, the processes by which a drug is absorbed, distributed, metabolized, and eliminated (ADME) in the body, and pharmacodynamics, which relates drug concentration to its therapeutic effect. Through the integration of data from diverse sources, including preclinical studies and clinical trials,

pharmacometrics provides a comprehensive understanding of a drug's efficacy and safety profile [1].

This discipline plays a crucial role in drug development, facilitating a transition from empirical observations to quantitative insights. By applying sophisticated mathematical models to drug data, pharmacometricians can help predict dose-exposure-response relationships across various patient populations. This predictive ability aids in optimizing drug dosing, minimizing adverse effects, and enhancing drug efficacy [2].

Pharmaceutical and biotechnological companies are investing significantly in pharmacometrics to refine their understanding of drug mechanisms and to develop therapies that are more effective and safer for specific patient groups [3]. This article aims to highlight the methodologies utilized in pharmacometrics in the development of mRNA therapeutics and vaccines, demonstrating their impactful role during their development.

PHARMACOMETRICS FOR LNP-BASED mRNA THERAPEUTICS

Lipid nanoparticles (LNPs) have emerged as an effective delivery solution for mRNA therapeutics and vaccines. Given the novel nature of LNP-based delivery [4], pharmacometric models must be adapted or developed anew to account for the unique ADME characteristics and biological interactions of these systems. The pharmacokinetics (PK) of mRNA therapeutics encapsulated in LNPs involve the evaluation of various components, including the encapsulated mRNA, the ionizable lipid that facilitates endosomal escape, and the polyethylene glycol (PEG) that stabilizes the nanoparticle [5–7]. Each component plays a crucial role in the delivery and effectiveness of the therapeutic mRNA. mRNA delivery to target cell types leads to expression of the encoded protein, which then drives the intended downstream pharmacology. In developing PK/PD models, mRNA PK is linked to dynamics of expressed protein and other biomarkers to help build dose-exposure-response relationships.

Translational pharmacokinetic/pharmacodynamic (PK/PD) models are powerful tools in drug development. They mathematically represent the complex interplay between PK and PD of a drug. These models incorporate knowledge of the drug's mechanism of action, along with experimental data, to describe how drug levels translate into therapeutic responses mathematically using algebraic and ordinary differential equations.

A key challenge in drug development is successfully translating findings from animal studies to humans. Differences in physiology and body size can significantly impact drug behavior. PK/PD models often utilize allometric scaling to address this challenge. Allometric scaling involves mathematical adjustments based on body size to predict how drug exposure and effects might change across species [8].

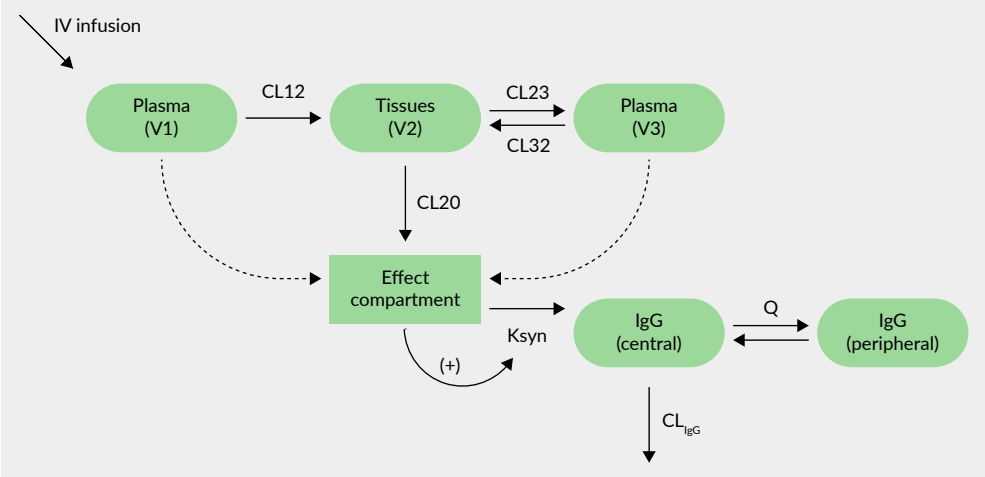
Translational PK/PD models have wide-ranging applications in drug development. They can be used to understand a drug's mechanism of action, simulate different dosing scenarios, and most importantly, inform dose selection for clinical trials. By integrating preclinical data and mathematical principles, these models support the design of safe and effective human studies, accelerating the path towards new treatment options [9,10].

