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SPOTLIGHT ON
mRNA processing and analysis

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CONTENTS

SPOTLIGHT mRNA processing and analysis

NUCLEIC ACID INSIGHTS

mRNA PROCESSING AND ANALYSIS

SPOTLIGHT

EXPERT INSIGHT

MHRA regulatory considerations for the quality of mRNA products

Ka-Wai Wan and Francis Galaway

Medicinal products that use messenger RNA (mRNA) as an active substance have great potential for treating many conditions. They offer an adaptable response to changing diseases and the promise of more personalized medicines for patients. Whilst supporting innovation, the Medicines and Healthcare products Regulatory Agency (MHRA) must ensure the quality, safety, and efficacy of the product for patients. Currently, many global regulatory authorities are discussing providing specific regulatory guidance to support developers in the mRNA field. In this article, we discuss some of the key considerations for the MHRA when assessing the quality of mRNA-based medicinal products.

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INTRODUCTION

The use of mRNA in medicinal products has already delivered a rapid vaccine solution to the COVID-19 pandemic saving millions of lives. The expectation is that advances in mRNA technology and nanomedicines will provide a great opportunity to quickly adapt medicines to the ever-changing diseases and new zoonoses. The versatility of mRNA in medicines is certainly a clear advantage when compared with other modalities. There is also a hope that personalized immunotherapies may breakthrough as an option for many patients where a unique set of mRNA drug substances may be required. A challenge for innovators in the growing mRNA field seeking to reach patients is the diverse applicability of the technologies as well as a lack of understanding of the current regulatory

landscape and relevant guidance specific for different products.

The MHRA and other regulatory authorities recognize the benefits of the rapid end-to-end manufacturing that mRNA could potentially offer to patients and our role in supporting innovation in this rapidly evolving field. Nevertheless, regulatory experience relevant to RNA-based therapeutics is currently limited to antisense oligonucleotide (ASOs), RNA interference (RNAi) and a very small number of mRNA-based COVID-19 vaccines. Specific guidance is required to help support industry for development and transition to clinic. There are basic tenets that can be applied to ensure safety and efficacy, but an enhanced approach is particularly important for mRNA applications, where the developer is required to demonstrate excellent knowledge of their product. From this understanding, a product-specific control strategy for manufacture can be developed to provide the best outcomes for patients over the full life cycle of a mRNA medicinal product.

A key part of the MHRA mandate is to help innovative medicines reach patients in the UK. We believe there is an opportunity for innovators in exploring the space afforded by existing regulatory frameworks. How these are applied to emerging mRNA technologies requires continuous dialogues between regulatory authorities and developers, as well as clinicians and patients.

ENSURING SAFETY AND EFFICACY

Stringent criteria for all inputs into manufacturing should be implemented even when terminal sterilization may not be possible for a mRNA medicinal product. Indeed, the same bioburden and sterility controls are expected for any injectable products. The regulatory starting materials for the mRNA-based products are the plasmids and nucleotides. As with all biotechnological products, the starting material controls must be stringent and well documented. It may cover physical characteristics, identity, purity and impurities, stability, functionality, adventitious agent and TSE risk, and storage as applicable. The regulatory position is that mRNA made from a plasmid is a biological product. When a cell-free manufacturing process for mRNA—one that uses a synthetic DNA template—is in place in the future, the designation of the starting material(s) and controls would still be highly scrutinized.

The best applications for marketing authorization benefit from continuous risk assessment. The assessment of risk for all aspects of starting materials and manufacture is updated regularly as knowledge improves. The comprehensive characterization of all attributes using orthogonal methods from early on in development of a mRNA active substance and the drug product provides invaluable knowledge. In addition, to justify the control strategy to the regulatory authority, this knowledge base can be used to save resources later in development and post-marketing. The methods used for characterization should also be appropriate to provide adequate understanding of the active substance and final drug product.

The impact of impurities is particularly poorly understood for mRNA. Therefore, detailed characterization of the drug substance and drug product is vital. For instance, how the abundance of double stranded RNA species relates to immunogenicity and reactogenicity requires investigation. For a given product, a single well-defined species of dsRNA that is easily controlled could be responsible for reactogenicity observed clinically. This is just the impurities associated with the mRNA. The impact of changes made in the untranslated region (UTR) and potential frameshifting events following administration of the drug product require further investigation. It should also be noted that following formulation with LNPs, the potential of RNA-lipid adducts as impurities and their biological impact should be considered. The analytical methods used in control of the commercial manufacture will need to be adequately validated and adhere to ICH Q2,

namely specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, and robustness validation. It is possible to justify fewer controls and fewer analytical methods as understanding and manufacturing experience improves.

Early in development, compendial methods covering physical characteristics and safety might be the only tests with established acceptance criteria and so represent the basis for control. In our assessment, we typically expect to see mRNA active substance release tests to cover attributes of appearance, particles, pH, endotoxin, bioburden, entire nucleotide sequence, RNA concentration, capping efficiency, poly(A) tail, integrity, residual template/enzymes/solvents/nucleotides, and protein expression. We recommend gathering as much information about the drug substance and drug product as part of release testing as resources will allow from early in development. These could then be used in the necessary comparative studies in later phase development or for other product developments. It is acknowledged that some analytical methods may not be further developed for commercial release, as it may be justifiable to propose a reduced number of tests at licensing based on the knowledge gained during development. Yet, the early phase data is often useful to help create a knowledge 'space' in which the foundations of studies are built upon. The acceptance criteria should be derived from the batch results with clinically qualified material. Specifications will be tightened during development as manufacturing process control improves.

The mRNA active substance itself cannot be considered alone without the drug delivery system for an effective delivery of the mRNA material. Encapsulation of the mRNA drug substance with the drug delivery system is part of the drug product manufacturing process, which should be included in the drug product section of the dossier. Both the active substance and drug product (or drug product intermediate) have their unique attributes and challenges for development. The lipids are excipients that are formulated into lipid nanoparticles (LNPs), to help encapsulate the mRNA. The LNPs are considered as a drug delivery system, as part of the drug product. The choice of the lipid excipients for the formation of LNPs, or the use of other drug delivery systems should also be explored. Currently, only LNPs have been used for the delivery of the mRNA active substance(s) in COVID-19 vaccines. However, there are other nano-based drug delivery systems that could also be explored for the delivery of mRNA. It is acknowledged that non-viral vectors for gene delivery have been in development for decades, though it is the realization of the COVID-19 vaccines that have brought nanomedicines to the limelight. Certainly, lessons from liposomal and polymer-drug conjugates could be applied to mRNA-LNP systems. There are certainly specific quality considerations for the mRNA-LNP products. For instance, particle size and polydispersity, morphological characteristics, surface properties, distribution of the mRNA drug substance in the mRNA-LNP, and encapsulation efficiency. The physicochemical properties of the mRNA-LNP system would have an impact on cellular uptake and intracellular interactions/delivery, affecting pre-clinical and biological outcomes. Where these are considered critical quality attributes of the drug product, they would be expected to be included in the finished product specifications (for release/stability).

A full range of release tests along with container closure integrity is often used for stability studies, but assessors are most concerned with stability-indicating attributes, where deviation from the necessary specification limits could potentially lead to product failure. The MHRA requires that real-time stability data is used to justify the proposed shelf-life, in line with ICH Q5C. Stability modelling data can only be considered as supportive for development. The number of containers, bags versus vials, storage conditions, and administration system require carefully considered in-use shelf-life studies. For

instance, when a multidose vial is employed, the in-use conditions should be verified and supported by relevant microbiological and physico-chemical stability data in order to ensure patient safety under the proposed in-use conditions. The instability of many mRNA products may warrant more scrutiny by a regulator. Stability can be considered in different ways: during manufacturing, temporary storage, long-term storage, as well as in-use storage. These are usually product-specific and take account of the manufacturing process and a wide range of clinical scenarios. We are keen to have data on in-use scenarios such as temperature excursions and photostability, as well as transportation simulation stability data. Essentially, consideration of the whole cold chain storage through to the clinic is required.

'TRADITIONAL' VERSUS 'ENHANCED' APPROACH

The preferred approach for ensuring the quality of a mRNA medicinal product from the MHRA's perspective is a product-specific one. This requires a very detailed knowledge of the product and manufacturing process with reference to the principles—such as Quality by Design—in ICH Q8 and Q14 [1]. The changes to analytical methods and manufacturing process are planned for with a strategy to bridge or demonstrate comparability. The early effort in analysis of product and process can save on the testing intensity at later stages of development or for the marketed product. However, a traditional validation approach where three process performance qualification batches are manufactured is still preferred over continuous process validation.

Medicinal products that use mRNA are very complex and require an extensive analysis with a broad range of methods. A recurring problem we see is that the selected sequencing methods are relatively inadequate at calling bases where there are stretches of modified sequences, raising concerns over identity. The applicant will then be challenged to develop a method suited to their product. Due to the nature of the molecule, heterogeneity in mRNA-based medicinal products is not unexpected, but this must be characterized, and their potential biological impact is satisfactorily assessed. It is, therefore, best to quantify the heterogeneity and to quantify the impurities in as much detail as possible. Together with data from clinical trial studies, this allows specifications to be chosen and acceptance criteria set. The absence of specifications or routine controls for certain impurities and aspects of identity or purity can then be justified, supported by the relevant data, in licensing applications. This approach should cover all components of the final drug product; including excipients used to form the LNP drug carrier system.

The early implementation of measurements, especially potency assays, can be really beneficial. The developer may discover that one method gives excellent correlation with patient outcomes allowing tight control of product efficacy at the manufacturing level. In the case of mRNA vaccines, the term 'potency assay' may be considered misleading by some, as it may be better served as a 'functional' assay, confirming the functionality of the mRNA-LNP drug product. We are hoping to see further developments in these 'functionality'/potency assays for mRNA medicines, as it is acknowledged that protein expression in an *in vitro* cell model may not always be satisfactory for the purposes of controlling efficacy. However, at present our opinion is that it is still a critical test parameter to ensure the encapsulated mRNA can be effectively translated upon intracellular uptake into cells. Some may argue that the physicochemical control in mRNA integrity, purity, the 5' cap, poly(A) tail, and % RNA encapsulation would be sufficient as a combined set of quality attributes to support the removal of the *in vitro* expression control test. However, these physicochemical parameters—though measurable with minimal variability in a tightly controlled product specification—have yet to confirm the

correlation with clinical efficacy as one would expect to see for any biological products, such as a vaccine.

