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COMMENTARY

Self-amplifying RNA—opportunities and challenges

Rachel Groppo

Messenger (m)RNA vaccines have made great strides in the past 5 years, highlighted by the rapid development of effective vaccines against SARS-CoV-2. mRNA vaccines have distinct advantages over other vaccine platforms, including modular design, rapid development, cell free manufacture, and the ability to express antigen genes of interest *in situ* resulting in efficacious immunogenicity, including for traditionally challenging membrane proteins [1]. The insights and technologies behind mRNA vaccines are now expanding to additional platform technologies, including other large RNA modalities such as self-amplifying RNA (saRNA). The first saRNA vaccine human drug product was recently approved, demonstrating the progress of this platform for clinical applications [2,3]. This commentary will focus on design and performance differences between conventional mRNA, as a reference point, and saRNA. Current challenges will be highlighted along with ongoing areas of improvement including saRNA design, production, and innate activation.

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INTRODUCTION

Conventional mRNA vaccines consist of a single-stranded RNA molecule that encodes a gene of interest (GOI) for a target infectious disease antigen. The GOI is translated to a functional protein after the RNA is introduced into a cell, resulting in antigen expression to drive immunogenic responses. Aspects of the design and manufacture of a mRNA impact the ability to produce the protein of interest. This includes recognition of the 5′ cap structure and the poly(A) tail at the 3′ end of mRNA by cellular ribosome complexes, enabling translation of mRNA molecules and production of the antigen protein. Additionally, the 5′ and 3′ untranslated region (UTR) sequence can impact RNA secondary structure, interactions with other regulatory proteins/elements, and overall

stability of mRNA [4]. Self-amplifying RNAs also consist of a single-stranded RNA molecule, including a 5′ cap, 5′ and 3′ UTRs, and a poly(A) tail [5,6]. However, saRNAs are more complex in that they encode not only the GOI but also proteins that drive replication of the input RNA and amplification of the GOI (Figure 1). While conventional mRNA vaccines result in direct translation of the incoming RNA molecule, introduction of saRNAs into the cytoplasm of a cell initiates continuing synthesis of RNA due to the encoded replicase machinery [5]. This results in increased levels of GOI (antigen) expression and duration [2,7–9].

saRNA DESIGN

Most saRNAs are derived from alphaviruses (single-stranded, positive-sense, RNA viruses) whereby the genes encoding the structural proteins have been removed and replaced by the GOI [10]. Removal of the structural genes renders the saRNA non-infectious. Similar to conventional mRNA vaccines, saRNAs delivered to a cell are translated by cellular ribosomes owing to the positive polarity of the RNA and the presence of a 5′ cap and poly(A) tail. The first pass of saRNA translation expresses genes that encode the viral nonstructural proteins (nsPs) which form a replication complex (Figure 1) [5,10–12]. The replicase complex mediates the transcription of a negative-sense copy of the positive-sense RNA. This negative-sense RNA then serves as a template for positive-sense RNA production. Replication cycles progress, resulting in many copies of the initial input saRNA. The replicase complex also recognizes a specific region within the negative-sense saRNA, the subgenomic promoter. Subgenomic protomer driven replicase transcription produces subgenomic RNAs (sgRNA), which include the GOI. Preferential subgenomic promoter replicase transcription generates many capped, polyadenylated, translation-competent sgRNAs resulting in high levels of GOI expression. Synthesis of all three RNA species—positive-sense saRNA, negative-sense saRNA, and sgRNA—is asymmetric and highly regulated. Replication is coordinated by several conserved sequence elements (CSE) for both negative and positive strand saRNA synthesis. For example, the 5′ UTR contains promoter sequence elements recognized by the encoded replicase complex leading to the generation of positive-sense RNAs from negative-strand intermediates [11,12]. In the 3′ untranslated region, a 19 nucleotide CSE immediately precedes the 3′ terminal poly(A) tail and is important for initiation of negative-strand RNA synthesis [13]. Additional saRNA regulatory sequence elements continue to be refined [5,6].