The development of a translational PK/PD model for mRNA-3927 [9], aimed at treating propionic acidemia, exemplifies a comprehensive approach to guiding first-in-human (FIH) dose selection. This model was constructed using preclinical data from mice, rats, and cynomolgus monkeys, showcasing the integration of mRNA PK data and protein PD responses.

The PK component of the model incorporated the kinetics of PCCA and PCCB mRNAs. The semi-mechanistic PK model developed for PCCA/B mRNA incorporated distribution and redistribution components, suggesting that the mRNA moves beyond the bloodstream into tissues and then partially

FIGURE 1

Semi-mechanistic PK/PD model for mRNA-1944 [11].



returns to circulation. This pattern is consistent with the observed delayed peak plasma mRNA concentration several hours after administration in non-human primates. The PK model with the inclusion of these distribution and redistribution components adequately captured the observed time-course of PCCA/B mRNA.

The PD component included dynamics of the protein encoded by the administered mRNA. This included quantifying the hepatic production of the PCC protein and assessing its impact on primary disease markers like 2-methyl citrate, 3-hydroxypropionate, and the propionyl carnitine to acetyl carnitine ratio (C3/C2). The PD model aimed to establish a quantitative link between mRNA levels and the subsequent protein expression and its pharmacological effects.

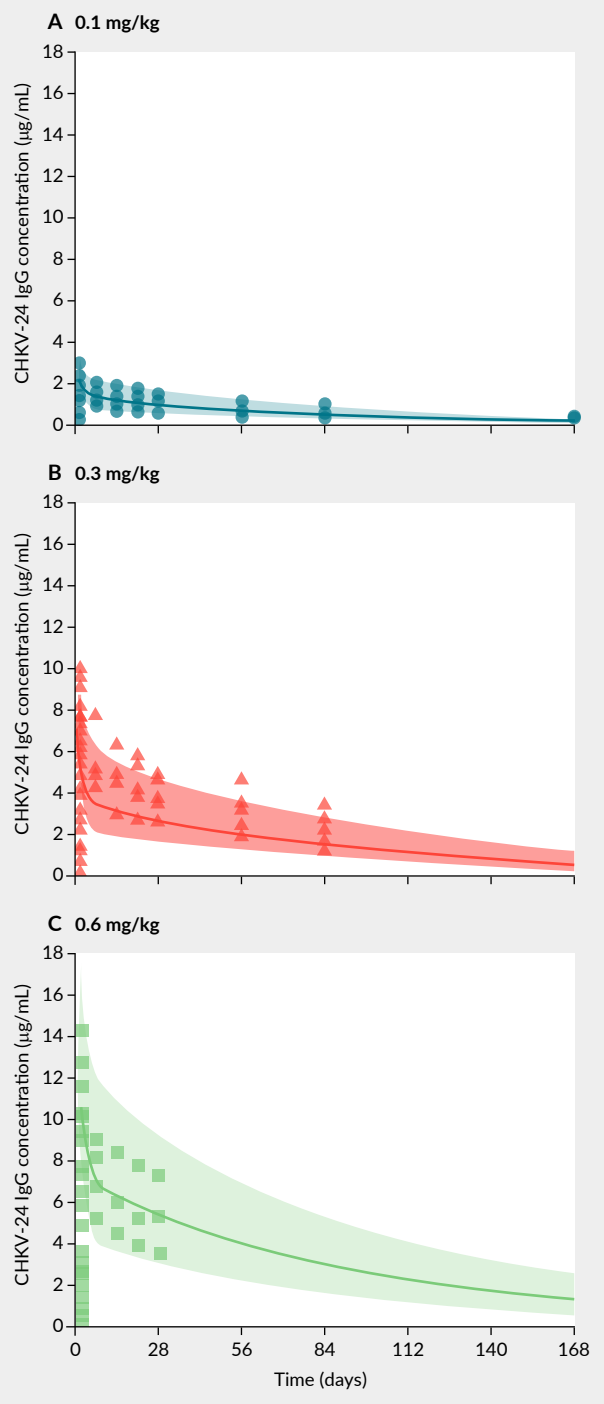
The integration of PK and PD components into a semi-mechanistic model provided a comprehensive framework to predict how mRNA-3927 behaves in the body and its impact on disease biomarkers. This model facilitated the prediction of dose-response relationships in humans, via allometric scaling of model parameters, guiding the selection of an appropriate dose range for the Phase 1 clinical trial [9].

