OCTOBER 2024 Volume 1, Issue 8

NUCLEIC ACID INSIGHTS

BIOINSIGHTS

CONTENTS

Content pillars

mRNA

Formulation and delivery

Plasmid DNA

Oligonucleotides

NUCLEIC ACID INSIGHTS

mRNA: PROCESSING

SPOTLIGHT

INTERVIEW

Advancing mRNA production through platform and continuous process development

The field of mRNA therapeutics is undergoing transformative growth, driven by innovations in both process development and automation. However, with this progress comes a number of complex challenges. **David McCall**, Senior Editor, BioInsights, speaks with **Zoltán Kis**, Senior Lecturer at The University of Sheffield, about platform technologies, continuous process development, advanced automation, and the future of mRNA manufacturing.

Nucleic Acid Insights 2024; 1(8), 301–308

DOI: 10.18609/nai.2024.037

Q What are you working on right now?

ZK: I lead a research team focused on innovating and digitalizing manufacturing processes for producing mRNA vaccines and therapeutics to combat multiple diseases. Our goal is to create technologies that can be used both in 'peacetime' and during regional epidemics or larger global pandemics, so we can respond more efficiently to outbreaks.

As we saw in the recent COVID-19 pandemic, vaccines were not distributed equitably around the world. We are developing technologies that can be implemented globally to produce

"By nature, enzymatic synthesis is faster...It is a more efficient way to rapidly prototype and screen different products, and it is also more scalable."

these medical products as needed during outbreaks. Even in between outbreaks, we aim to establish infrastructure where these technologies can be used to create candidate vaccines and therapeutics for trials, which could then be developed into preventive or curative treatments.

Our work focuses on the technology side, particularly on streamlining manufacturing processes by increasing automation, improving product quality, and finding ways to use the same manufacturing process for multiple products rather than having to create a new process every time. The aim is to develop a single process that can efficiently produce a range of mRNA-based medicines.

Q Can you frame for us why innovating and digitalizing RNA vaccine and therapeutics production platform technologies is necessary for the field?

ZK: We view this as a very new and exciting technology. While it has been developed over decades through numerous trials and gradual improvements, it was only deployed at a large scale for the first time during the COVID-19 pandemic. There are now exciting opportunities to make this process more efficient for vaccine production. Unlike traditional methods, this is not a cell-based but a cell-free, enzymatic process. By nature, enzymatic synthesis is faster because you do not have to grow and maintain cells to produce your product. It is a more efficient way to rapidly prototype and screen different products, and it is also more scalable.

Additionally, we believe that this process can be implemented in a continuous flow format. Most vaccines are made in batch processes, which are inefficient since they do not make the best use of resources, space, or time. Batch processing involves multiple steps, with each step waiting for the previous one to finish. For example, in fermentation, all the purification equipment remains idle while the fermentation process runs through its stages. Alternatively, in a continuous process, materials flow continuously through the manufacturing process. This makes it far more efficient. In addition, in terms of productivity—how much product you generate per unit time and per unit scale—continuous processes for mRNA can be five to ten times more productive than traditional batch processes. This efficiency allows for the production of more doses in a smaller footprint, which is important because GMP facilities are expensive to build and maintain. Moreover, automation can further reduce the number of people required. In batch processing, for example, you might need at least two people for each step—one to perform the task and another to verify it in a GMP setting. With ten steps, that could mean twenty people per shift. A fully automated, continuous process, on the other hand,

could run with just a couple of people overseeing the entire system, drastically reducing labor requirements.

In short, maintaining surge manufacturing capacity with an automated continuous process is far more efficient and sustainable compared to the manual, multi-step batch process.

Q What are some of the key challenges currently standing in the way of manufacturing tech platform development and validation in the mRNA space?