Demonstrating comparability is always a major problem that besets development of medicinal products. This includes comparability between developmental, clinical, process qualification, and commercial batches; comparability for analytical methods throughout the development; and comparability from one mRNA-LNP product to another when one wants to exploit the 'platform' approach. Satisfactory comparability based on extended characterization would also need to be demonstrated when a 'platform' approach is applied. The novel mRNA medicines are particularly vulnerable due to a lack of knowledge over the impact of attributes on clinical outcomes. ICH Q5E states: "*A determination of comparability can be based on a combination of analytical testing, biological assays, and, in some cases, nonclinical and clinical data. If a manufacturer can provide assurance of comparability through analytical studies alone, non-clinical or clinical studies with the post-change product are not warranted*". With insufficient understanding of the product and process, any differences observed between batches cannot be explained without resorting to further clinical studies. The MHRA must be confident that the manufacturing process can consistently produce a product that is comparable to that manufactured for the pivotal clinical trials. The use of analytical methods and reference standards is key to demonstrating comparability. In-house reference standards and reagent development are highly important until international standards and reagents become available. Inappropriate reference standards will be a major objection in part because comparability cannot be demonstrated. If the 'functionality'/potency assays do not sufficiently reflect the mechanism of action, then comparability cannot be easily demonstrated. It is important to consider comparability from early in development, as it could be difficult to address regulators' objections that may arise at the later marketing authorization stage.

There may be several iterations of the mRNA and LNP manufacturing process over the product life cycle. The scale of manufacture may increase dramatically, particularly during a pandemic. Analytical methods will need to be updated as more sensitive instruments become available to help enhance characterization of the drug substance and drug product. These changes will continue post-authorization. The changing of a mRNA sequence is not a simple variation procedure. In the UK, a change of an mRNA sequence in a COVID-19 vaccine is a new line extension, rather than a variation like that adopted for the annual flu vaccine strain updates. We recommend an early dialogue with the MHRA to perform such a change to a medicinal product.

The manufacturing team needs to work closely with the clinical team to link attributes to desirable patient outcomes. Critical quality attributes must be controlled by process design, in-process controls, and release testing. The immunogenicity and reactogenicity of mRNA products are not sufficiently understood from the perspective of the MHRA. Considering that both the mRNA and the lipids themselves can be immunogenic, the immunogenicity of the mRNA-LNP product or other mRNA delivery systems must be carefully studied. Therefore, characterizing clinically qualified material is still essential. The approach to immunogenicity should be product-specific and seek to be 3Rs (replace, reduce, refine) compliant. Reactogenicity is an important risk to control. It depends on the administration route and characterization should link to clinical observations.

Understanding and controlling both reactogenicity and immunogenicity are areas where platform knowledge can be very helpful. Immunogenicity can be a desirable attribute and part of an intended biological effect (immune modulators), but it could also be undesirable (such as parts of the innate immunogenicity observed with vaccines [2]). We expect to see *in vitro* methods developed to

characterize and control the associated attributes. For instance, if modified nucleotides are incorporated to reduce immunogenicity, then this is a key attribute that needs to be controlled, possibly by a release test. The detailed characterization of dsRNA, excipients and other components of a drug product associated with reactogenicity is expected as these are possible risk factors, affecting clinical outcome.

Potency—or functionality—is a key area for improving efficacy of mRNA medicines with product-specific methods. For the MHRA, potency assay(s) for a biological product assure that: the active substance amount is sufficient to induce a meaningful response; the amount is consistent between batches; detect clinically meaningful changes in amount of active substance; there is comparability throughout development. Therefore, correlates of biological effect could be acceptable as potency assays if they fulfil the required role of quality assurance. However, for mRNA medicinal products, structural attributes such as integrity and capping alone would not be adequate to provide assurance of the biological activity of the drug product. A meaningful 'potency' assay remains to be determined for mRNA medicinal products.

Establishing an appropriate reference standard for the determination of potency/functionality of a mRNA medicinal product appears to be a challenging task. Potency assays generally rely on appropriate reference standards. These are clinically qualified or demonstrated to be comparable to efficacious clinical product. Consequently, they have a well-established potency. There should be an initially intensive characterization of a reference standard, but subsequent qualification can follow a protocol acceptable to regulators. The primary reference standard should be included in the characterization studies presented in marketing authorization applications. The early establishment of a reference standard can, therefore, be helpful in establishing comparability between commercial product and clinical trial batches. The MHRA would like to hear what reference standards and reference reagents innovators would like to see our national control laboratory develop. We believe that international reference standards and control reagents will accelerate the field of mRNA medicinal products.

SPACE FOR INNOVATION

The unknown significance of many mRNA attributes is a challenge, but there is also an opportunity for innovators to work with us to help develop the regulatory and scientific space. A 'platform' technology approach may be seen by many developers as a way to help speed up development, but careful consideration should be made on how this is applied for different mRNA-based products, particularly when different clinical applications are proposed, or many changes have been made to the processes during scale-up. The 'platform' concept can be treated as 'prior knowledge', which is similar to any medicinal product development, where relevant data can be used to support a newer product. Some may consider the mRNA manufacturing process as their 'platform', but some may propose the LNP as their 'platform' technology and believe they could be applied to all active substances. This may not necessarily be acceptable when insufficient data is available to confirm product-agnostic properties of the concerned product. The 'prior knowledge' approach is useful for certain aspects such as providing the background information for the overall processing design, but it is not a substitute for most aspects of quality such as process validation, analytical method validation and drug substance or product characterization, which should be product specific. Appropriate evidence that the desired product 'fits' the proposed 'platform' would be required. In general, these 'platform' data are effective in supporting an understanding of the product and processes, performing risk assessments, and supporting further stability studies, as part of any good pharmaceutical development program. With this in mind, developing a robust design space and correlating to the

relevant manufacturing, physicochemical, and clinical outcomes would be desirable.

An ongoing dialogue with regulatory authorities is the best approach to innovation in the development of mRNA medicines. We welcome innovation and continuously support better medicines for patients. We acknowledge the rapidly evolving development of this field. In addition, the MHRA has convened an Expert Working Group on cancer vaccines to advise assessors and the agency on how to regulate this dynamic field. The UK is also actively legislating to facilitate innovative products reaching patients. The legislation for point-of-care manufacture [3] has finished consultation. The MHRA is also working closely with other regulatory authorities internationally to harmonize and provide regulatory guidance, so that a consistent approach can be applied wherever feasible.

CONCLUSION

Since the authorization of mRNA-based COVID-19 vaccines, there is an apparent increase in public and scientific interest concerning mRNA. While further studies in understanding the potential clinical impact of impurities and the fundamental research on mRNA in general are warranted, its applicability to a wide range of diseases or clinical conditions, and a relatively rapid manufacturing process—in comparison to a protein—can offer potential benefits for patients requiring personalized treatment, as well as in response to a pandemic situation. Different types of mRNA, drug delivery systems, and advances in analytical methods are likely to further improve our understanding and quality of the product available to address many unmet clinical needs.

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AFFILIATIONS

Ka-Wai Wan
Medicines and Healthcare products Regulatory Agency

Francis Galaway
Medicines and Healthcare products Regulatory Agency

AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

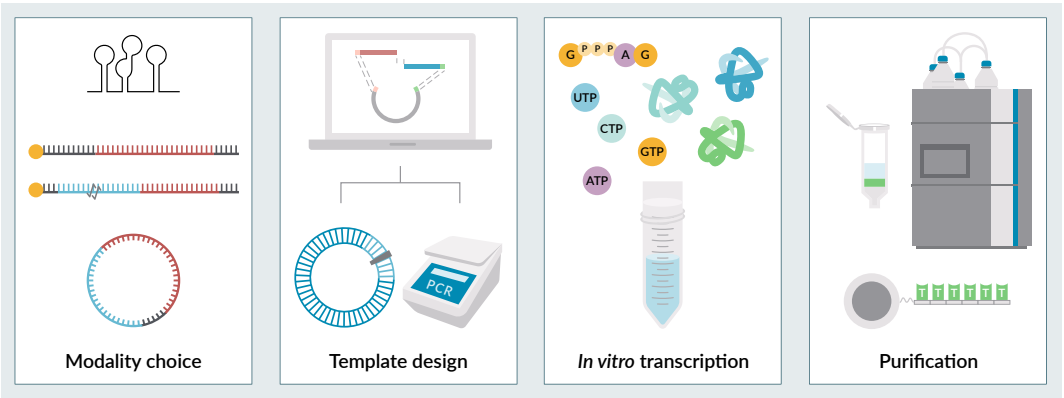
mRNA PROCESSING AND ANALYSIS

SPOTLIGHT

EXPERT INSIGHT

Insights into manufacturing lab-scale *in vitro* transcribed RNA modalities

Andrew Varley



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In the last decade, numerous RNA modalities have emerged as nucleic acid drug candidates. Thus far, small interfering RNA (siRNA), messenger RNA (mRNA), and self-amplifying RNA (saRNA) have found market approval. Such RNA can be broadly categorized as oligonucleotides or long RNA, which are primarily produced from chemical or enzymatic synthesis, respectively. Chemical synthesis is compatible with a plethora of RNA modifications, ranging from single atom replacement to completely distinct chemical structures. These modifications have been instrumental in the success of

the six FDA-approved siRNA drugs currently on the market (patisiran, givosiran, lumasiran, inclisiran, vutrisiran, and nedosiran). However, the number of synthetic steps required, and the associated challenges in purification from incomplete sequences, limits chemical synthesis of RNA to about one hundred bases long. Therefore, commercial production of long RNA relies on enzymatic synthesis via *in vitro* transcription (IVT), which generates affordable, high fidelity, long RNA transcripts. In contrast to chemical synthesis, the specificity of RNA polymerase excludes most non-canonical nucleotides. Three IVT-manufactured RNA products, all of which have been COVID-19 vaccines, have thus far reached market approval: Cominarty®, SpikeVax™, and ARCT-154. The former two are mRNA while the latter is saRNA. Notably, *in vivo* transcription offers a new paradigm for research or commercial RNA production. For example, recent *E. coli* engineering studies achieved >40-fold greater mRNA yields over non-engineered expression systems [1]. *In vivo* transcription prevents modified nucleotide addition and poses considerable purification challenges for pharmaceutical production, but has potential for affordable production as global mRNA manufacturing continues to scale.