Importantly, the model incorporated effects of the expressed protein within the context of propionic acidemia's disease biology in pediatric patients using data from adult animals. This integration was pivotal in predicting how mRNA-3927 could modulate disease-specific biomarkers in the target patient population and, by extension, the potential clinical biomarker outcomes in the target patient population. By employing this translational modeling approach, the clinical research team could make informed decisions regarding the FIH dose of mRNA-3927, optimizing the chances of therapeutic success while maintaining safety. This methodical process illustrates the critical role of translational PK/PD modeling in the development of novel therapeutics, particularly in the growing field of mRNA-based treatments [9].

The translational PK/PD modeling for mRNA-1944 [11] severs as another case example that involved integrating preclinical data from rats and monkeys to predict human responses. This semi-mechanistic model (Figure 1) was developed using the PK of mRNA encoding heavy and light chain of an antibody against chikungunya virus (CHKV-Ab) and the dynamics of the expressed protein—CHKV-Ab. Allometric exponents [8], which help scale PK and PD parameters based on body weight differences among species, were estimated for mRNA

FIGURE 2

Observed (symbols) and predicted CHKV-24 IgG in humans [11].



Solid line: median predicted; shaded area: 90% prediciton interval; shapes: individual observations.

and protein concentration-time profiles using data fitted across rats and monkeys.

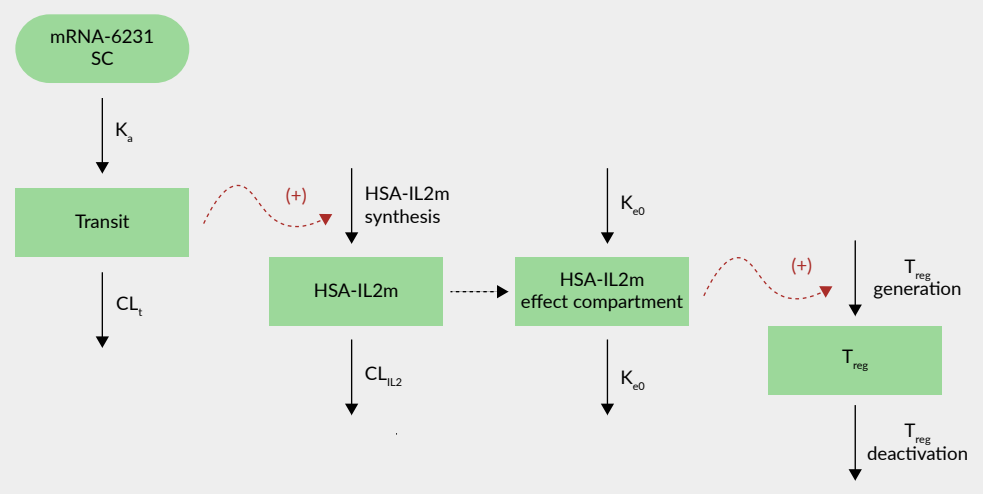
These estimated allometric exponents were then applied to scale the model to humans. This method allowed for the prediction of protein expression levels in humans *a priori*. When actual clinical data were available, they corroborated well with predictions from the model developed using animal data (Figure 2). The successful prediction of these clinical responses highlighted the utility of translational modeling in pre-emptively determining clinical outcomes based on preclinical data. This approach helps provide valuable insights that aids the clinical team in designing efficient clinical studies by enabling the selection of appropriate dose levels based on model informed pharmacology.

The application of MIDD and first-in-human translation has been exemplified through the development of mRNA-6231 [12]. This investigational therapy, designed to enhance Treg function, utilized a semi-mechanistic kinetic-pharmacodynamic model to connect subcutaneous dosing with Treg expansion in nonhuman primates (Figure 3). By employing allometric scaling, the model successfully predicted human pharmacodynamic responses, guiding dose selection for the FIH clinical study. The subsequent validation of these predictions (Figure 4) in the phase I trial underscores the critical role of MIDD in optimizing therapeutic strategies and enhancing the safety and efficacy of mRNA-based treatments. This robust modeling approach not only accelerates the drug development process but also ensures a higher probability of clinical success by leveraging detailed preclinical data modeling and simulations.

HSA-IL2m, human interleukin 2 mutein fused to human serum albumin, measured in plasma; SC, subcutaneous administration; Ka, absorption rate constant; CLt, clearance of mRNA-6231 from the transit compartment; HSA-IL2m synthesis, rate of synthesis of HSA-IL2m recombinant protein; CLIL2, clearance of HSA-IL2m; ke0, effect compartment rate constant; KPD, kinetic-pharmacodynamic; Treg, regulatory T measured in peripheral blood mononuclear cells.