ZK: The concept of platforms is relatively new and still evolving. Ideally, we want to combine prior knowledge from existing products with the same technologies to efficiently develop new products. With mRNA platforms, much remains the same when producing different RNA sequences. For example, your bioreactors, raw materials, analytical methods, and even DNA components can stay the same. In some cases, even the formulation might remain unchanged, depending on the product. We need to figure out how to leverage this knowledge to develop new products with less paperwork while still proving that the new product is safe and effective. One solution might be to start by developing different products for the same virus, such as for different strains of influenza or coronavirus. This is likely a lower-risk, simpler starting point. Switching between viruses—or from vaccines to therapeutics, for example—is much more complex because many things change, including delivery and formulation. Safety and efficacy are always top priorities, but there may be smarter ways to ensure them by using platform knowledge. If the process has consistently worked before, it is likely to work for similar products, reducing the need to fully revalidate everything.

The platform approach does come with some difficulties, and discussions with regulators around these are ongoing. Again, the ultimate challenge is ensuring product safety and efficacy for patients. To do that, we need to differentiate what is specific to the product and what is specific to the platform. The key is to validate the platform side and understand exactly what changes and what stays the same when moving from one product to the next. Once certain aspects are proven not to change, they can be considered part of the platform, allowing us to focus only on validating what does change. On the process side, we are mapping out the interactions between process parameters and how they affect product quality attributes. We use both experimental data and modeling to map these interactions, but there are many variables. There are over 20 quality attributes and numerous process parameters to consider, and they all impact the final product.

We map out what we call the 'design space' of the process parameters—essentially, a range of conditions under which the process produces a product of the desired quality. Each quality attribute has an acceptable range, and by staying within this design space, we ensure consistent product quality. This design space gives us the flexibility to make slight adjustments when switching from one product to another—for example, when dealing with RNA products of different lengths.

Our ultimate goal is to create a multi-product design space. This involves experimental work and modeling to understand the fundamental driving forces and conversions in the process (e.g., kinetics of enzymes and other processes, as well as mass balances within the system). By understanding how process parameters impact product quality, we can run the process more efficiently for multiple products without having to fully revalidate everything each time.

Q What are the key specific upstream and downstream process steps or technology areas on which you are particularly focused, and why? And what are the specific goals or benefits of a platform approach in these specific areas?

ZK: We are working on various different steps in the process, both upstream and downstream, as well as on formulation. On the upstream side, in batch production, a vessel is typically used to mix reagents and produce the product. In a continuous process, we use a continuous reactor, like a tubular reactor mimicking plug-flow conditions or a series of continuous stirred-tank reactors (CSTRs). We have tested various commercially available reactors, but not many were designed for mRNA production. Instead, they were often created for organic chemistry or flow synthesis. So, while they seemed suitable, we have found limitations and are now designing better reactors that outperform the commercial ones.

For instance, we have a reactor made up of small CSTRs stacked together, with ten reactors in a series. Material flows from the bottom, reacts, and moves to the next reactor. An impeller mixes the contents, allowing us to control the mixing independently from the flow rate. In other reactors, like tubular reactors with static mixers, the flow rate affects the mixing intensity. If you flow too slowly, mixing is weak; too fast, and there is not enough time for the reaction. Our goal is to design reactors that ensure good mixing, heat transfer, and product quality.

One interesting aspect we are exploring is residence time distribution, which measures how long molecules stay in the reactor. All molecules don't spend the same amount of time in the reactor—there is a distribution, with some molecules staying longer than others, which can impact product quality. If molecules spend too much time in the reactor, they can degrade or form by-products like double-stranded RNA (dsRNA), which is highly immunogenic and can cause side effects. We are trying to minimize this distribution to reduce degradation and dsRNA formation.

On the downstream side, we have set up a multi-column chromatography system. This system continuously purifies the material coming from the reactor. By using two columns in the loading zone, we can capture more product, increase the utilization of the resin and minimize losses compared to a batch process. The columns cycle in tandem, making the process more efficient and reducing product loss. We are also exploring continuous processes for filtration and lipid nanoparticle (LNP) formulation, optimizing each step to ensure the best possible product quality and process performance.