The landscape and potential for IVT-produced RNA drugs has been rapidly expanding and will likely soon out-pace oligo RNA drug development. mRNA made its debut with the ~4,200 base-long COVID-19 vaccines and the same principles can be applied for protein replacement therapies; however, there may be additional purification considerations to enable successful delivery of therapeutic RNA. More recently, new RNA modalities have also gained substantial interest. For example, translation-activating RNAs (taRNAs) are short, non-coding RNA that can target and drive translation of an mRNA. Another modality is circular RNA (circRNA), which is a covalently closed RNA strand with improved stability, but substantial purification challenges exist for its success. Lastly, saRNA encodes a replication cassette enabling self-amplification, leading to RNA extended expression profiles and strands as large as 20 kb. Not only do each of these modalities generally require different synthetic approaches, the application of interest will play a role in manufacturing and purity requirements for success.

This article aims to provide a high-level view of RNA production and modality-specific considerations to enable meaningful discussions to workflow designs, particularly for smaller, lab-scale production. These perspectives will be especially useful for those new to IVT or developing an unfamiliar modality, by identifying key elements to consider in their manufacturing processes. This will be achieved by discussing considerations for four unique RNA modalities and considerations for DNA production, RNA synthesis, and RNA purification. I remind the reader to appreciate that additional factors such as target cell type, applications of interest, and untranslated sequences are just a few of many factors that may add further criteria for therapeutic success.

KEY CHARACTERISTICS FOR EACH TYPE OF RNA DISCUSSED

The primary goal for most IVT RNA drugs is to facilitate expression of a protein of interest, which can be accomplished via direct or indirect routes. For instance, protein coding RNA (mRNA, saRNA, and circRNA) recruit ribosomes to directly act as a template for protein synthesis. In contrast, small RNA modalities can achieve expression through indirect mechanisms such as facilitating ribosome recruitment to an endogenous transcript (via taRNA; described shortly) or enabling stop-codon readthrough of nonsense mutations (via engineered tRNA) [2]. Thus, each RNA modality can bring unique characteristics and selection of the most appropriate modality is key for RNA drug success (**Figure 1** and **Figure 2**). To date, only three IVT drugs have reached market approval, all of which are in the vaccine

FIGURE 1

Key structural and functional elements of reach RNA modality in discussion.

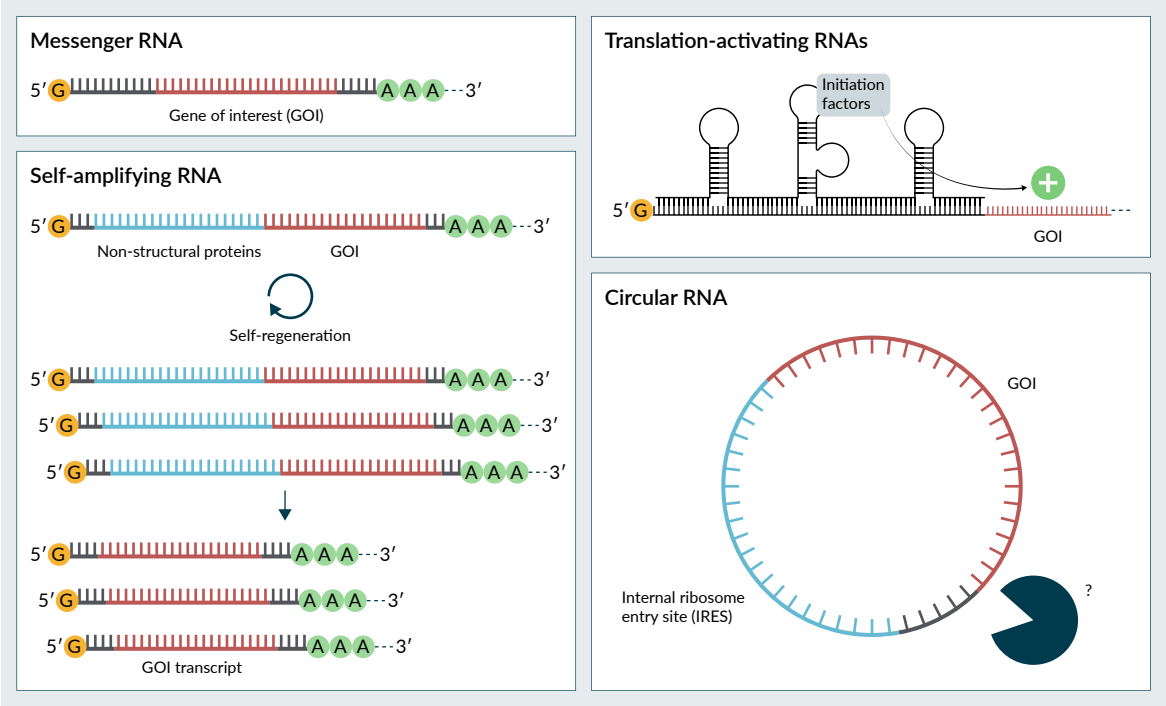
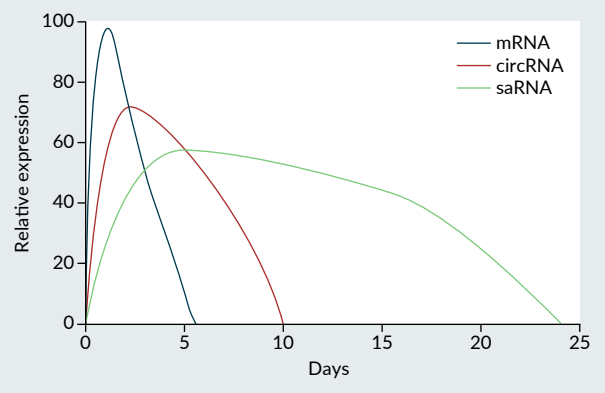


FIGURE 2

Representative expression profile for each translatable RNA modality.



For visualization only, not based on actual data.

space. However, a growing number of RNA drug candidates in development that span a range of modalities and applications suggest an upcoming surge in RNA drug approvals. To provide a foundation for the key factors in developing nearly any IVT product, four modalities will be described here.

mRNA typically ranges in length from several hundred to many thousands of bases, primarily determined by the gene of interest. A 5′ cap structure on mRNA is critical for both translation initiation and immune tolerance [3]. Untranslated regions (UTRs) are found upstream (5′) and downstream (3′) to the gene of interest, which play several roles including cellular stability, regulation, and translation. The 3′ poly(A) tail on exogenous mRNA facilitates translation initiation and acts as a buffer against 3′ exonuclease degradation, allowing it to persist longer in the cell. Selection of the UTRs and codon usage are two key considerations for mRNA sequence design, which have a relatively minor impact on manufacturing processes.

Translation-activating RNAs (taRNAs) are small transcripts and are unique from the other three gene-coding RNA discussed here. taRNA has been selected here to enable discussion of small IVT RNA products; however, many small non-coding RNA modalities

require similar synthetic criteria. Functionally, taRNA binds to a specific mRNA of interest and, via initiation factor recruitment, boosts translation of the associated transcript [4]. While this results in only modest upregulation, this approach holds promise for diseases such as SYNGAP1 haploinsufficiency, where mild upregulation and not overexpression is curative. taRNA has structured hairpins that may increase stability but more importantly, it does not carry a 5′ cap or poly-A tail. This is important, as these strands will be rapidly degraded by canonical exonuclease dependent pathways [5]. Additionally, methods to ensure 5′ and 3′ homogeneity could benefit these products [6–8], as undefined ends could alter binding kinetics.

saRNA transcript designs are traditionally derived from positive-sense RNA alphavirus genomes, where the structural proteins have been replaced with a gene of interest and are ~10 kb long [9]. The retained viral genome contains RNA-dependent RNA polymerases (RDRPs) and the structurally defined untranslated regions required for its self-amplification [10]. The RDRP uses the RNA as a template to synthesize a negative RNA strand, which itself acts as a template to:

1. Regenerate multiple copies of the positive strand; and

2. Use an internal, sub-genomic promoter to synthesize a ribosome-ready strand for the gene of interest.

Ongoing cycles of replication result in a copy-and-paste approach for RNA template persistence, providing an exceptionally long duration of expression. This allows saRNA vaccines to provide effective protection at lower doses than mRNA [11]; however, there are poorly understood factors that seem to play a large role in the cell-specific stability, amplification, or expression. Furthermore, self-amplification relies on the expression of viral non-structural proteins and a dsRNA intermediate that could illicit immune responses and limit functional repeated dosing [12]. Therefore, applicability of saRNA outside of the vaccines space may be narrower than mRNA.

As the name suggests, circRNAs are covalently closed, single stranded RNA transcripts. They can be synthesized by enzymatic ligation or template-encoded ribozymes for autocatalytic circularization [13]. The latter has gained traction in industrial production for its ease of production: simply adding GTP and heat after IVT. Within the last decade, circRNA has earned significant attention as a translation-competent RNA with high stability; however, there are several, less obvious applications for circRNA therapies such as miRNA-sponges or protein scaffolds [14]. Lacking any 5′ or 3′ termini for exonuclease activity, circRNA are naturally resistant to exonuclease-dependant RNA decay pathways, but can be rapidly degraded after hydrolysis or structure-mediated decay [15]. Additionally, in the absence of a 5′ cap for canonical translation, circRNA rely on highly structured internal ribosome entry sites (IRES) to recruit initiation factors and the ribosome. For this, many human and viral IRES sequences have been identified and engineered. The most effective IRESs are typically of viral origin and several potent IRES sequences are approximately 700 nt long [13,16]. Since cap-mediated translation initiation is substantially more potent than that from an IRES, circRNA stands to excel in applications when expression longevity is more important than peak expression. An important consideration for IRES-mediated translation is that the RNA structures necessary for function prohibit extensive inclusion of modified RNA nucleotides [16]. Lastly, there is some discrepancy in literature regarding the immune tolerance of circRNA products, as sequence, structure, and purity are all key variables across these studies [17].

DNA PREPARATION

There are two generally accepted approaches to prepare DNA for IVT, regardless of the intended RNA modality.