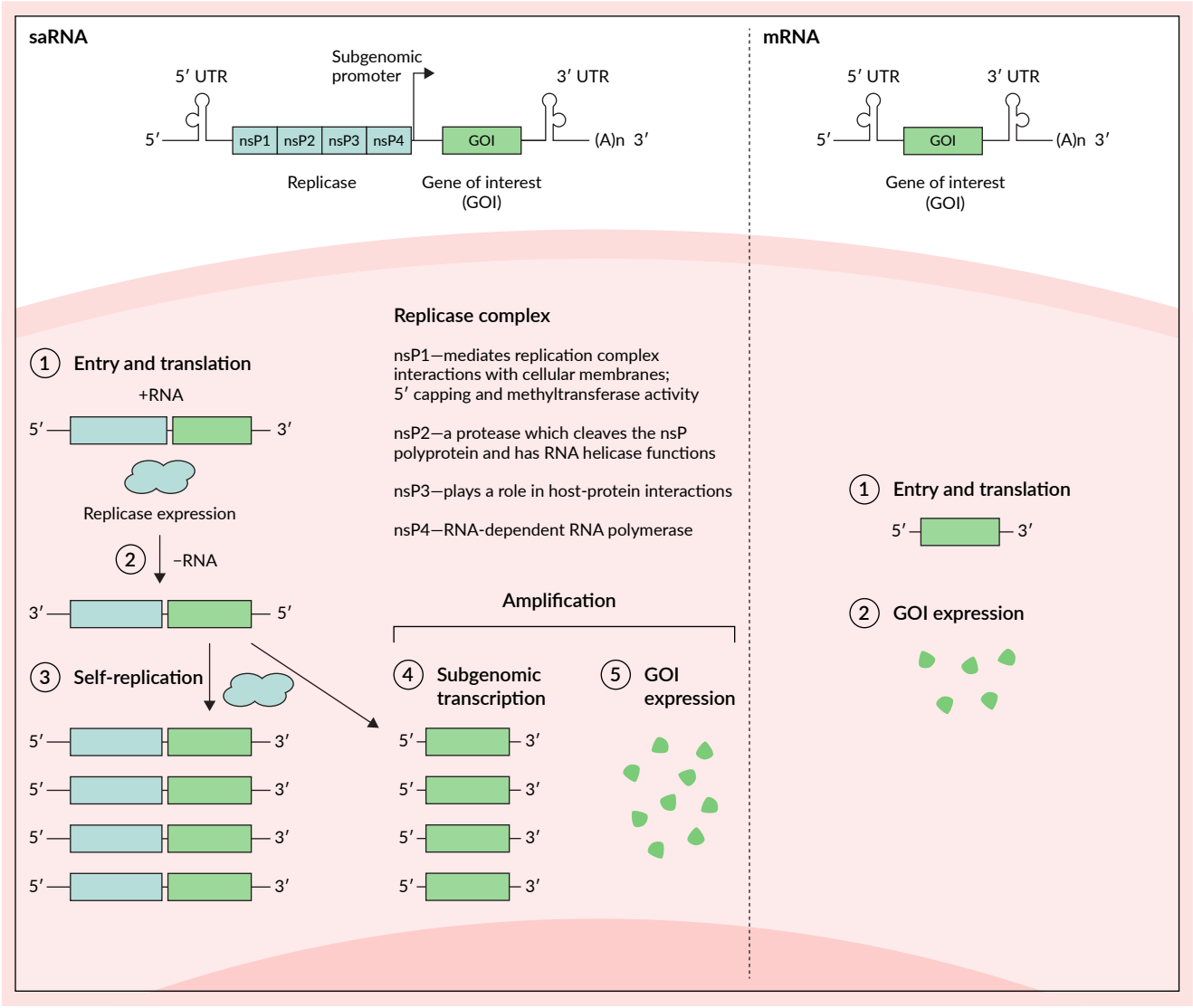
The resultant replication and amplification of input saRNA allows for both longer duration of transgene expression and reduced effective dose of saRNA versus conventional mRNA vaccines. Luciferase reporter expression from saRNA has been shown to last for 28–72 days in mouse models [7,14]. This is in contrast to most mRNA studies, in which similar reporter expression peaks at approximately 24 hours post administration and wanes over the course of the next several days [14–16]. Immunogenicity assessments in mice have shown saRNA vaccines to be effective at eliciting protective immune responses at 64-fold lower dose than mRNA vaccines [8]. Recent clinical data, in a booster vaccination setting, has also shown noninferior immune responses for saRNA vaccination at 6-fold lower dose when compared to a conventional mRNA vaccine [2]. An additional feature of saRNA that impacts vaccine efficacy is the self-adjuvating nature of these molecules via induction of innate antipathogen pathways.

INNATE ACTIVATION

Cells contain innate immune pathogen recognition receptors (PRR) to identify foreign agents, typically viral or bacterial pathogens, through recognition of non-self nucleic acids. PRRs include membrane bound Toll-like receptors (TLRs) in the endoplasmic

FIGURE 1

Schematic of GOI expression from saRNA versus mRNA.



Alphavirus-based saRNA molecule including 5′ cap, 5′ UTR, non-structural protein (nsP) genes that compose the replicase complex, subgenomic promoter, GOI, 3′ UTR, poly(A) tail. Following entry, translation of the positive-sense saRNA molecule nsP genes results in expression of the replication complex (1). The replicase complex transcribes a negative-sense RNA copy of the input saRNA molecule (2). This negative-sense copy serves as a template for replicase driven (3) transcription of positive-sense copies leading to self-replication, and (4) transcription of subgenomic RNAs as mediated from the subgenomic promoter. These subgenomic RNAs are translated by cellular ribosomes, leading to (5) expression of the GOI. Specific functions of the nsP genes are highlighted [5,10–12].
mRNA molecule including 5′ cap, 5′ UTR, GOI, 3′ UTR, and poly(A) tail. Following entry into a cell (1), the mRNA molecule is translated by cellular ribosomes to produce the GOI (2).

reticulum and various cytoplasmic sensor proteins (RIG-I, MDA-5) [17,18]. TLR3, RIG-I and MDA-5 can recognize double-stranded RNA (dsRNA); TLR7/8 recognizes single-stranded RNA (ssRNA) [18,19]. Activation of these sensors leads to induction of signal transduction pathways and the production of proinflammatory cytokines and interferon. A consequence of innate activation is a reduction in mRNA translation as a mechanism to reduce the production of infectious pathogens and limit their spread [18].