FIGURE 3

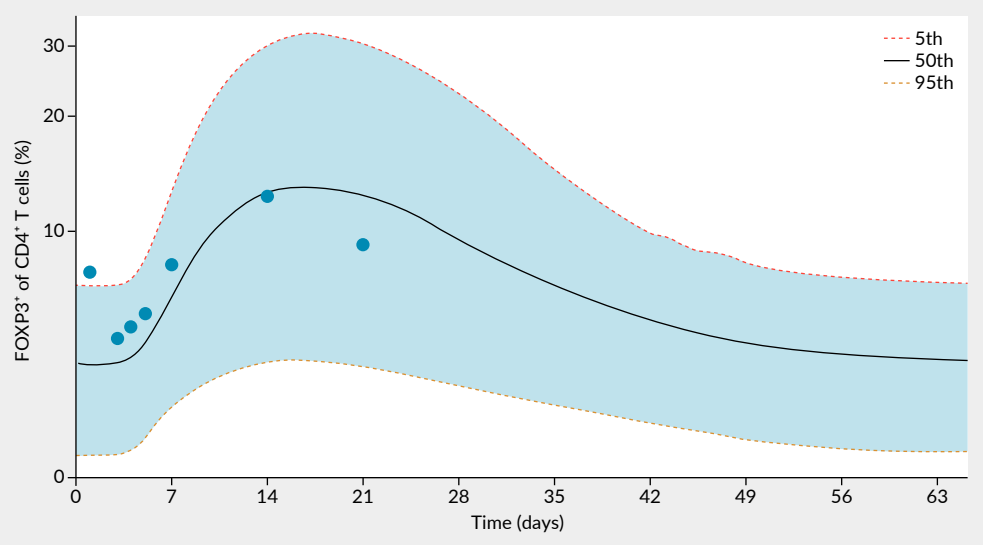
KPD model of mRNA-6231 in nonhuman primates.



CLIL2: Clearance of HSA-IL2m; CLt: Clearance of mRNA-6231 from the transit compartment; HSA-IL2m: Human interleukin 2 mutein fused to human serum albumin, measured in plasma; HSA-IL2m synthesis: Rate of synthesis of HSA-IL2m recombinant protein; Ka: Absorption rate constant; ke0: Effect compartment rate constant; KPD: Kinetic-pharmacodynamic; SC: Subcutaneous administration; Treg: Regulatory T measured in peripheral blood mononuclear cells.

FIGURE 4

External validation of human predictions.



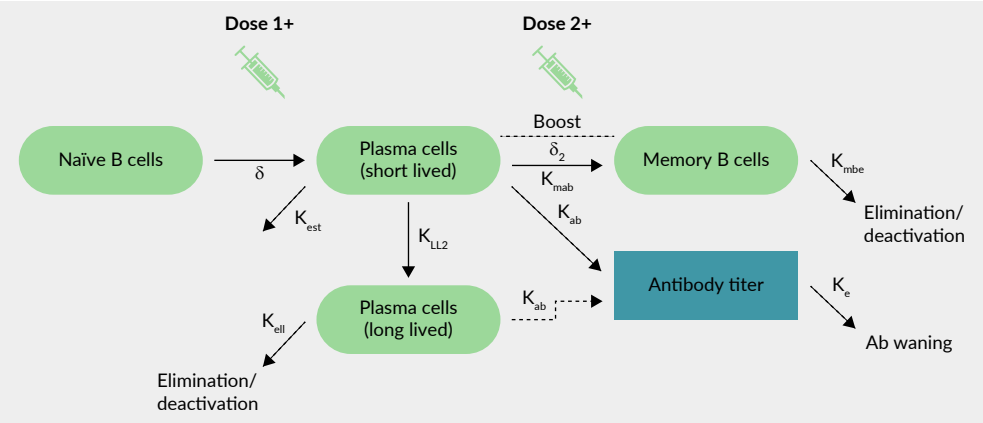
Predicted (lines and shaded area) and observed (blue datapoints) percentage of Treg profiles in humans.

PHARMACOMETRICS FOR VACCINES

Unlike drug development for therapeutics, vaccine development has not widely adopted pharmacometric or PK/PD model-based methods for dose decision-making during clinical development [13]. Implementing similar quantitative approaches in vaccine development, could significantly enhance the

FIGURE 5

Schematic representation of IS/ID model [15].



translation of dose-response data from animal models to humans, thereby accelerating vaccine development [14].