1. A common and scalable approach is through bacterial amplification of a DNA plasmid that encodes the desired transcript. To prepare a plasmid for high quality, custom RNA synthesis, this process requires gene synthesis, cloning into a backbone, transformation into bacteria, screening, bacterial growth, extraction, and linearization of the DNA. Although cumbersome, the process is well developed and ample template can be prepared. The time-consuming bottlenecks with bacterial amplification can be largely circumvented through the second approach;

2. PCR, which when combined with gene synthesis rapidly affords transcription-competent linear DNA templates. PCR production aligns well with high-throughput, low yield approaches by eliminating the need for cloning, screening, bacterial growth, extraction, and linearization. This was recently exemplified through collaborations between the BASE mRNA facility and Integrated DNA Technologies (IDT), allowing for the high-throughput production of poly(A)-tailed templates by long overhang primer PCR.

Importantly, the RNA workflow must be considered early in DNA template design. For example, enzymatic 5′ capping approaches generally use a T7 promoter followed by 'GG' for efficient transcription initiation, whereas cap-analogues such as TriLink's CleanCap® require an 'AG' (mRNA) or 'AU' (saRNA) at the same location. There is a hefty price associated with cap-analogues and they can provide lower capping efficiency than enzymatic capping, but they expand chemical control over the 5′ cap and compared to enzymatic capping, which requires additional purification steps, reduce the number of steps for synthesis. To address the latter, New England Biolabs is developing high efficiency enzymatic co-transcriptional capping systems for 1-pot synthesis and capping.

Another critical factor for template design is the poly(A) tail, which can be template encoded or enzymatically synthesized. Poly(A) tails tend to shorten during bacterial amplification, with recombinase negative bacterial strains and segmented poly(A) tails attempting to circumvent this issue [18]. The alternative to template encoded tails is the post-transcriptional addition of a poly(A) tail via poly(A) polymerase. However, apart from the additional step, this is avoided in pharmaceutical production for two key reasons:

1. Template encoded tails provide a discrete poly(A) tail length whereas enzymatic tailing results in heterogenous tail lengths; and

2. Improved purification, as poly(dT) capture purification will not be able to discriminate against aborted/truncated RNA transcripts if they are post-transcriptionally tailed.

Template amplification via bacterial fermentation (plasmids) or PCR can result in undesirable mutations or unexpected challenges. A single base change could completely abolish activity, and even in small quantities, a mutant gene could impact immune sensitive applications. Companies such as Plasmidsaurus and Flow Genomics, which leverage Oxford Nanopore Technology, have made the identification of mutations easy and affordable through whole plasmid sequencing. Repeats, polynucleotide runs, and host-homologues sequences are just some traits that can increase mutation frequency. For an efficient and clean PCR with high-fidelity polymerases, concern for mutation is low, but plasmid amplification can be more sensitive to sequence specific challenges. For example, even in the absence of typically concerning traits, some sequences seem to be inexplicably error prone or toxic. When these sequences exist within coding sequences, this can be solved by changing codon usage.

Lastly, plasmid structural quality is a significant consideration, typically evaluated by percent of supercoiled DNA. Supercoiled plasmids are free from template-strand nicks, which result in an array of truncated products due to early run-off termination.

Transcription is intentionally terminated by one of two means: template run-off or a terminator sequence. Run-off transcription requires the template to be linearized such that the T7 polymerase 'falls off' the end of the transcript. For this, type II restriction enzymes, which cut outside of their recognition site, are commonly used to ensure a pure poly(A) terminus. The restriction enzyme recognition site must be unique to the transcript to avoid unintentional cleavage. If present, recoding via codon optimization (if present within the coding sequence) or an alternative enzyme can be used. High purity, linear DNA is recommended for high quality RNA products; however, unpurified linear templates from restriction digestion reactions can be used after complete heat denaturation of the restriction enzyme. This is ideal for high-throughput and discovery research where scale, efficiency, and fidelity are less of a concern.

In contrast to run-off transcription, which uses a linear template, a T7 terminator can be used for polymerase release. However, run-off transcription is preferred over transcription terminator sequences. One reason for this is to avoid synthesis of extended or heterogenous transcripts that arise from terminator readthrough [19,20]. This issue could be solved by encoding self-cleaving ribozymes, such as the hepatitis delta virus or Twister into the 3′ terminus of the transcript, but incomplete hydrolysis and removal of cleavage fragments present additional considerations [8,21]. Secondly, sequence-specific termination is associated with pausing, likely leading to reduced RNA polymerase (RNAP) turnover and reduced reaction rates [22]. Nonetheless, T7 terminators may provide an ideal option for high-throughput applications, or when readthrough or reaction time is less of a concern.

IN VITRO TRANSCRIPTION OF RNA MODALITIES

Commercially available RNA kits offer convenient and effective RNA synthesis workflows; however, a fundamental understanding of each variable will offer the best opportunity to produce an effective and scalable product. Furthermore, these kits are designed for the average mRNA transcript, rather than small, large, or circular varieties. Here, in an easy-to-digest format, each topic will be introduced and the role it plays in modalities-specific production will be discussed. It is important to remember that these are general approaches and each individual product requires fine-tuning to achieve optimal yield and quality.

Trait: RNase contamination

Brief: RNase contamination is the bane of RNA production. First and foremost, proper sample handling (aseptic technique) and use of tight-fitting, RNase-free gloves is essential to prevent aberrant RNase contamination in samples. A misconception is that wiping your gloves or tools with 70% ethanol will reduce contamination, but RNases are robust enzymes that persist in these conditions. Instead, a nuclease decontamination solution is needed, such as RNase Zap™, RNase Away™, or even a mixture of bleach, NaOH, and detergent. Whenever possible, use filter tips to prevent aerosolized contaminants, reserved tools/instruments specifically for RNA work, and a RNase-free laminar flow hood or biosafety cabinet (cleaned with RNase decontaminating solutions, not 70% ethanol).

It is common for labs to conduct plasmids extractions and RNA production in the same space; however, bacteria are a major source of RNases and physical separation of these workspaces is ideal. Lastly, the elevated temperatures

and salt concentrations in an IVT reaction provide an ideal environment for RNase digestion and RNase inhibitors are an effective control. There are a wide variety of RNase families that threaten RNA production; however, commercially available RNase inhibitors (origin: murine, human placenta) only inactivate the commonly encountered superfamilies of RNase A, B, and C. Researchers should therefore always handle all upstream components with the same care as an RNA product.

Modality considerations: no degree of RNase contamination is acceptable for any RNA modality; however, circRNA is resistant to any exonuclease contaminants.

Trait: RNA stability

Brief: RNA is a naturally unstable molecule due to nucleophilic attack of the 2′ hydroxyl on the 3′ phosphate, which is be catalyzed by alkaline conditions. It is therefore useful to store RNA in a slightly acidic buffer and avoid elevated temperatures. Many researchers may mistakenly attribute degradation to autohydrolysis when it is really due to RNase contamination. Our group has found that within the span of one day in divalent cation-free, pH ~6.5 aqueous samples, autohydrolysis is measurable only with the most sensitive capillary electrophoresis assays. Long-term storage requires ultracold freezer temperatures of at least –60 °C and slow thawing samples on ice is recommended, so planning ahead is key. Limit free-thaw cycles by freezing appropriately sized aliquots.

Modality considerations: The longer the RNA molecule, the more functionally sensitive it is to degradation. For example, 10 strands of a 1,000 nt long RNA transcript would require a minimum of 10 hydrolysis events to become inactive. However, a single 10,000 nt long strand of equal mass requires a single hydrolysis event to become inactive. Therefore, the longer and less structured the transcript, the more care is required. Furthermore, longer strands are more susceptible to shear stresses and it is likely that vigorous vortexing or peristaltic pumps could be detrimental to long strands. As circRNA typically exhibit higher structure, they may exhibit improved resistance to shear stresses.

Trait: product length/sequence

Brief: many commercial kits are optimized for transcripts of about one to several thousand bases long and assume uniform nucleotide inclusion. Any transcripts with a disproportional nucleotide usage will therefore be reagent-limited. In these cases, adjusting the ratio of NTPs used the IVT reaction can significantly improve yield. Protocols for short RNA often suggest extended reaction times; as long as overnight. This is likely due to the increased number of transcription initiation/termination events required for synthesis. Additional template and polymerase could be added to improve reaction rates, but it is important to consider the total nucleic acid-to-Mg^{2+} ratio in solutions when doing so.

Modality considerations: RNA polymerases tend to abort early in transcription. A consequence of the numerous transcription initiation events that occur for the synthesis of small RNA is the relatively high number of very small, aborted transcripts that accumulate. Therefore, for a given mass of taRNA, there is a much higher ratio of aborted RNA strands and 5′ termini compared to mRNA. Lastly, autocatalytic ribozymes for circRNA synthesis are active during the IVT reaction, and concatenation increases with circRNA length. Therefore, lower concentration/yield reactions are preferable to favor intramolecular vs intermolecular interactions for circularization.

Trait: reaction time

Brief: low yield reactions are often the result of low reaction rates, where increasing reaction time is a reasonable option. Factors such as low DNA concentrations, modified nucleotides, and shorter transcript length will require elongated reaction times. Since IVT

conditions are rich in Mg^{2+}, slightly alkaline, and elevated temperatures, RNA hydrolysis does occur. However, the minimal degradation resulting from increasing reaction time is generally acceptable for discovery or preclinical work. In our practice, doubling the DNA template concentration can help compensate for the reduced rate incorporated for modified nucleotides.

Modality considerations: most IVT reactions are typically complete within 2 to 4 hours. Overnight incubation can provide optimal yield for IVT of short RNA products, but this will inevitably come at some cost for integrity. For long RNA, there are diminishing returns for longer reactions times, as these strands are functionally more sensitive to degradation. Time-course assays to evaluate the yield plateau are easy to conduct and are encouraged during early scale-up for any modality.

Trait: temperature

Brief: reaction time and temperature are inversely correlated. For instance, the use of thermostable T7 RNAP enables high temperature reactions that increases reaction rate and weakens the strength of stable (high GC) regions that resist elongation. Higher temperatures also reduce dsRNA formation by preventing effective annealing and 3′ extension [23,24]. However, higher temperatures also increase the rate of RNA degradation and may other factors such as fidelity. Unless reactions are on the microscale (~20–100 µL), where temperature changes are rapid, consistency can be improved by ensuring the entire reaction mixture is brought to temperature before adding the RNAP.

Modality considerations: the integrity of long strands such as saRNA benefit from reduced temperatures [25]; however, the impact on dsRNA is not yet explored. For circRNA, the reaction temperature plays a role in ribozyme activity, as the ribozymes are brought together via 5′ and 3′ annealing. Therefore, reaction temperature likely plays a role in co-transcriptional circularization efficiency.