Innate sensing pathways can be activated by mRNA vaccines in various ways. Double

stranded RNA byproducts that may be present following *in vitro* RNA synthesis can activate TLR3, RIG-I and MDA-5 [18]. The recognition of exogenous ssRNA by TLR7/8 can be reduced by the incorporation of various modified triphosphate nucleotides, notably pseudouridine, into mRNA therapeutics during *in vitro* transcription RNA synthesis [19]. A combination of both *N*1-methyl-psuedouridine substitution for conventional uridine triphosphate as well as purification to reduce dsRNA in the final mRNA drug product was shown to have the largest impact on preventing innate immune activation and hence maximize therapeutic protein expression *in vitro* and *in vivo* [18].

saRNA vaccines may benefit from removal of dsRNA from the drug product, ensuring robust launch of saRNA through initial rounds of translation and replication in the absence, or at least reduced, induction of innate sensing. The incorporation of *N*1-methyl-psuedouridine into saRNAs during synthesis has been shown to inhibit saRNA replication, likely through interfering with the nsP4 RNA-dependent RNA polymerase recognition of the *in vitro* synthesized *N*1-methyl-psuedouridine containing RNA [14,20]. The impact of incorporating other modified nucleotides on saRNA activity is an active area of investigation by several groups [14,20,21]. The inherent ability of saRNA to replicate in the cytoplasm of cells generates large amounts of dsRNA, which activates innate sensing pathways and results in decreased translation, including reductions in saRNA GOI expression, thereby potentially limiting efficacy [22]. However, the ability of saRNA to replicate quickly, within the first 4–6 hours following transfection [23] combined with amplification of subgenomic RNA provides conditions in which innate activation, and subsequent reduction in translation, may be competitively mitigated at least for a relevant period of time to allow robust transgene expression. Many alphaviruses employ various mechanisms to subvert innate activation and reduce deleterious impacts on saRNA expression including RNA structural elements, such as the downstream loop (DLP), which promotes translation under conditions of innate sensing-induced eIF2a phosphorylation [24,25]. The nsP2 protein has also been implicated in modulating the host cell innate response [26–28].

In this way, saRNA vaccines may subvert aspects of innate sensing detrimental to GOI/antigen expression but maintain features that may serve an adjuvant function such as production of interferon and proinflammatory cytokines. Activation of innate signaling pathways is characteristic of other potent vaccine vectors which induce strong immune responses and activate multiple innate signaling pathways [29]. The 'gold standard' of live attenuated vaccines, yellow fever 17D vaccine, activates many features of the host immune system including the innate immune system, resulting in optimal immunogenicity and efficacy [30,31].

CHALLENGES

Alphavirus-based saRNAs are typically a minimum of 8,000 nucleotides in length due to the encoded replicase genes and necessary control elements. Depending on their length, GOIs can add several thousand additional nucleotides. Thus the overall size of a saRNA molecule is several thousand nucleotides longer than most conventional mRNAs and presents challenges in terms of production, yield, stability, and analytical assessments [5]. Optimal *in vitro* transcription (IVT) reaction conditions for saRNA synthesis may differ from conditions typically used for conventional mRNAs [32]. Modification of the IVT reaction can greatly reduce subsequent downstream purification and polishing steps to remove truncated transcripts and other byproducts, such as dsRNA [33].

Hydrolytic stability of RNA is inherently low owing to the presence of a 2′ hydroxyl group on the ribose sugar [5]. As saRNAs require an intact full-length RNA molecule for successful replication, hydrolysis anywhere

along the RNA can impact performance. Packer *et al.* showed RNAs and formulation components from lipid nanoparticles can interact to form adduct species that result in reduced mRNA translation [34]. The impact of this adduct formation was more notable with longer RNA length. The development of both robust potency assays together with analytical measures can help assess the impact of these deleterious alterations through manufacturing steps and on the final drug product.

Improved analytical assessments are being developed with better resolution for long RNAs [35,36]. Capillary gel electrophoresis (CGE) is currently the most widely used method but provides minimal single-stranded RNA resolution beyond 9,000 nucleotides [37]. Improved separation gels, run protocols, and the development of appropriate sizing standards will positively impact assessments of IVT conditions, purification, and formulation on the integrity of saRNA molecules. Analytical methods beyond CGE can provide greater resolution and insights to saRNA drug product quality and consistency [34,35,37].

Both conventional mRNA and saRNA are challenged by delivery to target cells. Conventional mRNAs are large negatively charged molecules that don't readily traverse cellular membranes; saRNAs are even larger. Unformulated ('naked') saRNA mixed in buffer and delivered intramuscularly induced immune responses in mice, however relatively large doses were needed [7]. Overall potency of saRNA vaccines is improved by formulations which protect the labile RNA from RNAses present in tissues and facilitate cellular uptake. Much of the focus on formulation has been on the use of lipid nanoparticles (LNPs) given their success as a clinically validated approach for conventional mRNA delivery as used in the approved mRNA SARS-CoV-2 vaccines [38]. When formulated into LNPs, saRNA vaccines demonstrated increased immunogenicity versus unformulated dosing regimens [7]. Similarly, overall *in vivo* reporter protein expression was higher when saRNA was formulated [7,8]. As for conventional mRNAs, development is ongoing to compare different types of saRNA delivery vehicles, including cationic polymers, lipoplexes, dendrimers and other approaches [37–39].

FUTURE AREAS OF INTEREST

As noted above, a critical challenge of saRNA vaccine development is the long length of these molecules and the impact on manufacture and stability. One way to shorten saRNA molecules is to find ways to reduce the length of the replicase coding region. As Comes et al. suggest, this could be accomplished by protein engineering to streamline the overall design of the replicase complex and/or encode replicase machinery from related viruses that are more compact [39]. Elucidation of RNA regulatory sequences that mediate saRNA replication and host protein interactions across a variety of cell types will facilitate improved, increasingly rationally designed, new streamlined vectors [7].