"The idea is to use the experimental data to build mechanistic models that simulate thousands of scenarios quickly."

For each of these steps, we conduct experimental and modeling work. The idea is to use the experimental data to build mechanistic models that simulate thousands of scenarios quickly. This saves time and resources compared to running physical experiments for each scenario. Once we identify optimal conditions via modeling, we validate them experimentally and refine the models if needed.

These models can even run in real-time during production. By measuring parameters like ultra-violet light, pH, and conductivity, the models can predict outcomes like yield or product quality ahead of time, allowing us to take preventive actions if issues are predicted. For instance, if the model suggests that the yield might drop out of spec in the next 10 minutes, we can adjust parameters to prevent the issue before it occurs. This approach allows for automated, proactive control of the process, improving efficiency and product quality.

Q Would that be a digital twin approach?

ZK: Exactly. When people talk about digital twins, they often mean different things. For some, a digital twin is simply a model that runs independently of the process, but for us, that is not the true definition. In our view, a digital twin is a model that continuously receives real-time data from the process through an automated data flow, eliminating the need for manual input. This data flows from the equipment into the model, which replicates the process digitally in the computer.

The key aspect is two-way communication. Not only does the model receive data, but it also sends control actions back to the process equipment. This is what makes it a true digital twin—an interactive digital copy of the physical process. If there is only one-way communication, where the model receives data but does not send anything back, we call it a soft sensor. A soft sensor processes data and calculates performance indicators or quality metrics that cannot be measured directly. While useful for monitoring, it does not control the process.

The digital twin, however, is designed for both monitoring and advanced process control. Specifically, it enables feed-forward control, where issues are corrected before they happen, as opposed to conventional feedback control (e.g., through Proportional—Integral—Derivative controllers, which only respond after a problem has occurred). Feed-forward control is more proactive and advanced, helping prevent errors and reducing the need for manual oversight.

In essence, this advanced automation reduces the need for constant human intervention, making the process more efficient and robust.

Q Looking to the future, what will be some critical next steps in mRNA manufacturing technology and process innovation as the field continues to expand into new application areas and indications?

ZK: As you can imagine, there is a lot happening in this rapidly expanding field. While we primarily focus on the process side, there is much more beyond that. On the product side, different RNA modalities such as mRNA, self-amplifying (sa)RNA, and circular (circ)RNA are being developed, each offering distinct advantages for various applications as vaccines or therapeutics. There is also significant work being done to improve RNA targeted delivery into various cell types and tissues in the body. There is additional work on stabilizing RNA to make it thermally stable and more efficiently deliverable to cells. This will enhance efficacy and safety at lower dosages. Additionally, there is innovation around new reagents for the process—enzymes, capping reagents, and formulation components.

From our perspective, we are especially excited about the platform concept and how it can be applied to create products targeting different diseases. We are developing new equipment and kits tailored specifically for RNA production. Most of the current equipment was originally designed for proteins and then adapted for RNA, and it is not always optimal. Now, we are focused on creating purpose-built tools that address the unique needs of RNA production.

One of our major projects, funded by Innovate UK, is the development of what we call the 'RNA box'—a benchtop, end-to-end continuous system for RNA synthesis, purification, formulation, and sterile filtration, resulting in a final drug product. We started less than a year ago and are still in the process of building it, but we are confident that once completed, this continuous and automated process will be transformative. It will not only make production more efficient but also accelerate development. Researchers could use this box to quickly prototype different RNA sequences for vaccines or therapeutics without the need for outsourcing or large teams.

This approach will enable rapid research, quicker product development, and eventually, large-scale GMP production. The beauty of the RNA box is that it scales predictably, making it suitable for everything from early-phase clinical trials to large-scale manufacturing. This will eventually be an output that, while we might publish findings on it, will primarily be available as a device for purchase and use. The goal is to make this technology accessible globally, so countries can easily adopt it and start producing products tailored to their local needs.

Q What else should we be looking out for from your lab in the near future?