Trait: modified nucleotides

Brief: modified nucleotides, such as N1-methyl pseudouridine ($m^1\Psi$), have proven essential for the success of current mRNA vaccines; a discovery that led to the Nobel Prize award to Katalin Karikó and Drew Weissman [26]. They are incorporated at a slower rate, for which a longer reaction time or higher DNA template concentration can compensate. They can also impact fidelity. For example, $m^1\Psi$ is incorporated with greater fidelity than pseudouridine (Ψ) [27]. Importantly, recent studies have revealed that N1-methylpseudouridine results in ribosomal frameshifting, potentially impacting immune sensitive RNA applications [28]. However, inclusion of alternative modified nucleotides abolished this phenomenon.

Several IVT-compatible modified nucleotides can be used to improve stability and limit immune recognition, such as m5C, m5U, m6A, ΨTP, and s2U [29,30]. It is also possible to expand the type of modifications to include 2′-O-methyl NTPs using T7 variants, but this modification is not suitable for protein coding RNA as it results in tRNA rejection during translation [31,32].

Modality considerations: while mRNA has benefitted greatly from modified nucleotides, they are not universally applicable to all modalities. Some modifications abolish saRNA activity, possibly due to disruption of secondary structure, while others can enhance saRNA potency [33,34]. Similarly, autocatalytic circularization and IRES activity in circRNA are heavily impacted by modifications, due to changes to the necessary structural conformation for their activity. Partial incorporation is tolerated in circRNA, but for m6A, only minor stability benefits were observed [16]. Lastly, taRNA function through IRES-like mechanisms

for the recruitment of initiation factors, and therefore share a similar structural challenge with modified nucleotides.

Enzymes

Brief: the core essential enzyme in IVT is the RNAP, of which the T7, T3, and SP6 polymerases are effective options for RNA synthesis. The T7 RNAP is the most widely employed RNAP due to it being a simple, single-subunit enzyme with high usage, specificity, and activity [35]. Guanosine residues immediately following the promoter are required for efficient canonical transcription initiation, but different sequences can be used to preferentially incorporate cap-analogues.

Multiple groups are working on improving T7 characteristics such as incorporation efficiency of cap-analogues and limiting production of immunogenic products, which decrease the cost and purification needs of RNA [36,37]. The T7 RNAP starts transcription by recognizing the promoter, TAATACGACTCACTATA, and the adjacent downstream base is used to begin transcription. Novel T7 mutants, such as those developed by Codexis, can increase capping efficiency and reduce dsRNA contamination, thereby reducing cap-analogue costs and purification needs; however, there is a substantial cost associated with these enzymes.

Lastly, the triphosphate to monophosphate conversion provides energy required for elongation; however, it releases stoichiometric amounts of inorganic pyrophosphate. This by-product forms a magnesium salt that, in high yield reactions, reaches its solubility limit and precipitates. The addition of a pyrophosphatase prevents this by hydrolysing the inorganic pyrophosphate into the more soluble inorganic monophosphate.

Modality considerations: commonly used trinucleotide capping analogues such as TriLink's m7(3′OMeG)(5′)ppp(5′)(2′OMeA)pG or CleanCap AG require an 'AG' instead of 'GG' following the T7 promotoer and are effective for mRNA. To preserve the authentic alphavirus 5′ end, saRNA benefit from AU versions of the cap-analogue, while circRNA and most small RNA do not require any cap structure.

Buffer composition

Brief: the term buffer will be used broadly here to discuss the remaining components of the IVT reaction. Several commercially available buffers (such as those available from NEB, Promega, and Hongene) agree on three key components in their standard buffers: 40 mM Tris-HCl, 6 mM $MgCl_2$, and 2 mM spermidine (pH 7.9). The buffering agent itself, typically Tris-HCl, serves to maintain a slightly alkaline pH. However, improved yield was reported by Thermo Fisher Scientific when using HEPES from pH 7.6 to 7.9 [38]. This is in agreeance with a technical report from Roche, which also found HEPES slightly improved yield under their conditions [39].

The concentration of NTPs and Mg^{2+}, as well as the molar ratio of NTPs to Mg^{2+}, play a key role in reaction yield and quality. Typically, commercially available kit-based protocols recommend NTP concentrations of 5 to 8 mM NTP. In a 20 µL reaction with 1 µg of DNA, this typically affords 100–180 µg. In our hands, these 5 mM reactions have scaled well to least 2.5 mL (>12 mg yield), with agitation of 300–800 rpm and ensuring no bubble formation. While the source of magnesium for many commercial buffers is $MgCl_2$, acetate (via MgOAc) is a preferred counterion due to the inhibitory effect of chloride on recognition of the T7 promoter [40]. Perhaps more important is the ratio of Mg^{2+} to NTPs in solution. The ideal Mg^{2+} concentration is unique to each template and both reagent and goal-dependant. For example, maximum yield is typically achieved with Mg^{2+}/NTP ratios ranging from 1.25 to 1.875 [41–43]. However, reduced Mg^{2+} concentrations have been shown to reduce dsRNA generation [23]. Regardless, when batch feeding NTPs, it is important to

TABLE 1

Highly accessible purification methods for lab-scale production of RNA.

Technique	Pros	Cons	Comments
Silica spin columns	Rapid, kit-based, can separate high and low MW, good for low concentrations	Does not scale well	Consistent orientation of columns in fixed angle rotors is important
LiCl	High solubility of LiCl in 70% ethanol makes LiCl removal effective, preferentially precipitates RNA over DNA, protein	Time consuming resuspension for ~1 mg or greater, small RNA (200 nt) may not precipitate	Improved scalability over spin columns, but resuspension becomes challenging with masses over 10 mg
Ammonium/sodium acetate	Precipitates shorter strands	Time consuming resuspension for ~1 mg or greater, less selective precipitation	Phenol/chloroform can be used upstream to remove proteins
Magnetic beads	Removes incomplete transcripts, proteins, nucleotides, dsDNA, and can be automated for high-throughput	Beads can be expensive, can require more handling and steps	Primarily benefits RNA with template encoded poly(A) tail
Cellulose slurry	Scalable, removes double stranded RNA	Does not effectively remove dsRNA under 30 bp, no kits currently available	Typically used as a secondary, polishing step

also add Mg^{2+} to maintain similar Mg^{2+}/NTP ratios for activity.

DTT is regularly added as a reducing agent to combat protein oxidation (in the form of disulfide bonds) that inactivates enzymatic activity [44,45]. Enzymes repeatedly exposed to oxygen will require more DTT to reduce the disulfide bonds. Thus, DTT is typically added in excess, often 5 mM, as a precautionary measure. It is stable for years as a refrigerated solid and in our practice, with limited freeze-thaw cycles, frozen aqueous DTT solutions remain potent for at least a year. However, increasing pH and temperature dramatically reduces its half-life [46].

Lastly, spermidine is typically considered a critical additive that is thought to improve synthesis by interacting with inhibitory anions and improving polymerase dissociation after each round of elongation [47]. The addition of chaotropic agents such as urea can be added to limit dsRNA formation by nearly 80% with nearly no impact on yield, while improving the generation of desired-length transcripts [48,49].

Modality considerations: most modalities will face similar buffer optimization challenges, such as optimizing Mg^{2+} concentrations; however, chaotropic agents may play an interesting role in autocatalytic circularization of RNA. At sufficient concentrations, it is possible that these agents will alter IVT co-circularization efficiency due to modified secondary structure kinetics. If co-circularization could be effectively inhibited in these conditions, downstream changes in concentration, temperature, and buffer could improve control circularization efficiency. Similarly, urea could provide substantial benefit to saRNA, as the challenge to produce full-length transcripts increases with size.

PURIFICATION TECHNIQUES

Purification remains an under-appreciated challenge facing preclinical RNA production. The improper removal of ions, abnormal mRNA, DNA, or proteins can limit expression and pose potential safety hazards, especially outside of the vaccine space. This section will be divided into three parts: highly accessible (summarized in **Table 1**), capital intensive, and enzyme-based approaches.

Accessible approaches

At sub-mg scale, commercially available spin columns offer a very convenient strategy for purification. These columns bind RNA to

a silica bed and adjust stringency via buffer composition (salt, pH, ethanol) to selectively capture, wash, and release the RNA. While this is effective for buffer exchange and protein removal, incompletely transcribed RNA and dsRNA is not removed. As these kits are typically designed for tens to hundreds of microgram scales, they become impractical at multi-mg scales as the number of columns required per sample increases. When using fixed-angle rotors, it is important to ensure that the columns are always placed in the same orientation, as centrifugal forces focus samples and washing agents against the column walls. A swing bucket or vacuum apparatus effectively negates this concern.

A semi-scalable approach for purification is lithium chloride (LiCl) precipitation. This is a very simple method that relies on the ability for LiCl to preferentially precipitate RNA away from nucleotides, DNA, and protein. IVT reactions are typically high yield and readily precipitate in concentrations as low as 0.5 M LiCl [50]. In our lab, 5-minute incubation at –20 °C in 2.5 M LiCl, followed by 10 minutes at 16,000 xg, is sufficient for full recovery. It is commonly accepted that LiCl does not effectively precipitate small RNA strands such as taRNA and may not effectively precipitate dilute samples [51]. For circRNA synthesis, it may be possible to fine-tune this inability to precipitate small strands to effectively remove the small intronic by-products from autocatalytic approaches. The key drawback to LiCl purification is the time it takes to resuspend samples, especially once exceeding 10 mg. We have found that shaking samples at 25 °C to 37 °C until the pellet becomes translucent, followed by infrequent, careful, and gentle pipetting with a large bore tip to manually break apart the pellet, dramatically quickens the process with no appreciable loss in integrity. Similar to spin columns, LiCl does not remove incompletely transcribed RNA or dsRNA.

Sodium acetate (NaOAc) or ammonium acetate (NH4OAc), often in combination with ethanol or isopropanol, are alternative salt-based methods to precipitate RNA, but these salts also tend to precipitate proteins. Proteins can be removed via upstream phenol/chloroform extraction, but this adds the risk of phenol contamination and additional steps. A key advantage for these salts is that they effectively precipitate smaller RNA species and NaOAc is less inhibitory for downstream enzymatic treatments [52]. Low concentrations/small amounts of RNA can be more effectively precipitated with the addition of 1 µg/µL glycogen by increasing yield and ensuring a more visible pellet.