Recently, transreplicon systems have been described in which the two open reading frames encoded in an saRNA are separated into two RNA molecules—one encoding the replicase genes, the other the GOI [40]. Care is taken to retain sequence elements important for replication, amplification, and function. In transreplicon systems, each RNA molecule is closer in size to conventional mRNAs (several thousand nucleotides), aiding synthesis, purification, and analysis [12]. This system also enables a single 'universal' replicase encoding RNA molecule that can then be paired with a variety of different GOI encoding transreplicase molecules. However, for maximal activity, both transreplicon RNA molecules (replicase and GOI) would be delivered to the same cell. Ongoing development and assessments will compare the performance of transreplicon systems to conventional (cis) saRNA platforms [40].

For vaccine applications, the strong induction of innate pathways by saRNAs through both endosomal and cytosolic sensors may be advantageous by providing an adjuvant

function. However, this can also lead to translation inhibition and a reduction in the level of saRNA GOI expression thereby limiting efficacy and potential applications beyond vaccines [6]. A further extension of the alphavirus innate subversion strategies mentioned above is to express other viral immune evasion proteins that robustly counteract cellular innate sensing pathways, which is being investigated by several groups [41,42]. Initial evaluations have shown a positive impact of various viral evasion proteins, such as NS1, E2, K3, and others, on saRNA expression and immunogenicity [41,43,44]. Expression of these evasion proteins can be accomplished either by encoding them in cis within the saRNA or as a separate RNA molecule. When expressed in trans, from separate RNA molecules, the impact of different immune evasion proteins can be evaluated more easily, including dosing levels of these proteins. However, the impact of these immune evasion proteins is most advantageous when they are delivered to the same cell as the saRNA.

FINAL THOUGHTS

The first approved saRNA vaccine for COVID-19, ARCT-154, has demonstrated the utility of this platform for vaccine applications [9]. Clinical evaluation of a booster dose of ARCT-154 was noninferior to a conventional mRNA vaccine [2]. Additional analysis showed superior breadth and duration of neutralizing antibody responses in saRNA vaccines [9]. This immune profile was accomplished using 6× lower dose for the saRNA vaccine vs the standard mRNA vaccine. No significant differences in safety were observed. Ongoing clinical trials for other infectious disease vaccine targets have also shown promising immunogenicity at lower doses than conventional mRNA vaccines [11,45]. Conventional mRNAs are being more widely evaluated beyond infectious disease vaccines, such as for therapeutic cancer vaccine approaches [46]. Here too saRNA based vaccines are also being evaluated [47]. As additional data from clinical evaluations becomes available, it will be important to understand the translation of preclinical model evaluations, including mouse and NHP models of immunogenicity, safety, and efficacy, to clinical performance. Ongoing and future work will aid saRNA developers to refine current vector designs, delivery formulations, and manufacturing strategies with the goal of improving and expanding the use of this platform.

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NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES: ANALYTICS AND CMC

SPOTLIGHT

INTERVIEW

Exploring issues and opportunities in the development of platform technologies for oligonucleotide manufacturing

David McCall, Senior Editor, *Nucleic Acid Insights*, speaks to **Jeske Smink**, Senior Director, Head of Drug Substance, about Silence Therapeutics' platform-based approach to developing targeted short interfering RNA-based therapies. They discuss the current state-of-the-art in oligonucleotide manufacturing and CMC, and explore the emerging alternatives to the traditional solid-phase synthesis approach.

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Q What are you working on right now?

JS: Currently, my team and I are working on several programs in early-stage development. We take short interfering RNA (siRNA) candidates that have been selected by our drug discovery team as lead compounds to the development pathway for clinical Phase 1. We are working on analytical development and process development for the first non-GMP batches

intended for nonclinical studies, as well as the first clinical batches for Phase 1 studies. In addition, we are performing scale-up work on our proprietary starting materials that are used to enable targeting of our siRNAs specifically to the liver. Finally, we also support our other CMC colleagues in preparing late-stage projects for regulatory filing for clinical Phase 2 and 3 studies.

Q Can you tell us more about Silence Therapeutics' platform/approach and R&D pipeline? What differentiates it?

JS: At Silence, we have developed our own GalNAc platform, known as the mRNAi GalNAc Oligonucleotide Discovery (mRNAi GOLD™) platform. GOLD contains a specific proprietary GalNAc conjugate that is attached to our siRNA molecules with a proprietary linker. The GalNAc conjugate, a type of conjugate widely used in the oligonucleotide field, is responsible for targeting our siRNA molecules specifically to the liver.

In our case, the GalNAc modality is designed to have particular advantages in manufacturing. We attach our proprietary linker and GalNAc conjugate in the last step of the oligonucleotide drug substance synthesis to the siRNA molecule, whereas some of our competitors attach their GalNAc modality as first building blocks to the solid phase resin. This order of attachment during the synthesis provides us with greater flexibility in manufacturing. We also have a chemical toolbox at our disposal—our siRNA molecules have a specific chemical backbone structure that allows us to improve the molecular design and stability of our compounds for more specific targeting and minimal off-target effects.

Silence Therapeutics' R&D pipeline includes several early-phase programs. We have a proprietary pipeline, but we also collaborate with various pharma partners on early-stage programs that are then taken over by our partners at a later stage of development. Moreover, Silence currently has three programs from our proprietary mRNAi GOLD platform in the clinic (of which two are fully owned).

Q What, for you, are the key issues and talking points in oligonucleotide therapeutics manufacture and CMC currently?