The immunostimulatory (IS)/immunodynamic (ID) model (IS/ID), akin to PK/PD modeling, employs mathematical models to delineate the mechanisms of immune response stimulation (IS) and the ensuing immune response dynamics (ID) post-vaccination. Although mathematical models have been developed that represent immune responses to infection and vaccination, they have not yet been widely integrated into a framework for vaccine dose prediction informing predictions during their clinical development. Utilizing the IS/ID model to describe and predict vaccine immune response data can help inform clinical dose selection for vaccines during clinical development.

In a pioneering effort to advance vaccine dose selection, an immunostimulatory/immunodynamic (IS/ID) semi-mechanistic mathematical model (Figure 5) was developed, adapted from model developed by Rhodes *et al.* to inform the Phase 3 dose for the investigational mRNA-based cytomegalovirus (CMV) vaccine, mRNA-1647 [15].

Utilizing data from the Phase 1 clinical study, the model predicted responses at doses evaluated in the Phase 2 study, aligning well with the observed data upon their availability. This success highlighted the model's capability to support clinical predictions and guide dose decisions. Moreover, the model was employed to simulate neutralizing antibody (nAb) responses at various dose levels, aiding in the prediction of scenarios not evaluated in clinical trials such as varying dose levels and dosing intervals; thereby serving as a mathematical tool for dose-response prediction.

Building on this foundation, a groundbreaking study focused on optimizing pediatric vaccine dose selection—utilized a similar IS/ID model, this time for the mRNA-1273 COVID-19 vaccine. By quantitatively characterizing nAb responses from three previous clinical trials, the model successfully predicted that a 25 μg dose would be most suitable for young children and infants. This prediction was later validated by the outcomes of pediatric clinical trials, reinforcing the model's utility in enhancing dose precision, especially for special populations [15].

TRANSLATIONAL INSIGHT

Pharmacometrics plays a transformative role in drug development. This discipline uses mathematical modeling to analyze complex data, providing insights into drug behavior, dosing, and how drugs interact with the body. Pharmacometrics is rapidly becoming indispensable for developing innovative

mRNA-based therapeutics and vaccines, where understanding intricate delivery mechanisms and immune responses is crucial.

In mRNA therapeutics, pharmacometrics tackles the complexities of LNP based delivery systems, predicting the behavior of the drug in relation to disease biology. This is critical for understanding dose-response relationships. Translational PK/PD models bridge preclinical and clinical studies, enabling researchers to project how drugs will perform in humans. This powerful tool guides early dose selection for first-in-human trials, promoting data-driven decision-making, safety, and efficiency in the development process.

Pharmacometrics is emerging as an important discipline in vaccine development. Immunostimulatory/immunodynamic models mathematically simulate how vaccines trigger immune responses. This allows scientists to predict immunogenicity and safety outcomes at different dose levels and schedules for investigational vaccines, including across diverse population subgroups. IS/ID modeling has successfully aided in Phase 3 dose selection for an mRNA-based vaccines and optimized the dose of an mRNA COVID-19 vaccine for children, demonstrating the potential of pharmacometrics to improve vaccine development.

As the field of mRNA-based therapeutics and vaccines continues to expand, pharmacometrics will play an increasingly vital role. By integrating sophisticated modeling approaches with biological knowledge, pharmacometrics enables researchers to unlock the full potential of these groundbreaking treatments. This data-driven approach holds the key to optimizing drug delivery, dosing strategies, and the prediction of both safety and efficacy outcomes, eventually leading to more optimized and effective therapies.

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NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: STABILITY

SPOTLIGHT

INTERVIEW

Delving into the unknowns of mRNA-LNP formulation and characterization

As nanoparticle-based delivery systems continue to gain traction in the nucleic acids space, the need to address lingering issues, such as stability, becomes ever more pressing. **David McCall**, Senior Editor, BioInsights, asks **Ramesh Marasini**, Scientist, Lead, mRNA–LNP Drug Product Development with CAMRIS International LLC at the Pilot Bioproduction Facility, Walter Reed Army Institute of Research (WRAIR), for his thoughts on the key knowledge gaps threatening to impede the continued growth of the field.

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Q Tell us about yourself and your current work

RM: My degree is in organic chemistry, specializing in nanomedicine. My PhD research focused on designing and developing novel drug delivery technologies using proteins, peptides, biomimetics, liposomes, and polymer-liquid hybrid nanoparticles for intracellular delivery of nucleic acids, small molecules, and diagnostic agents.