ZK: In addition to the RNAbox project and our work on advanced automation and continuous process development, we are focused on developing faster methods for analyzing product quality—something that is crucial in continuous processing. Ideally, we want to

predict product quality, but real-time analysis is impossible for multiple quality attributes and manufacturing performance indicators. It is not acceptable to run a process for 10 days, only to find out something went wrong on day 1. That wastes resources, time, and money. Therefore, real-time or predictive measurements (e.g., future, model-based readouts) of product quality and performance indicators are vital.

As you can imagine, we are quite busy with all of this, but it is exciting work that has the potential to transform the field.

BIOGRAPHY

ZOLTÁN KIS is a Senior Lecturer (Associate Professor) at the School of Chemical, Materials and Biological Engineering at the University of Sheffield, Sheffield, UK and an Honorary Lecturer at the Department of Chemical Engineering, Imperial College London, London, UK. Zoltán is leading a multidisciplinary team that is innovating and digitalizing RNA vaccine and therapeutics production platform technologies. His work is addressing the challenges of producing large volumes of RNA-based vaccines and therapeutics, rapidly, at high quality and at low cost in a disease-agnostic manner. Zoltán has previously worked as a Research Associate in the Future Vaccine Manufacturing Hub at Imperial College London. He obtained his PhD in Bioengineering from Imperial College London and holds an MSc in Applied Biotechnology and a BEng in Chemical with Biochemical Engineering.

AFFILIATION

Zoltán Kis
Senior Lecturer,
School of Chemical, Materials and Biological Engineering,
University of Sheffield,
Sheffield, UK

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

Funding declaration: The author has received funding from the Biotechnology and Biological Sciences Research Council (BBSRC), Innovate UK, the UK Department of Health and Social Care and Engineering and Physical Sciences Research Council (EPSRC), and the Wellcome Leap RNA Readiness and Response (R3).

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Interview conducted: Aug 28, 2024; **Revised manuscript received:** Sep 23, 2024;

Publication date: Sep 25, 2024.

NUCLEIC ACID INSIGHTS

mRNA: PROCESSING

SPOTLIGHT

INNOVATOR INSIGHT

Process optimization of mRNA purification for vaccines and therapeutic applications

Chantelle Gaskin

Critical to bringing mRNA therapeutics to patients is an efficient, effective, and scalable downstream production process. This article explores the development, characterization, and optimization of an mRNA capture step using POROS™ Oligo (dT)25 Affinity Resin. This resin provides an affinity solution that leverages the rigid POROS backbone with its linear pressure-flow relationship, enabling a consistent purification platform from process development through to clinical manufacturing.

Nucleic Acid Insights 2024; 1(8), 263–278

DOI: 10.18609/nai.2024.033

INTRODUCTION

Synthetic mRNA has multiple applications, from enhancing allergen tolerance to treating serious diseases including cancer, genetic disorders, and infectious diseases. The current main methods of mRNA therapy delivery include direct injection, *ex vivo* injection of transfected cells, and transfection of genome editing enzymes. These diverse applications have led to the industrialization of mRNA therapy.

Novel biotherapeutics typically follow four stages of growth **(Figure 1)**. The COVID-19 pandemic has quickly moved mRNA past the 'nascent' and 'growth' phases and into the 'emerging modality' phase. However, in order to reach the 'mature technology' phase, considerable investment is needed into optimizing mRNA production. More specifically, a robust and easy-to-use purification platform is a prerequisite to drive the future success of mRNA's diverse applications and methods of delivery.

IMPROVING THE mRNA THERAPEUTICS PROCESS WORKFLOW

A typical mRNA process workflow includes DNA template preparation (including plasmid production and purification, which can be completed in-house or outsourced), mRNA synthesis, and downstream purification (Figure 2). Thermo Fisher Scientific's POROS™ products can be used for both the plasmid and mRNA purification steps.

FIGURE 1

Stages of biotherapeutic development.