JS: Since the COVID-19 pandemic, the RNA therapy field has drawn a lot of attention and is growing fast. However, for oligonucleotides there are still no specific regulatory guidelines. The European Medicines Agency (EMA) is currently working on a specific guidance but for now, there is a lack of clarity. This allows on the one hand for a lot of freedom, but also results in a few challenges for the industry.

If we look more into the technical aspects of oligonucleotide manufacturing, there are several important issues. One major challenge is that oligonucleotides have a complicated impurity profile, which means it is difficult to separate individual impurities. However, a key CMC criterion for these products is to have a good understanding of the impurity profile.

Platform technologies are another topic of discussion in the field. Oligonucleotide manufacturing is suitable for extending knowledge and experience to all molecules using the same manufacturing platform technology. However, as there are currently no regulatory guidelines in this area, it is important to initiate discussions with the regulatory authorities to align on this issue. If we can do this successfully, we might potentially be able to use platform knowledge

"Since the COVID-19 pandemic, the RNA therapy field has drawn a lot of attention and is growing fast. However, for oligonucleotides there are still no specific regulatory guidelines."

to accelerate regulatory approval of novel siRNA compounds and get our drugs to patients faster. To this end, there are several collaborations between industry partners underway in the oligonucleotides space, including some consortia—for example, The European Pharma Oligonucleotide Consortium (EPOC), which aims to harmonize the regulatory CMC side of oligonucleotide development and manufacture.

Oligonucleotides are synthesized using solid-phase synthesis and this process has certain limitations, specifically regarding its scalability and the use of large amounts of solvents. Therefore, solid-phase synthesis is likely not very suitable for large indications in the future. This is the reason why another topic currently drawing a lot of attention in the field is the evaluation of novel manufacturing technologies for oligonucleotides.

Q What is your analysis of the various emerging alternatives to the traditional approach to oligos synthesis, and what can we say so far about the benefits and considerations/challenges they present?

JS: Over recent years, there have been many initiatives established to tackle the limitations of solid-phase synthesis and to look for new manufacturing technologies. These initiatives are frequently organized through consortia, which are often combinations of governmental institutes, industry partners, and sometimes also include CMOs. This shows the high degree of interest in and focus on this topic, which is mainly concentrated on addressing specific issues of scalability as well as product quality specifically considering impurity profiles.

A primary focus of these consortia has been on liquid-phase technology, which would offer the advantages of being more easily scalable and reducing the use of reagents and thus, reducing costs. For liquid-phase synthesis of oligonucleotides, the synthesis process would be different from solid-phase synthesis—however, the subsequent purification process could be the same as for solid-phase synthesis.

Although liquid-phase synthesis is currently a key focus area, the technique seems to be progressing slowly. Therefore, people are now also looking at other alternatives, such as enzymatic approaches. This could hold promise but is dependent on the availability and suitability of different enzymes that would be used—for example, if there are certain enzymes required that are proprietary, then the exchange of those between different companies could pose a challenge.

An additional challenge for both these novel techniques is the use of specific chemical building blocks in oligonucleotide synthesis. The chemical modification of oligonucleotides is essential to ensure good bioavailability for targeting the liver, ensure product stability, and reduce immunogenicity, among other things. One limitation of the new techniques is the currently unanswered question of how well these chemical building blocks can be implemented and used. Moreover, both novel techniques would result in different process- and product-related impurities as solid-phase synthesis. Therefore, it may be some time before these novel techniques are ready to be implemented in products that can reach the market.

"Often, we cannot fully separate and characterize all individual [oligonucleotide] impurities with a single analytical method, which means that for some impurities, a different strategy must be applied."

A combination of the different techniques could potentially be an option. For example, some developers are considering 'blockmers', where one produces smaller fragments of the oligonucleotide and combines them later on to make the full-length product. These blockmers could be produced by either the liquid-phase technology or by the standard solid-phase synthesis, and then combined using the enzymatic approach. In this way, one might be able to combine the advantages of the different manufacturing techniques.

In general, though, employing novel manufacturing technologies requires a large effort and acceptance from the entire field, including the regulatory authorities. Some discussions are ongoing with regulatory authorities around accommodating within their guidelines a degree of flexibility in the used manufacturing technology for these types of modalities.

Independent of the manufacturing technologies themselves, there are more opportunities to further optimize oligonucleotide production. For example, there is some focus now on using liquid active pharmaceutical ingredients (APIs). Today, at the end of the oligonucleotide manufacturing process, the drug substance is lyophilized before the lyophilized powder is dissolved again into a liquid drug product. There are currently ongoing discussions to see how feasible it would be to have a liquid API and thereby avoid this lyophilization step in the drug substance manufacturing.

Q Can you expand on the key remaining questions around impurities characterization, specifically, and what are some best practices for oligonucleotide therapeutic developers in light of current uncertainty?

JS: As I mentioned earlier, oligonucleotides have a complicated impurity profile. To analyze this, one needs high-quality analytical methods, which are challenging to develop. Often, we cannot fully separate and characterize all individual impurities with a single analytical method, which means that for some impurities, a different strategy must be applied. A common practice in the oligonucleotide field is to group impurities. One can do so based on shared characteristics—for example, their relative retention time or structural class.

Grouping impurities is the standard control strategy, but there are ongoing discussions about the most appropriate way of doing so and how to properly justify it. Currently, regulatory authorities agree on the grouping approach as long as a good justification is provided. In the future, this may change, and with increasing resolution of analytical methods, we may be able to better separate impurities. The level of impurity characterization might increase depending on the analytical methods available in the future.