Typically, spin column or LiCl precipitation is sufficient for small-scale studies (e.g., <1 mg) and can be scaled up using multiple columns in parallel. If the removal of incomplete transcripts is desired, poly(dT) magnetic beads provide an affordable, low-scale option. Recall that poly(dT) affinity purification is best applied with template-encoded poly-A tails, to ensure that only full-length transcripts are captured. Of those discussed thus far, poly(dT) bead purification is the most complete purification approach, as it selectively retains polyadenylated RNA, thereby removing nucleotides, aborted transcripts, proteins, and salts. Poly(dT) bead purification is also translatable into highly scalable HPLC methods. The key drawback to poly(dT) bead purification, however, is the extra steps and proper handling required for effective purification. The method works well for poly(A) tailed RNA [53], but the highly structured nature of circRNA (containing poly(A) tracts) may limit its ability to be captured by poly(dT) beads.

The removal of dsRNA is important to limit immune responses; however, it remains possible that incomplete removal of dsRNA could provide beneficial adjuvant properties for vaccines. Progress in IVT design, via additives or enzyme mutation, is an effective method to reduce dsRNA production, but post-IVT dsRNA removal (polishing) will likely remain an important step for RNA drugs. Removal of dsRNA can easily be accomplished via

the cellulose chromatography method [54]. Currently, there are no kits available for this procedure and components must be individually sourced and prepared, but product recovery is at least 65% with over 90% dsRNA removal; whether the 35% loss sample is simply the result of abundant dsRNA removal or whether significant loss of ssRNA occurs is somewhat unclear. Regardless, this process is attractive for dsRNA removal for its purity and scalability. When applied for saRNA vaccine production, it drastically reduced innate immunity and improve efficacy of an saRNA vaccine [55].

Whenever purified RNA is recovered in a non-ideal buffer, either NaOAc precipitation, ultrafiltration columns, or benchtop tangential flow filtration (TFF) systems are effective methods for buffer exchange or concentrating samples. As IVT RNA molecules are large, it is easy to find columns with sufficient molecular weight cut-off (MWCO) to retain RNA.

Capital intensive purification

Investment into specialized equipment for RNA purification unlocks various options for automation, throughput, repeatability, and/or scalability. For example, magnetic bead purification methods can be effectively automated using a Kingfisher™ (ThermoFisher) instrument in a 96-well format. Combined with oligo(dT) beads, high-throughput and highly purified mRNA or saRNA can be achieved.

In contrast, HPLC or FPLC instruments offer lower throughput, but provide hands-free purification with incredible scalability and efficacy. Indeed, chromatography is considered the gold standard for mRNA purification, but due to the expense and the need for method development, is less commonly employed for small-scale production. It is also important to remember that each application may have its own purity requirements. For example, vaccines may benefit from some residual dsRNA. The following is a high-level discussion of widely accepted chromatography approaches, with modality specific considerations. The reader is recommended to also explore recent reviews that provide a more comprehensive description of chromatography methods [56,57].

Oligo(dT) affinity chromatography is fundamentally similar to bead-based purification. It is widely applied for mRNA and saRNA purification, which uses resin-anchored oligo(dT) strands to anneal poly(A) tailed RNA. If RNA is produced from a template with an encoded poly(A) tail, this purification method effectively removes incomplete strands, short RNA fragments, and many promiscuously initiated T7 transcripts. While this removes most undesirable compounds, cis-dsRNA remains as it is generated from 3' extensions after the poly(A) tail. In contrast, the absence of a poly(A) tail on taRNA prevents oligo(dT) purification. Instead, a custom oligo that matches the 3′ terminus of taRNA can be developed. Interestingly, some circRNA designs do carry a poly(A) tract [16]; however, oligo(dT) purification of circRNA has not yet been documented in literature. This may be due to higher structural stability associated with circRNA that could result in poor access and therefore, poor capture of these sequences. Currently, the utility for affinity purification of circRNA is not clearly defined.

If dsRNA removal is necessary after oligo(dT) purification, RNA can undergo a 'polishing' step using either hydrophobic interaction, reverse phase, or ion exchange chromatography. The latter is particularly appealing due to its ability to concentrate samples, tolerability to harsh washing conditions, and reduced sensitivity to fouling by proteins and aggregates [58–60]. A challenge for anion exchange chromatography is the need for elevated temperatures (50 °C to 70 °C) to effectively elute the RNA [61]. These methods have been well developed for mRNA and, by extension, most other RNA modalities.

Autocatalytic circRNA faces a unique challenge as the immunogenicity and ability of HPLC purification to sufficiently

purify circRNA is poorly defined [16,62–64]. Interestingly, the 3′ extensions that form cis-dsRNA are cleaved during autocatalysis, simplifying the removal of such species. However, if the circRNA strands lack a poly(A) tract, they simply cannot be purified using oligo(dT) capture. Size exclusion chromatography (SEC) of circRNA has proven challenging due to co-elution of immunogenic products and by nature, SEC does not scale well [62]. Regardless of which chromatography chemistry is used, the greatest challenge in circRNA purification is the removal of linear strands of similar length, often generated by hydrolysis of circRNA or the failed circularization of precursors.

Lastly, shear or other physical stresses are a considerable concern for RNA integrity, especially for saRNA. High turbidity and flow rates have the potential to cause shear stresses on RNA that physically cleave the molecules. This is a selling point for the monolithic columns which operate in laminar flow conditions and therefore, should be gentler on the RNA. Within the same concept, when filtration systems such as TFF must be used, it may be wise to avoid peristaltic pumps as they can create significant shear forces.

Enzymatic approaches

A commonly applied enzyme for RNA purification is DNase I, which is used to digest the template DNA present in the IVT reaction. DNase I can be post-transcriptionally added directly into the reaction mixture; however, the buffer and viscosity are important to consider as high yielding IVT reactions become viscous and limit efficacy of the enzyme. While this is easily solved by dilution, the nature of the diluent is important as DNase I requires both Ca^{2+} and Mg^{2+} for optimal activity [65]. For discovery or early preclinical work, electrophoresis is sufficient to confirm bulk DNA removal, but qPCR can be employed when higher sensitivity is required.

As previously mentioned, cap-analogues are attractive for reducing processing steps; however, whereas the vaccinia virus capping systems can reach nearly 100% capping efficiency, cap-analogues are incorporated with variable efficiency and their functional utility is impacted by their chemical structure [66,67]. Importantly, any uncapped RNA carries an immunostimulatory 5′ triphosphate, recognized by immune sensors such as RIG-I or MDA5 [68]. Cleavage of a short 5′ oligo (via RNase H, ribozyme, or DNAzyme) from RNA, followed by polyacrylamide gel electrophoresis, can be used to evaluate capping efficiency. Alternatively, capillary electrophoresis or mass spectrometry can offer high resolution analysis [69]. The use of a phosphatase ensures that any 5′ phosphorylated RNA have been immunogenically silenced.

Circular RNA purification benefits greatly from enzymatic treatment in two ways. Firstly, RNase R is a 3′ to 5′ exonuclease that specifically degrades linear RNA, which can be otherwise quite difficult to remove. Additionally, RNase R degrades much of the short intronic by-products that are formed during the circularization process; however, their partial dsRNA nature or structure seem to resist complete degradation. Further chromatographic purification helps remove the introns, but the treatment also results in significant nicking of strands. A phosphatase has sometimes been used to improve immune evasion from any phosphorylated RNA, which can arise from nicking or incomplete circularization [62,64].

FUTURE DIRECTIONS AND CONCLUSIONS

One cannot discuss RNA drugs without mentioning the challenge associated with both masking immunogenic RNA during delivery and facilitating cytoplasmic entry of these massive, polyanionic macromolecules. Lipid nanoparticles have largely addressed this challenge for vaccines via intramuscular injection, and Alnylam's patisiran drug effectively delivers siRNA to the liver following intravascular infusion, but precise and non-toxic delivery to

extrahepatic tissues remains a major challenge. While this topic is well beyond the focus of this article, the size and overall structure of unique RNA modalities play a role in encapsulation efficiency. For instance, extra long RNA (saRNA) require unique formulations from mRNA [70]. Organ-, tissue-, and cell-specific delivery is under intense investigation and with it, the potential for RNA drugs to treat an unprecedented number of diseases.

In the last decade, advancements in DNA synthesis have dramatically reduced the investments required to evaluate and synthesize RNA, and further automation of RNA platforms has the potential to similarly impact research- or preclinical-grade RNA production. Companies such as TelesisBio have already made progress towards this with systems such as the BioXp™. Continued development of such tools, especially with developing pre-set conditions for modality specific synthesis and automated purification has the potential to unlock very affordable, small-scale RNA synthesis. It is practically attainable to develop a platform that requires input of a purified template and delivers an effectively purified RNA product for nearly any modality.

To date, mRNA and saRNA vaccines are the only IVT-manufactured RNA drugs to reach market. While the design and synthesis of mRNA has become routine, purification is often an unappreciated challenge for discovery and preclinical work. For many reasons outlined in the 'Purification techniques' section, key purification factors can have major impacts on the success of new approaches. Therefore, developing a strong RNA production, purification, and QC platform is critical for the success of many RNA studies.

Here, gross challenges in developing new RNA modalities have been addressed. However, expanding RNA synthesis approaches will continue to broaden the success of current and emerging RNA modalities. A combinatorial approach that integrates both enzymatic and chemical RNA synthesis is of great interest for two reasons:

1. There are relatively few chemically modified nucleotides compatible with IVT; and

2. Incorporation of modifications during IVT is random, whereas site-specific modifications hold promise for improving RNA.

For example, site or region-specific modification of sgRNA significantly improves efficacy; however, chemical synthesis of these large strands can be prohibitively expensive. An effective approach in combining these two methods would dramatically reduce synthesis costs and unlock new possibilities for long RNA that depend on site/region specific or IVT-incompatible modifications. Additionally, there are many chemical or structural modifications that may extend cellular RNA half-life, and some examples already exist [71,72].

We are on the cusp of numerous, exciting translational medicine approaches that will redefine how we treat a vast number of diseases. Success of these projects will depend in part on effective workflows that balance purity needs and production costs for their specific modality and application. With each modality presenting its own unique characteristics and challenges, this discussion aimed to provide the foundation for these further discussions in the complex RNA landscape. As the field continues to evolve, so too will the strategies and technologies that enable these interventions, paving the way for new RNA drugs.