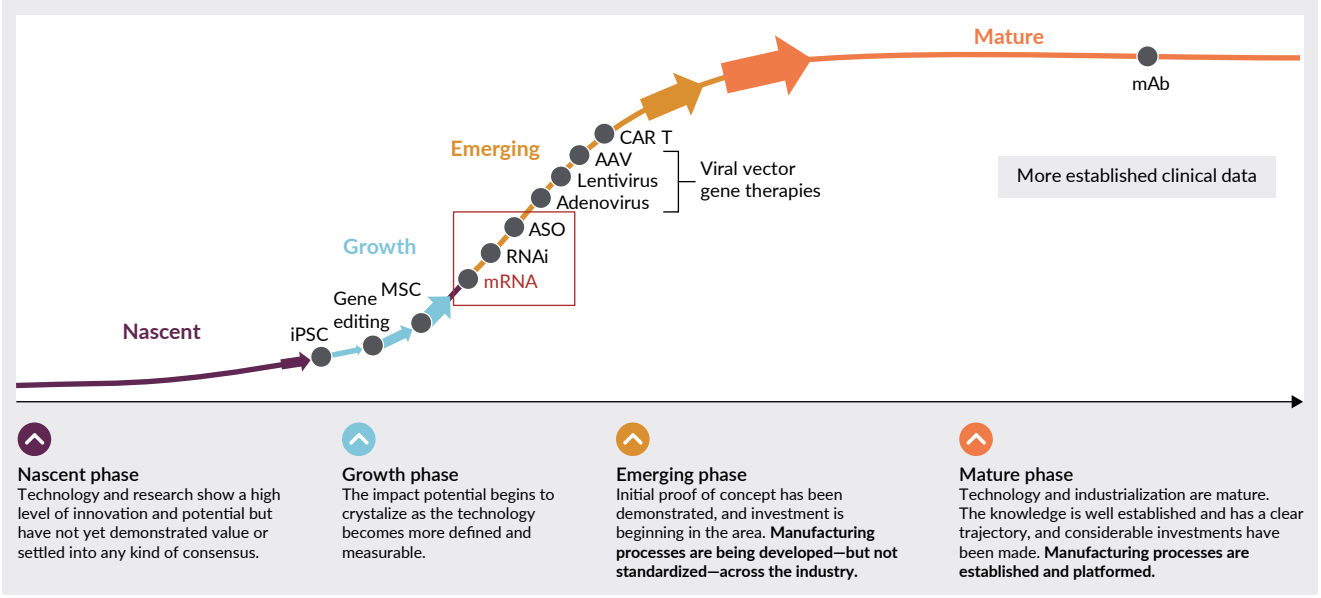


FIGURE 2

mRNA therapeutics process workflow overview.