Q Speaking of the future, what for you are the promising technological pathways forward to deliver the scalability required to meet rapidly growing demand for oligonucleotide therapeutics on a global basis?

JS: I believe the most promising approaches will combine the already proven solid-phase synthesis with new and emerging types of manufacturing technologies. A combination of blockmers and enzymatic technology, for example, might be a favorable way forward, as this allows the creation of fragments of the full-length product that will have fewer impurities due to a shorter synthesis cycle. This will also require fewer raw materials. If these fragments are combined by enzymatic ligation, fewer downstream process steps will be required. This will enable a reduction in costs and will result in improved purity, with fewer impurities that are closely related to the parental molecule. An additional advantage of that technique is that it allows us to combine the existing solid-phase technique with a new enzymatic technology. This may help to get the manufacturing technology ready for the market quicker and ease the regulatory acceptance process.

Q Lastly, can you sum up one or two key goals and priorities, both for yourself in your own role and for Silence Therapeutics as a whole, over the foreseeable future?

JS: Silence Therapeutics has been around for over two decades, but especially in the last couple of years, we have been able to accelerate our development programs considerably. In addition to the three programs from our proprietary mRNAi GOLD platform that are currently in the clinic, we have several more promising targets in the pipeline. Our aim is to bring more of these to the clinic and eventually, to the market in the coming years.

Oligonucleotide manufacturing processes are suitable for using platform technology. Prior knowledge can be applied to new compounds and that provides many opportunities for accelerating drug development. The goal for my team and I is to deepen even further our current platform knowledge, related to both the manufacturing process for and product characteristics of our compounds. By further expanding our manufacturing platform knowledge, we can further accelerate programs to bring these drugs to patients faster.

As I mentioned earlier, Silence has over two decades of experience and incredible know-how in the siRNA arena. We believe we are at the early stages of what we can do with our technology.

BIOGRAPHY

JESKE SMINK is a biologist with over 20 years' experience in the biotech industry and academic research. She has expertise in CMC aspects in different areas, including cell therapies, biologics, and RNA therapies. After 7 years in academic research focusing on molecular mechanisms in bone development and diseases, she moved to co.don AG as Head of Scientific Affairs in 2012. Here her work focused on preclinical development and quality aspects of cartilage cell transplants, contributing to the EMA central market authorization

of a cartilage cell therapy. Following, she joined ProBioGen as CMC project leader, where she was overseeing customer projects, focusing on process and analytical development, scale-up, and large scale GMP manufacturing of biologics for clinical Phase 1 studies. In 2020, Jeske joined Silence Therapeutics, Berlin, Germany, as Senior Director, Head of Drug Substance overseeing CMC development of early stage development projects, including process and analytical development, scale-up and clinical product manufacturing of siRNA compounds, and contributing to quality sections of regulatory documentation. Her work includes managing a team consisting of several CMC project leaders, a starting material lead, and analytical chemists.

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NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: STABILITY

SPOTLIGHT

INTERVIEW

Taking a high-throughput approach to enhancing the target specificity and stability of nanoparticles

David McCall, Senior Editor, BioInsights, speaks to Genentech's **Chun-Wan Yen**, Senior Principal Scientist, and **Yuchen Fan**, Principal Scientist, about current and future innovations in nucleic acid formulation and delivery utilizing nanoparticles. They explore challenges and advances in achieving targeted extrahepatic delivery of nucleic acids *in vivo*, and discuss the application of high-throughput screening and AI/ML tools to extend the capabilities and reach of LNP-based drug delivery.

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Q What are you working on right now?

YF: We are both based in the Synthetic Molecule Pharmaceutical Science Department at Genentech. My team focuses on automation and high-throughput screening efforts to support development of formulation and drug delivery systems. In recent years, we have developed a screening workflow for lipid nanoparticle (LNP) formulation, in collaboration with Chun-Wan's team.

Prior to joining Genentech, I obtained a PhD in Pharmaceutical Science at the University of Michigan. I have been in pharmaceutical science and drug delivery for over a decade, with passion for nanoparticle formulation development and additional experience gained over my previous research in immuno-engineering.

CY: I joined Genentech about 6 years ago. My team is working on preclinical and early-phase formulation development in support of IND enabling toxicity studies. We are using new technologies to increase the solubility both of new drugs and of their delivery vehicles, including LNPs for mRNA delivery. We are also working on different modalities, including small molecules, peptides, oligonucleotides, and mRNAs. We are also working on a parenteral preparation to support a Phase 1 clinical trial.

Before joining Genentech, I was at Merck, and prior to my industrial career, I worked as a postdoc on nanoparticles. I have always been passionate about using nanoparticles for drug delivery.

Q Can you describe for us the current state-of-the-art in nucleic acid delivery utilizing nanoparticles as you witness and experience it today—what can and can't we do?

YF: The nucleic acid space became a hot topic because of the COVID-19 pandemic. Now, we are seeing mRNA therapeutics expand beyond infectious disease vaccines and into other therapeutic areas. For example, mRNA-based cancer vaccines are now in clinical trials and mRNA therapeutics are also being employed in the gene therapy world to enable the delivery of gene editing tools. The first-generation delivery systems were lipid-based, but we now see some promising results with other materials such as polypeptides or polymers. It will be interesting to see how these compare with lipid-based drug delivery systems moving forward.