After completing my postdoc at Johns Hopkins University School of Medicine, I joined Advanced RNA Vaccine Technologies (ARV Technologies, Inc.), where I worked on designing novel ionizable lipid candidates for targeted delivery, developing novel LNPs formulations involving single or multiple nucleic acid cargos, and comprehensive characterization of formulations [1]. I also learned about immunogen design and contributed significantly to the production of multiple nucleic acid cargo constructs from plasmid generation through *in vitro* transcription and their downstream selection. Additionally, I played a key role, as an organic chemist, in generating intellectual property around ionizable lipids, formulation recipes, and targeted delivery systems.

During my time at ARV Tech, while working in the tight-knit and scientifically stimulating team, I helped establish a pipeline that included vaccine candidates against herpes simplex virus, and COVID-19—the latter at the peak of the pandemic. However, due to fundraising challenges for IND applications, I transition to my current position with CAMRIS International LLC as an mRNA scientist and lead for developing mRNA vaccine manufacturing at the Walter Reed Army Institute of Research (WRAIR).

At WRAIR, I work with a team to translate our R&D work into GMP-compliant processes for early-phase vaccine trials. We have a GMP facility, the Pilot Bioproduction Facility, inside WRAIR for early-phase development of vaccines. We are working on mRNA vaccines for HIV, dengue, and malaria. Additionally, we have established processes and are known for producing traditional vaccine candidates against HIV, malaria, dengue virus, and other infectious diseases of military relevance. Recently, we have also added further newer vaccine candidates using self-amplifying mRNA to our pipeline. Additionally, we support government agencies and private partners alike through cooperative research and development agreements (CRADA) offering our expertise and GMP capacity to translate their technologies.

Q You recently Chaired a conference workshop around the status and challenges of LNP formulation and manufacturing—what were your take-homes from that meeting regarding the current state-of-art of LNPs utilized in nucleic acid delivery?

RM: I led a pre-conference workshop in Boston titled 'Current Challenges with Process Development and LNP Drug Product Manufacturing'. Over the course of the conference, a lot of issues surrounding mRNA-LNPs developed as vaccines and therapeutics against various indications were covered. The key messages from these discussions could be categorized into a few sections.

Firstly, there is the selection of quality critical raw materials. You have plasmid DNA, which is required as a template for the mRNA *in vitro* transcription. There are also enzymes, such as the T7 polymerase required to transcribe mRNA during the *in vitro* transcription process. Then there are the lipids; the primary lipid being ionizable lipid, and the other helper lipids and PEGylated lipid. Important considerations include quality, impurities, solubility of lipids, phase transition of lipids, and thermostability of lipids.

"...the question of how these blebs relates specifically to the final potency is largely unanswered. There is still work to be done to understand the structural impact.

Another key issue discussed was the lack of a uniform approach or consensus among the key players for in-process monitoring—how to correctly trace process-related impurities, how to characterize them, and whether we have the necessary tools to monitor processes in real time. Additionally, some discussion was focused on how to make formulations more stable, maintain quality during processing, especially from encapsulation to tangential flow filtration and fill-finish, and design to deliver where they are intended to go—for instance, liver versus lungs, or cell-specific delivery. On the side, intellectual property constraints in both the mRNA and LNP spaces were also touched upon.

There was a lot of debate on the selection of buffers and whether this has a direct impact on the oxidation of lipids—especially the ionizable lipid—leading to adduct formation that lowers the potency as well as stability in different storage conditions, for example, during freeze-thaw cycles. The question of what issues this might cause in terms of the stability of the LNP as a structure, or the stability of mRNA as the API, was raised.

Another topic of discussion was different structures or morphologies—for example, 'bleb' structures reported in the literature. The question arose of whether these blebs are good or bad, and how they correlate with potency. The impact of empty LNP versus encapsulated LNP on potency was also considered. There was a debate on whether these characteristics should be considered critical quality attributes (CQAs).

There was unanimous agreement among the community that there is a need for continuous manufacturing to enhance robustness and controllability from the manufacturing standpoint. However, the manufacturing of LNPs is always challenging. One particular issue is the scaling of LNPs utilizing different mixing technologies, such as T-mixing, Y-mixing, or impingement jet mixing (IJM), and the lack of in-process monitoring technologies. Some of the shared experiences showed that whatever is claimed and marketed by the original equipment manufacturers (OEMs), such as scaling-up capabilities, is often not accurate.