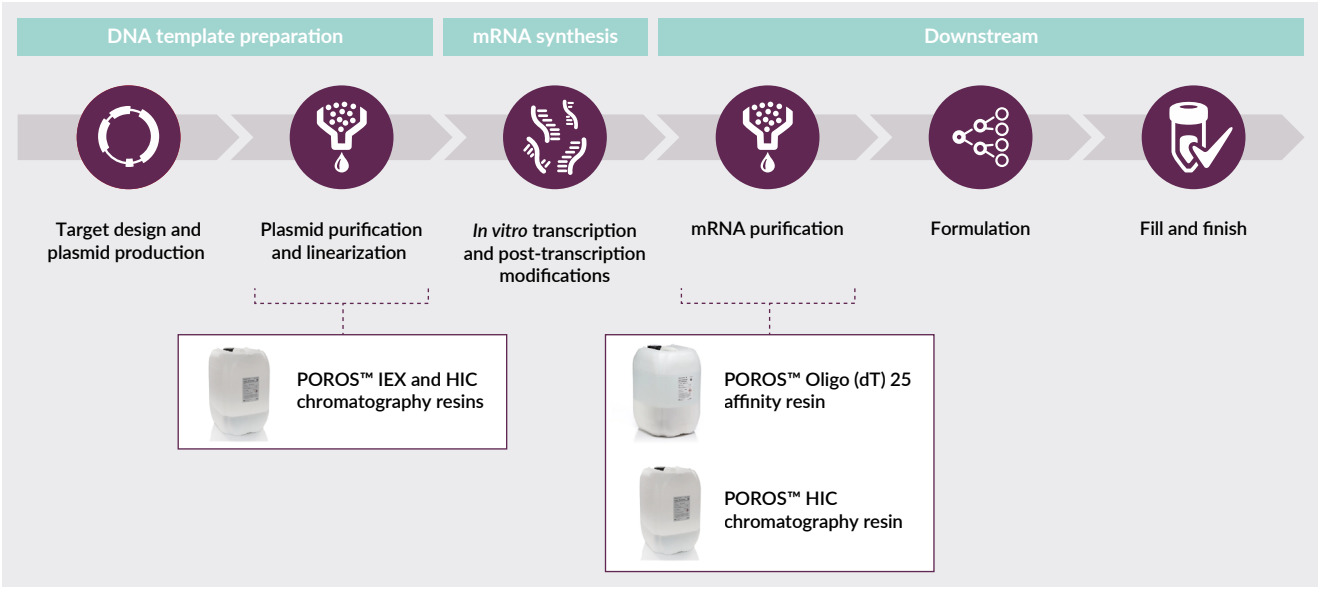


FIGURE 3

mRNA purification workflow overview.

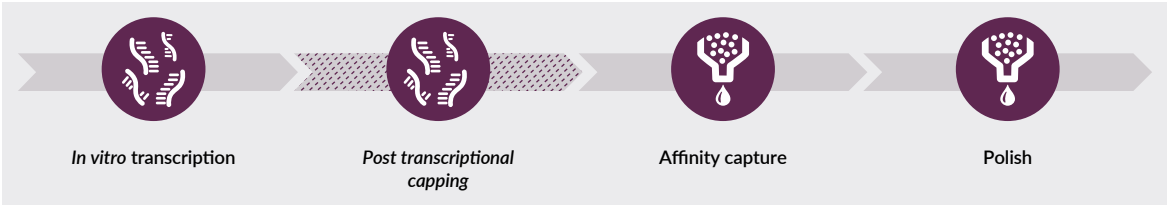


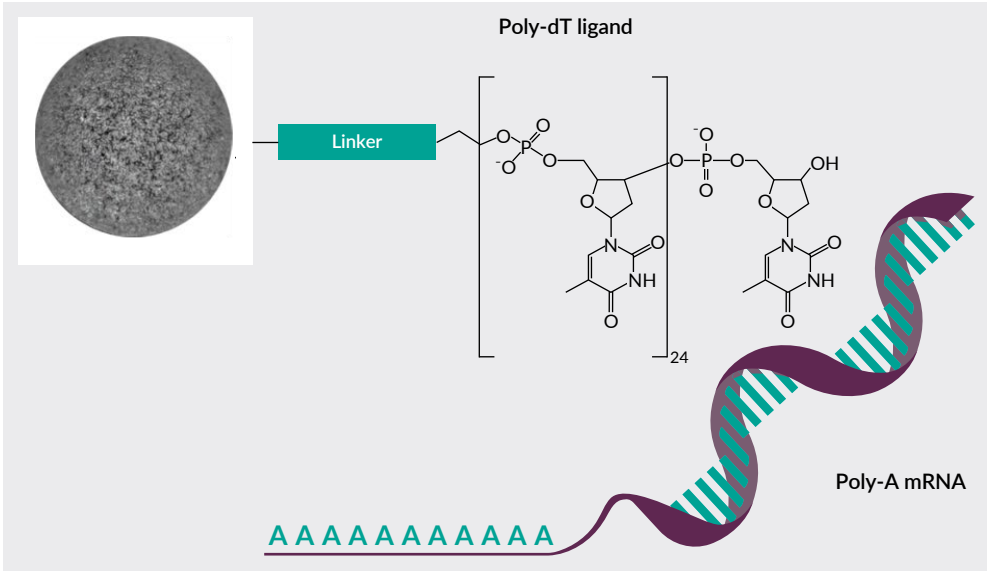
Figure 3 zooms in on the mRNA purification workflow, specifically, where the POROS™ Oligo (dt)25 Affinity Resin can be used for affinity purification. After the *in vitro* transcription (IVT) reaction (or following post-translational capping, if it is performed as a separate, subsequent upstream step), the full-length mRNA is selectively captured by the Poly(A) tail with the POROS Oligo (dT)25 Affinity Resin. Because of the specificity of the resin to the Poly(A) tail, only the full-length mRNA remains following the affinity capture step. Furthermore, this step enables the removal of process-related impurities such as DNA template, nucleotides, enzymes, and buffer components. An additional polish step can be conducted using POROS™ Benzyl Hydrophobic Interaction Chromatography (HIC) Resin, aiding in the removal of double-stranded (ds)RNA, uncapped RNA, and secondary RNA structures from the final product.