One of the main challenges for the field right now is tissue-specific delivery, and how to better reach the target organs if the administrative route is systemic. In general, IV delivery will lead to the majority of nanoparticles ending up in the liver or the other reticuloendothelial system (RES) organs. However, certain indications will require targeted CNS delivery, or

"...there are still many unknowns regarding different lipid compositions and different excipients."

delivery to a tumor elsewhere in the body. Beyond tissue-specific delivery, targeted delivery to a specific cell population, for example, certain antigen-presenting immune cell subtypes, would be preferred.

Q Can you go deeper on the challenges relating to the stability of nanoparticle-based therapeutics? And what are some promising approaches towards alleviating this issue, for you?

CY: Although we have gained more knowledge in recent years, our understanding is still largely based on the COVID-19 vaccine mRNA-LNP technology. There, the compositions are more fixed, helping us to understand the stability of those specific formulations. Nevertheless, there are still many unknowns regarding different lipid compositions and different excipients. In addition, the question of how to characterize them is still largely unanswered. While we do now have more tools available that enable measurement of physicochemical properties or of *in vitro* expression, for example, we are yet to understand how that physicochemical characterization or *in vitro* data will correlate with *in vivo* findings, which we know can be highly cell-specific or disease-specific.

For the past year, we have worked together on elucidating the structure of LNPs. Despite similar performance across the particle size distribution, as LNPs and nanoparticles in general are soft materials, they can undergo subtle structural changes upon storage or transportation. As a result, while there may still be similar performance in the cell line, it will be hard to translate this subtle structural impact to *in vivo* performance. Nevertheless, this structural change could further trigger some immunogenicity. This poses a challenge to researchers—not least because the current major database is so limited and focused on the COVID-19 mRNA-LNP vaccines.

Secondly, the tools we do have are not yet comprehensive enough, although there is a great deal of activity in this area. For example, much of the work that I have seen published recently involves freeze-drying technology such as lyophilization. The existing COVID-19 vaccines require ultra-cold storage—-70 °C storage in the case of the BioNTech vaccine. However, if we were to freeze dry LNPs, they could perhaps be stored at a much more feasible temperature, especially for developing countries. Some researchers are even exploring the possibilities of storage at room temperature. For vaccines, easier storage would be a huge benefit for global accessibility. Additionally, spray drying, which is a commonly used technique for small molecules, could perhaps be scaled up to larger quantities in order to work on vaccines.

Q What is your commentary on current exploration of routes of administration in the nucleic acid therapeutics space—particularly to help achieve extrahepatic delivery *in vivo*? What are some of the key challenges and breakthroughs here that you have encountered through your work?

CY: Many nucleic acid therapeutics, such as antisense oligonucleotides (ASO) and siRNA therapeutics, reach targets in the central nervous system through local administration, including methods such as direct intrathecal delivery. However, despite the fact they help ensure on-target delivery, patient compliance is not high with these methods because they can be highly invasive and painful. We are seeing more development in this area now—for example, oligonucleotide-conjugated antibodies potentially for targeting brain delivery via systematic IV administration. But from a CMC perspective, controlling the conjugation and assessing the stability of that approach is a challenge.

In terms of local delivery, two specific areas have received a lot of interest recently. One is the pulmonary system and most notably, siRNA or mRNA-LNP delivery to the lungs. This region poses some challenges—for example, the sheer and mechanical stress from a nebulizer may change the performance of the LNP or cargos. In addition, nebulization takes longer than an inhaler. Dry powder inhalation may be a viable alternative, but it requires more development work due to the need to engineer particle size. There have also been challenges due to dry powder's unwanted ability to reach deeper parts of the lungs, as well as stability-related CMC challenges.

Secondly, intravitreal injection for the eye has become another key area of research for nucleic acid therapeutics, largely due to the successes of gene therapy in this particular region. Again, local injection to the eye is an appealing idea because reaching the target can be guaranteed. However, there is a higher bar in terms of the associated toxicity challenges due to the high sensitivity of the ocular region.

The Selective Organ Targeting (SORT) LNP platform is engineered with a fifth distinct lipid, as opposed to the usual four, which adds the ability for the protein corona to reach different parts of the tissue. This could hold potential, but it is still early days and the toxicity compared to that of the COVID-19 vaccines is still unknown. It is certainly a technology to keep an eye on, though.

YF: The SORT strategy opens up a field in which people can intentionally utilize protein coronas in the formulation design. They can tune their surface properties and incorporate tissue specificity for their synthetic carriers. In addition, people may tune nanoparticle properties, or leverage molecular designs with intrinsic tissue tropism or tissue specificity of AAVs. One recent example is VLP or virus-derived protein cages. So, rather than using a synthetic particle, we can potentially explore intrinsic carriers and biomimetic delivery approaches. Another technology advance is the barcode strategy, which we believe is a useful tool to enable the screening of different formulations *in vivo* to identify molecular and/or composition designs that can achieve specific organ targeting.

"...in addition to characterizing the bulk for an average value, high-res analytics may better capture the distribution profile of nanoparticle sub-populations and better minimize any batch-to-batch differences."

However, one of the challenges in exploring different delivery routes is the selection of an appropriate model at the preclinical stage, and working out the result variability among different pre-clinical models, and how that can be translated into clinical trials.

Q Can you go deeper on the specific enabling tools and technologies that you utilize in your work—what important insights are they delivering now, and where would you personally like to see future innovation moving in this regard?