Finally, there was much discussion around the pressing need for guidance from the major regulatory authorities such as the US FDA and EMA. This field is in a very fluid stage of development, and we are still waiting to see what emerges in terms of more comprehensive guidance from regulatory agencies.

Q Can you go deeper into the specific challenges relating to the stability of nanoparticle-based drug products?

RM: This is a key question and a significant topic of debate within the scientific community, which is frequently discussed in literature, and of course, throughout the recent

conference. When we talk about stability, does that mean the stability of the entire drug product, or stability of just the lipid nanoparticle morphology, or just the stability of the API alone? If stability issues result in problems such as structural impurities, how do we characterize and address these?

Going one step further, what impact do these morphological characteristics such as blebs have on the mRNA integrity and leakage from LNP structures? If they have a huge impact, then why don't we consider them as CQAs themselves? On the other hand, regarding process control, the adage 'process is the product' underscores the importance of minimizing or eliminating some of the byproducts that can arise during the manufacturing process and/or from storage conditions. There was a very good publication showing that the choice of buffer—PBS versus Tris-HCl, for instance—may result in major variations in adduct formation [2]. The adduct here is the oxidation of the ionizable lipid, and dramatic changes in the oxidation of the ionizable lipid affect the structural integrity and hence, the potency of the final drug product.

Considering stability as a broader category should involve evaluating critical factors like critical raw material sources, structural elements of the cargo, manufacturing process, storage conditions, and transportation logistics.

As mRNA also tends to hydrolyze by breaking its phosphodiester bond, how does that correlate with the breaking or oxidation of the lipids, and leakage of the mRNA during the structural changes in the LNP? As a part of stability evaluation, how do we account for the intramolecular base pairing of the mRNA or higher-order structures?

We don't have sufficient data, at least in the public domain, to show we are keeping track of the higher-order structures of the mRNAs, such as hairpin structures, during the storage or freeze/thaw cycle. How does that impact the final potency of the drug product? We are still trying to figure out the right assays to measure and understand the mechanisms behind the stability of the mRNA drug product itself.

Q What are some promising approaches towards alleviating this particular issue, for you?

RM: Regarding stability, the use of one technique to understand product stability does not fit all and does not provide all of the information. It requires multiple orthogonal techniques for comprehensive characterization. Some of these techniques and their interpretation of the results from bulk LNPs are still fluid or likely to be misinterpreted. For example, the use of dynamic light scattering for particle size and distribution monitoring, and cryo-electron microscopy (cryo-EM) to monitor bleb formation after different storage conditions or thaw cycles. Again, the question of how this bleb formation relates specifically to the final potency is largely unanswered. There is still work to be done to understand the structural impact.

For in-process stability control, we need to know what the limits are for impurities. The setting of these specifications is crucial and needs to be both phase-appropriate and based on the specific application. Then you need functional testing—if you know that a given impurity is there,

"I think the future lies in more targeted, more tissue-specific delivery. We know that LNPs primarily target and deposit in the liver—we now need to tune them in tissue-specific or cell-specific ways."

then you need to prove that it does or does not have an impact safety of the final drug product. At the same time, for formulation using different mixing technologies, you need to know what a good size is, and what your size cut-off should be. There are some technologies available—for example, in-line size measurement—although they are not real-time. Nevertheless, it is very helpful to keep track of the size measurement as a part of the formulation process.

I also mentioned above the importance of buffer selection to avoid issues with final drug product stability. Again, it is important to understand this early in the program. Carrying out some short-term stability studies to aid in the selection of the right buffer will help to avoid this problem. Finally, it is crucial to identify the proper assays to characterize impurities during the process development work, or even during R&D.

As a community, we should establish quality systems from the beginning, and consider commercialization of products from early on—in the lead identification process, ideally. This enables the implementation of quality systems for the raw materials, like DNA, enzymes, and lipids, and reevaluating our approach to process development and R&D work. For example, efforts should focus on integrating more aseptic processes, implementing periodic monitoring of endonuclease activity, monitoring endotoxin levels in lipids, and enzymes, use of environmentally friendly solvent systems, and incorporating scalable purification systems like columns or small-scale tangential flow filtration (TFF) systems. I believe these proactive measures will help ensure consistency, quality, and scalability as products progress toward commercialization.

The list is long and not to forget, we must ensure we have enough people well-trained to handle the GMP environment. This is an evolving field, and not everyone in the community will have all of the expertise that is needed. Early engagement of manufacturing personnel will help to minimize some of the manufacturing issues.