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AFFILIATION

Andrew Varley
Director
The University of British Columbia,
Vancouver, BC, Canada

AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

Production of mRNA by *in vitro* transcription utilizing a single-use system

David Sokolowski, Global Workflow Manager, Nucleic Acid Therapeutics, Cytiva

***In vitro* transcription (IVT) is an important process within the mRNA manufacturing workflow, and scale-up of this process needs to be carefully considered. This poster explores a study demonstrating IVT reactions in the ReadyToProcess WAVE™ 25 bioreactor, which can be used in small-scale mRNA manufacturing workflows and can be scaled up where needed.**

In *in vitro* transcription (IVT) reactions, the DNA template, RNA polymerase, nucleoside triphosphates (NTPs), pyrophosphatase, and IVT buffer are combined to produce mRNA. **Table 1** provides an overview of the IVT process.

Optimization of the IVT step is critical for RNA quality. Optimization ensures stability of the mRNA, minimizing undesirable truncated or double-stranded products, and allows for successful scale-up. The reaction constituents and ratios need to be optimized, while the manufacturing conditions such as temperature and time need to be precisely controlled. This optimization can be achieved using the ReadyToProcess WAVE™ 25 bioreactor system.

SCALING UP AND REDUCING RNASE CONTAMINATION IN IVT

The ReadyToProcess WAVE bioreactor system and single-use bags provide a controlled environment with efficient mixing and temperature control in a closed system. Single-use Cellbag™ bioreactor containers with Bioclear™ film make the process scalable from process development to manufacturing in a GMP environment. WAVE™ bioreactor bags, which are presterilized and disposable, require no cleaning and minimize the risk of cross-contamination. Furthermore, the manufacturing process data generated can form the basis of electronic batch records, providing in-line data for GMP-certified processes.

Table 1. Overview of the IVT step and considerations to be made to ensure success of the process.

Enzymatic synthesis and capping of mRNA	
Overview	**Considerations**
• Chemical synthesis of RNA is limited to less than 200 base pairs. The discovery of RNA polymerases allows the synthesis of long RNA transcripts through IVT. • In IVT, the DNA template, RNA polymerase, nucleoside triphosphates (NTPs), pyrophosphatase, and IVT buffer, are combined to produce mRNA. RNase inhibitor is often used but not required for the reaction itself. • RNA polymerase copies the DNA to RNA. • DNA templates can be a plasmid product, PCR, or cell free production methods. • mRNA is capped to protect and promote function. • During transcription, a poly(A) tail can be added (for stability) by designing it into the template. Otherwise, it would be added enzymatically post transcription.	• Capping can occur co-transcriptionally and post-transcriptionally—both via different methods (e.g., enzymatic, chemical), which will impact on other steps. • Careful control of reagent quality and processes is needed to ensure stability of the mRNA, minimize undesirable products, and ensure successful scale-up. • Reduce RNase wherever possible with careful processes, nuclease-free components where possible, and single-use consumables. • Consider automating steps as much as possible for GMP processing. Manual methods are generally not scalable for manufacturing.

IVT CASE STUDY

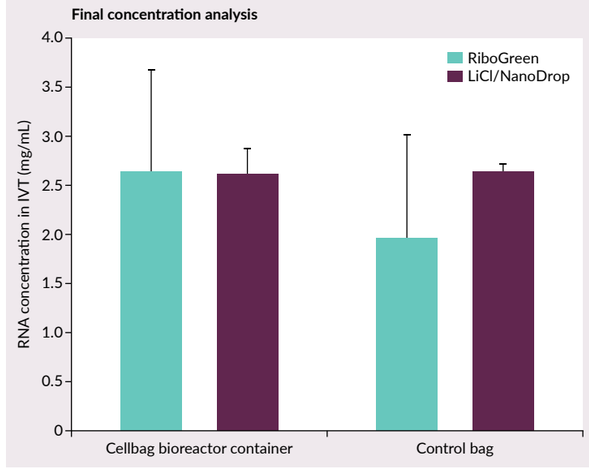
In a case study, IVT reactions (3×150 mL) were performed with the WAVE 25 rocker under the following conditions:

- Reaction volume: 150 mL
- Angle degrees: 6° to 7°
- Rocking speed: 40 rpm
- Target temp: 37°C
- Heat-up time: 30 to 35 min

The entire process spanned 5 to 6 hours, encompassing setup, reaction, and DNA digestion. IVT incubation lasted 2 to 4 hours, which was contingent upon the construct, followed by a 30-minute template digestion step. EDTA was introduced to halt enzymatic reactions.

Analysis of the final concentration of mRNA after IVT, template digestion, EDTA addition, and 5× dilution is shown in **Figure 1**. Triplicate IVT reactions were performed for each condition. The RiboGreen assay demonstrated higher variability than the lithium chloride (LiCl)/spectrophotometric method. Moreover, RiboGreen testing showed no significant difference between the Cellbag bioreactor container and control IVT reactions (*t*-test, $p>0.05$). The LiCl/spectrophotometric method also showed no significant difference in final mRNA concentration between the Cellbag bioreactor container and control IVT reactions (*t*-test, $p>0.05$).

Figure 1. Scale up of IVT using ReadyToProcess WAVE 25 bioreactor



The final concentration in the 2 L Cellbag bioreactor container is 5× lower than directly after the IVT reaction. This difference is due to buffer addition for template digestion and EDTA addition to reduce the magnesium pyrophosphate complex formed during the reaction.

In conclusion, high quality RNA can be successfully generated within the WAVE 25 bioreactor for further purification downstream.

NUCLEIC ACID INSIGHTS

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NUCLEIC ACID INSIGHTS

mRNA PROCESSING AND ANALYSIS

SPOTLIGHT

INTERVIEW

Moving mRNA–LNP therapeutics towards the clinic: overcoming myths and misconceptions to solve the specificity challenge

The COVID-19 pandemic catapulted mRNA into the spotlight—but what wider impacts did this success have on the field? **David McCall**, Senior Editor, *Nucleic Acid Insights*, speaks to **Ansgar Santel**, CEO and **Jörg Kaufmann**, CSO, of **Pantherna Therapeutics** about the latest developments in the mRNA–LNP space, tackling myths and safety concerns surrounding cationic lipids, and Pantherna's approach to solving the issue of moving beyond hepatocyte targeting.

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Q What are you working on right now?

JK: Our major goal is making mRNA a reality as a new modality of therapeutic. Everybody watched the success of the COVID-19 vaccination programs, but we had already been working on using mRNAs as drugs in therapeutic settings for some time. The delivery side of the scientific problem is the major hurdle, and that is the primary focus of our research.

There are challenges to address associated with finding the indications that best fit the modality, and also with limitations of the current delivery systems. Additionally, there are manufacturing problems because mRNAs and LNPs are very complex and expensive to produce, especially for small biotech companies. And there are obviously also some toxicity issues associated with LNPs to overcome.

Our lead program targets acute respiratory distress syndrome (ARDS). We purposely avoided going into a chronic field to begin with, preferring instead to target an acute disease indication where a course of only one to two treatments is required.

AS: Jörg and I both come from the RNAi field, so we are very familiar with the challenge of delivering nucleic acid to a desired cell type. Due to our 20 years of experience, we are aware of all the problems we are facing. Some we believe we have already solved, but there is of course room for further improvement. For example, the latest developments in the field, especially around ionizable lipids, are helping us to better understand how LNPs need to be constructed.

This mitigates the risk of running into toxicity problems at an early stage. It was important to us that we learn our lessons first within an acute setting. Nevertheless, ARDS is a big challenge. It is a difficult disease scenario with many different pathologies contributing to the process. We are currently moving our lead therapeutic program—PAN004—for ARDS toward IND-enabling studies. PAN004 is an mRNA expressing a TIE2 agonist in the lung endothelium, for preventing vascular leakage during lung infection. We are using a delivery system based on positively charged LNPs, with targeting specificity for the capillary endothelial cells of the lung.

On the technology side we are expanding our LNP delivery platform (PTX-LNPs) by exploring new LNPs in different *in vivo* settings.

Q Tell us more about your approach—what differentiates it?

JK: The 'classical' LNPs in the field are neutral and based on ionizable cationic lipids. We are taking an additional, different approach because we want to move away from hepatocyte targeting, especially for systemic treatment, as neutral LNPs based on ionizable cationic lipids predominantly target the liver. We are working with non-ionizable cationic lipids in addition to ionizable, and our goal is to achieve safer cell type-specific targeting in other organs.

We are experimenting with the surface charge of the particles—we can have positive, negative, and neutral surface charges. These lead to different distributions in the body, and uptake by different cell types. This is our edge, because the rest of the field is mainly working with neutral LNPs. There is a belief that they are safer, but they do have the limitation that for different cell types they are not very potent because they are always shuttled into the liver.

The wider field is starting to realize that you have to identify the cell type you want to target, and this cell type needs to fit the indication. If you want to treat cancer, delivery to hepatocytes doesn't work. Despite this, historically, a lot of people did try this approach. People thought

> "The wider field is starting to realize that you have to identify the cell type you want to target, and this cell type needs to fit the indication. If you want to treat cancer, delivery to hepatocytes doesn't work."

that classical neutral LNPs would fit all indications, and that is something that needs to be reconsidered by the whole field.

AS: The defined composition of the lipids used to build an LNP do make a difference and can lead to a kind of cell-selective delivery. We are addressing this, mainly in preclinical *in vivo* studies, to get a direct idea of which ones can be applied for a certain disease and a certain tissue when you know that targeting a defined cell type is critical for the outcome.

Q As two people who were actively working in the mRNA–LNPs space prior to the COVID-19 pandemic, how do you reflect today on the impact that the success of the COVID-19 mRNA vaccines has had on the space—for instance, in terms of some of the misconceptions that currently exist in the field?

JK: For us, as a company working on delivery research, the key misconception coming from the success story of COVID-19 is that people who are new to the field think that the delivery problem is solved. They believe that here is a wonderful, proven, safe delivery system that they can apply to everything. At the same time, manufacturing organizations saw the money generated from pandemic production of vaccines and moved into this field—it appears to us that some are now offering solutions for *in vivo* delivery without having done a single experiment in this context. They are basically repeating the production of neutral LNPs and claiming it can be used for various applications. This is exactly the opposite of what we are claiming. I am not saying that these first-generation LNPs are not useful; they clearly are useful for—for example, in classical vaccination applications. However, we would argue that in the therapeutic context, you have to work primarily and specifically on developing a delivery system that fits with your target cell type and indication.