YF: One advantage of high-throughput screening is that it can help address the iterations of the molecular design of RNA formulations. Libraries of lipids or other polymeric materials can be screened to figure out molecular traits associated with different delivery efficiency. This strategy increases efficiency of formulation preparation and reduces material consumption, thereby increasing productivity. An important emerging use of high-throughput screening would be to build a large database to enable artificial intelligence (AI) and machine learning (ML). This will help with, for example, structural activity relationship, and better design for future generations of formulations.

Another field in which people are becoming more interested is high-resolution analytics. We know that nanoparticles are typically heterogeneous, even within a single batch, meaning that they may show a distribution of physicochemical attributes—for example, size or cargo loading. So, in addition to characterizing the bulk for an average value, high-res analytics may better capture the distribution profile of nanoparticle sub-populations and better minimize any batch-to-batch differences. There are emerging technologies, such as field-flow fractionation (FFF), that enable nanoparticle fractionation by size followed by multi-mode characterizations; or single particle-based analytics. These will help to enable better quality control of nanoparticle formulations.

CY: Yuchen and I designed the concept for high-throughput screening for LNPs in late 2019/early 2020. Now, everyone has adapted this concept for LNP screening.

It is important to note that this isn't just for use in formulation preparation; the downstream process also needs to be streamlined. We do now have more characterization tools that can support high-throughput approaches. For example, we have recently been working with the Lawrence Berkeley National Laboratory to support high-throughput structural elucidation.

Eventually, we hope there will be a streamlined workflow including the physical property structure or single particle measurements, *in vitro* readouts, and even *in vivo* readouts. Furthermore, AI/ML can help accelerate our understanding and design in the space.

Q Where are you seeing promise in terms of emerging innovation in the nanoparticle technology field, and what will be some important next steps in this area to further enhance nucleic acid therapeutics delivery?

CY: The fields of RNA therapeutics and AI/ML are emerging simultaneously. The timing here is key and will hopefully lead to some exciting applications. The nanoparticle design space is so vast that we need tools like AI/ML to identify the best formulations and correlations. With the help of high-throughput screening, we can further enable the design of the right properties for a lipid, including considerations of immunogenicity and toxicity.

In addition, we can move beyond the current, conservative four-lipid component paradigm. Can we go beyond the current four-lipid component? Can we even add active transporter conjugations to the LNP? CMC is a huge challenge there, but maybe there will be greater benefit in terms of therapeutic index.

Lastly, looking beyond just adding further components, I think that fundamentally redesigning nanoparticles to change their behavior could be the future, particularly for tackling un-druggable targets.

YF: Follow up on that, rather than increasing the number of components from four to five or more, we have seen some researchers doing the opposite—reducing the number to, for example, a single species for constructing the nanocarrier. This may lead to unexplored, potentially unique formulation properties, and relieve CMC challenges in complex formulation compositions. Furthermore, having fewer components could provide an innovative way for developers to establish their own technology advantage compared to the standard four-component systems.

Q Finally, what are some key goals and priorities for your work over the foreseeable future?

CY: We have touched on the fact that AI/ML will be a vital tool for the future of medicine. In this particular field, we are hoping to see more applications of this in the design space—for example, the use of AI/ML to identify what LNP compositions can target specific organs or cells. Again, developing a greater toolbox for instead of being limited to the usual four components will increase specificity and precision and could allow things like personalized

cancer vaccines. We still have a long way to go, but we cannot do that without the help of AI/ML—it is simply too big an area for us to explore alone. AI/ML could assist in the design of a greater number of unique lipid compositions, thus enabling developers to find a niche in the area and to work freely on more specific applications.

YF: I agree. We have seen increasing AI/ML-assisted investigations in therapeutic design space. We could apply that to the cargo nucleic acid design with the aim of increasing stability or achieving specific functionality. AI/ML could also be explored in our SAR and formulation optimization work. The application space is huge, and we believe an intelligent data-driven approach will further increase our development efficiency in the future.

BIOGRAPHIES

CHUN-WAN YEN received her Bachelor's degree in Chemistry from National Taiwan University, Taipei, Taiwan. She then pursued her PhD in Chemistry at the Georgia Institute of Technology, Atlanta, GA, USA. Following her PhD, she was awarded an ORISE fellowship and conducted postdoctoral research at the Massachusetts Institute of Technology, Cambridge, MA, USA. In 2015, Chun-Wan began her industrial career at Merck Research Laboratory from 2015–2018. She is currently a Senior Principal Scientist in the Synthetic Molecule Pharmaceutical Sciences at Genentech, San Fransisco, CA, USA. She is the CMC lead for parenteral formulation development. Her research interests include drug delivery for exotic modalities such as oligonucleotides, peptides, and mRNAs to support undruggable targets. Chun-Wan has published 40 peer-reviewed papers and holds three patents.

YUCHEN FAN is a Principal Scientist in the Synthetic Molecule Pharmaceutical Science department, Genentech, where he supports early-stage formulation screening and analytical development of small molecule, nucleic acid, and peptide therapeutics. He established and leads a functional team of automation and high-throughput screening (HTS) for pharmaceutical formulations and drug delivery platforms, particularly building HTS and high-content analysis workflows for lipid nanoparticle (LNP) formulations. Prior to his Genentech tenure in 2019, Yuchen received his PhD degree in Pharmaceutical Sciences at the University of Michigan Ann Arbor, MI, USA and MS and BS degrees both at Peking University, Beijing, China where he has gained expertise in pharmaceutics, drug delivery, nanomedicine, and immunoengineering.

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