Q As someone who is intimately involved in designing and building manufacturing processes and accompanying analytical toolkits for mRNA production, what for you have been some key recent advances in terms of enabling tools/equipment? And where is further innovation most pressingly needed in this regard?

RM: As we build capacity in this expanding field, we need to implement better orthogonal testing for both drug substance and drug product in a phase-appropriate manner, with,

of course, regulatory requirements becoming increasingly more stringent as one progresses from Phase 1 to Phase 3 and on to commercialization. For example, in the case of drug substance, we need more characterization tools for robust process understanding such as capillary gel electrophoresis, liquid chromatography (HPLC) systems, ELISA kits, and immunoblotting systems There is also the question of evaluating potency based on cell-based versus cell-free assays.

On the other hand, for final drug products, there are multiple ways to do encapsulation and quantitation of the mRNA such as RiboGreen assay or PCR-based assays, and each has its pros and cons. For the identification and concentration of lipids, liquid chromatography with charged aerosol detection (LC-CAD) is the go-to option. However, it would be nice to have liquid chromatography-mass spectrometry (LC-MS) for that process as well.

Optimizing the manufacturing process for mRNA products with the use of modular technologies to rapidly scale up/scale out, a closed continuous flow process that reduces manual actions, and the use of disposable technologies in a fully closed system will provide increased flexibility and efficiency which ultimately help to reduce cost and time of manufacturing, improvement in process reproducibility, and help to optimize qualified workforce occupation. To achieve the aforementioned streamline of the manufacturing process, I certainly see that there are emerging trends such as continuous-flow and end-to-end GMP manufacturing from *in vitro* transcription of RNA and its purification followed by the encapsulation into LNP are approaching the market. The integrated analytics for real-time in-process monitoring and seamless scaling from R&D quantities to commercial needs in a small and more economical footprint are exciting developments. Additionally, high throughput systems for screening LNP formulation to GMP production in a single technology with a modular footprint are coming to the market. All of these evolving technologies will help the revolutionary mRNA medicine for better reach from mass vaccination needs to therapeutics for many indications and personalized medicines that require small-scale production technologies.

As an institute, we are keeping track of the evolving technology in this exciting field and trying to accommodate newer tools and techniques in our workflow whenever feasible.

Q What is your vision for the future of nucleic acid medicines—particularly in terms of evolution in the formulation and delivery components?

RM: I think the future lies in more targeted, more tissue-specific delivery. We know that LNPs primarily target and deposit in the liver—we now need to tune them in tissue-specific or cell-specific ways.

Gaining access to proprietary lipids and equipment will also be key in continuing to grow the field. Governments or even some regulatory agencies can work to ease this situation, as was seen recently in the COVID-19 pandemic.

There is also a need for novel thermostable delivery components, as this greatly limits the equitable distribution of these important and highly effective medicines. Millions, if not billions, of people do not yet have access to the COVID-19 vaccines, because of inequitable distribution of vaccines caused in large part by cold chain requirements. Developing countries that don't have proper storage capacity at ultra-low temperatures are lagging far behind in accessing these life-saving medicines. As a scientific community, we must work together to address this issue.

Q Finally, can you share one or two key priorities, both for yourself in your own role and for the Walter Reed Army Institute of Research as a whole in the nucleic acid therapeutics space, over the foreseeable future?

RM: We will continue to work on more targeted delivery, and on driving broader availability of information. We will also be working on developing more thermostable delivery systems, especially for lipids, and on preparing for potential future epidemics or pandemics. At the same time, we keep advancing the lipid adjuvant system for many vaccine candidates for better efficacy and protection against many infectious diseases of military relevance.

Overall, we want to continue to develop our platform technologies in the mRNA space. We aim to contain potential pathogens quickly and to achieve vaccine turnaround than was the case with COVID-19, while also expanding mRNA technology to treat many infectious diseases of military relevance.

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RAMESH MARASINI is a Process Development Scientist and Project Lead for the mRNA program at the Pilot Bioproduction Facility, Walter Reed Army Institute of Research, Silver Spring, MD, USA. He completed his PhD in Chemistry at Kansas State University, Manhattan, KS, USA and subsequently held a postdoctoral research fellowship at Johns Hopkins University School of Medicine. Dr Marasini then joined ARV Tech, Inc. as a Scientist, where he significantly contributed to the development of novel ionizable lipid candidates and optimized tissue-specific LNP formulations for RNA delivery. His research and expertise focus on mRNA–LNP drug product development, bioprocessing, vaccine manufacturing, and

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