Another misconception is that people believe that only neutral LNPs are safe. At Pantherna, we had already worked with different charges on LNPs in another context, and we believe they are also safe. There are some old papers discussing the use of first-generation cationic lipids such as DOTMA and DOTAP. They showed some toxicity in mice, and this led to the historical misconception that you cannot use these non-ionizable, permanently charged lipids *in vivo*. As a consequence, almost everybody stopped using them. However, in our view, restricting the whole field to only neutral ionizable LNPs is not the right approach.

AS: From a translational perspective, the manufacturing and the CMC process remains a big bottleneck. If you plan to use neutral ionizable LNPs as the basis for a delivery vehicle and then enhance the potency by adding something else on top, this strategy leads to even more complicated LNPs and a more complicated mRNA–LNP drug product, which is even more difficult to manufacture. We want to make these LNPs simpler, not more complex.

For example, the standard vaccine mRNA–LNP consists of a four-lipid moiety. There are even companies who add a fifth lipid, in order to improve specificity. For our lead formulation, we actually reduce it so that we only need a three-lipid moiety. Our prototypes tell us that it makes sense to invest in the formulation because then translation into the clinic becomes easier and cheaper, especially with regards to manufacturing.

You can decorate these LNPs and play around with different approaches, but the bottom line is always: is this scalable, and what challenges will you face in order to manufacture it and test it in the clinic? Each new lipid is also a new chemical entity, which may have unexpected pharmacological activity. This is something many people who are new to the field tend to neglect.

Q Can you expand on the key limitations of LNP technology in its current state, particularly in the therapeutic setting?

JK: Currently, there is only one approved nucleic acid therapeutic utilizing an LNP—Onpattro from Alnylam. It uses a neutral LNP to target hepatocytes with a siRNA molecule. This is essentially the gold standard, and a lot of people are aiming to repeat this.

However, to go beyond hepatocyte targeting, we have to think more broadly in terms of different lipid systems and charges, and even adding ligands. The field essentially has to start over again (and not for the first time in its history...). It is an iterative process for the whole community, and we shouldn't restrict ourselves to working with neutral LNPs.

Another big limitation for the field is that we are working with *in vivo* delivery systems. In order to find out if a given system works, you need to go *in vivo*. This is very challenging because you cannot make some predictions from *in vitro* data and then just doing some high-throughput screening. At the end of the day, you have to inject it into an animal model. Then, even more challenging, you have to translate this data to non-human primates, and then to humans.

Some of these early successes in rodents might not be repeatable in non-human primate environments. It happens to us, and to others: someone develops a beautiful system for mice or rats, and then they move to the next step and it doesn't work, or shows very high toxicity. Alongside this, we are always under the restrictions of finding an approach that needs to be manufacturable, feasible, and sufficiently cost-effective. It is a big problem that contains four or five different smaller problems. If you solve three or four of them, but not the fifth, you still don't have a solution.

Looking at another aspect, you have two major problems with LNPs. In general, these LNPs are around 60–80 nm in size. Even though that sounds pretty small, it is still very large compared to small molecules and in itself, can limit penetration deeper into tissues because it cannot readily diffuse through the body. The other issue is associated with nanotechnology in general: you have a small volume but a large surface area. If you are injecting nanoparticles, you are in effect injecting square meters of foreign surfaces, which the body recognizes, reacts against, and toxicity is observed. People have now started to solve this last issue with premedication.

AS: An additional challenge that remains is the pharmacology of LNPs. As discussed earlier we are dealing with a multi-compound drug: a nucleic acid plus three, four, or five lipids. This is administered to a body, so especially with the systemic route, we are then dealing

"[Single-cell RNA sequencing] information, perhaps along with AI in the future, will help us to learn how a LNP can be designed effectively for cell selectivity."

with challenges relating to morphology, circulation, and how the body reacts to this non-viral intruder.

These lipid compositions have multiple components, and they all have some kind of intrinsic pharmacological activity. You have to figure this out in the solid state, but you also have to think about the disintegrated state once it is delivered. What happens after the cargo and the LNP are delivered to the cell, and what actually happens to all the LNPs that don't reach the target cells?

Next, you have to think about long-term usage. Applying these LNPs repeatedly is possibly the biggest challenge we face, and something we need to learn much more about.

Q What are the keys to addressing these limitations? For example, what emerging tools do we have available to enhance tissue tropism?

JK: Moving towards local administration will help to avoid systemic toxicity and achieve higher concentration at the site of injection. Another solution is better standardizing LNP particles, and avoiding heterogenous LNP preparations.

On the research side, we are also working on targeting ligands. There are a lot of efforts underway in the field to put ligands on the surface of the LNPs—antibodies, chemical ligands, or even nanobodies. We have some collaborations we are working on in this area. As we discussed previously, the danger to avoid is making the system more complicated. We need to see a significant pharmacodynamic and cell-type-specific targeting benefit by adding these ligands.

There is also the prospect of using AI or machine learning to look at the lipid system *in silico*, and generate a prediction of which cell type it goes into *in vivo*. Right now, in our view, the data sets are probably not homogeneous enough to feed into machine learning tools. We still have no good tools to predict if a particular LNP system will hit a certain cell type *in vivo*.

AS: It also depends on the scientific attitude—for example, how thoroughly you investigate cell-selective delivery—because most of the data sets we see are on the organ level, and there is very little on the cellular level. We believe single-cell RNA sequencing is a good readout to understand cell-selective delivery of the nucleic acid cargo because this is the method that offers the highest granularity in terms of delivery. This information, perhaps along with AI in the future, will help us to learn how a LNP can be designed effectively for cell selectivity. Or, to put it another way, for certain selectivity for a cell signature; it doesn't need to be only one cell type.

I have also noticed a bit of a change in thinking around mRNA therapies—for example, around starting with the idea of using gain of function in gene replacement. I believe that mRNA is seen by many as primarily being a non-viral delivery alternative to AAV in making genome editing a reality. For gene therapy, this might also circumvent or bypass many of the problems around dosing and administration.

Q How do you see the cationic versus ionizable lipids debate continuing to unfold moving forward, particularly in terms of their relative toxicity profiles?

JK: We are a small company with a variety of different LNPs. I would not even view it as ionizable versus non-ionizable; you need to test a lot of different LNPs with all the cationic lipids available. Every system is worth testing, including ionizable and non-ionizable. If you want to improve vaccination, you would probably stick to the neutral ones, maybe ionizable, or you can make them neutral without ionizable lipids. If you want to discover something new, though, you could try negative lipids in combination with positive.

As discussed earlier, there continues to be a belief that you cannot use anything except ionizable lipids, but that is a myth. We are quite happy with the tox profile for our program, which is a positively charged LNP targeting the lung endothelium. We have not seen any major toxicity issues to date.

AS: Additionally, improvements have been made with the new generation of cationic lipids, which, for instance, exhibit chemical structures that can spread the positive charge (e.g., guanidino groups), and lipids that even exhibit multiple positive charges. Generating this type of charge spread and charge density profile may even translate into less toxicity. We hope that we can get rid of these myths surrounding cationic lipids with our PAN004 program.

Additionally, it is important to bear in mind that the cationic lipid is only one of several components in these multi-component drugs that can contribute to toxicity. Ultimately, the dose makes the poison, and this is the aspect that you always have to figure out. Therefore, if you haven't done any pivotal toxicity studies, you cannot making a sweeping claim that cationic lipids are bad.

Q What are some key goals and priorities, both in your own roles and for Pantherna as a whole, over the foreseeable future?

AS: Our overall goal is to translate our technology and get one of our mRNA–LNP concepts into clinical use. With PAN004, our prototype, we are now close to moving this from the bench towards the bedside. Again, the big financial challenge is the manufacturing. Once we have overcome this obstacle, we are ready to go for IND-enabling studies, and all the way to the clinic. In addition, we have several ideas around developing new LNPs.

JK: Another major priority is to gain support. A lot of people are very interested in mRNA, especially in the therapeutic setting. But convincing people to fund R&D programs in the space is not easy, as big pharma remains skeptical. We want to convince people to help get our company in a growth mode and realize some of our visions.

AS: In some ways, this is an even bigger limitation than the limitations of LNPs. At the end of the day, we need the money to make this a reality, and we hope to raise awareness of our approach and secure funding. This will help us to translate all the different LNPs that we have developed, including those for other indications and for more local delivery. We are also open

to collaborations with companies that have a therapeutic concept that can employ and exploit an mRNA–LNP approach.

BIOGRAPHIES

ANSGAR SANTEL is CEO at Pantherna Therapeutics GmbH, Hennigsdorf, Germany and joined the company in November 2021 as Head of Translational Research and Alliance Management, leading the early development of Pantherna´s mRNA drug candidate PAN004. Previously, he acted as Technology Development Manager at Ascenion GmbH in life science innovation management and worked prior to that for more than 15 years in various roles in the biotech industry (Silence Therapeutics plc, Atugen AG) on the preclinical and clinical development of RNA therapeutics.

Santel studied biology and holds a PhD in Molecular Cell Biology from the Philipps-Universität Marburg, Marburg, Germany, and was postdoctoral fellow at the Stanford University School of Medicine, Stanford, CA, USA. He is author of more than 30 peer-reviewed research publications and named inventor on several patents/patent applications.

JÖRG KAUFMANN is the co-founder of Pantherna Therapeutics and CSO since the beginning of the company in 2017. Before this, he served as CSO of Silence Therapeutics plc, and he had senior management positions at Atugen AG and SR Pharma, after he had researched as senior scientist at Chiron Corporation in genomics, oncology, and new technologies, in the research field of nucleic acid therapy and oncology.

Kaufmann holds a PhD in Molecular Genetics from the Philipps-Universität Marburg. Then he was a post-doctoral fellow at the Howard Hughes Medical Institute, UCLA, Los Angeles, CA, USA followed by a group leader position at the Institute for Molecular Biology and Tumor Research (IMT) at Philipps-Universität Marburg. He is inventor on several patents and patent applications and is author of more than 30 peer-reviewed scientific publications.

AFFILIATIONS

Ansgar Santel
CEO,
Pantherna Therapeutics,
Hennigsdorf, Germany

Jörg Kaufmann
CSO,
Pantherna Therapeutics,
Hennigsdorf, Germany

AUTHORSHIP & CONFLICT OF INTEREST

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