POROS OLIGO (DT)25 AFFINITY RESIN AND THE POROS BEAD

The POROS Oligo (dT) resin has been specifically designed for the purification and isolation of mRNA from the IVT reaction. This is enabled by A-T base pair hybridization. As illustrated in Figure 4, a Poly-dT ligand with a proprietary linker is attached to a 50 µm POROS bead. The resin has a dynamic binding capacity of up to 5 mg/mL

FIGURE 4

dT-25 (poly-deoxythymidine) ligand with proprietary linker.



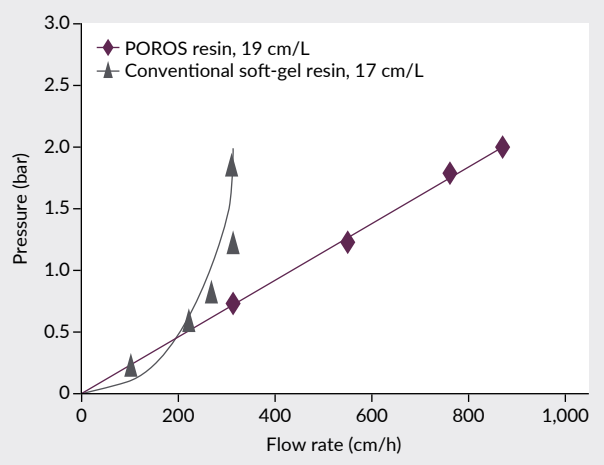
for 4,000 nucleotides (nt) of mRNA. With >90% recovery, the resin has excellent scalability and is also non-animal derived.

Additionally, the Oligo (dt)25 resin is offered in a variety of formats as a tool for early development, which can then be easily scaled for manufacturing. Conditions can be optimized with a 96-well plate, or with a POROS RoboColumn™ for a specific mRNA construct to maximize mRNA purification. Once the ideal conditions are determined, they can be verified with pre-packed columns at bench-scale. The optimal conditions thus established at small-scale can then be applied when scaling up mRNA purification to the liter+ scale.

The Oligo(dT) resin on the unique POROS bead has three main attributes that differentiate it from other chromatography resins. The first of these is that the bead is made of Polystyrene-divinylbenzene, which results in stable column beds with linear and scalable pressure. This enables the operation of high linear flow rates with modest pressure drops. For example, an operation at 800 cm/hour in a 20 cm column will result in a 2-bar pressure drop across the column **(Figure 5)**. This is a small change in comparison to a classic soft gel resin that shows a typical exponential increase in back pressure as linear velocity increases.

FIGURE 5

The POROS bead allows for high linear flow rates with modest pressure drops.



Additionally, due to the polymeric nature of the backbone and the robust covalent chemistries of the beads themselves, POROS beads exhibit strong physical and chemical stability. They are chemically stable at pH 1–14, in high-salt concentrations, and in the presence of detergents and denaturants, which enables the use of aggressive cleaning solutions and improves resin lifetime and reuse.

The second key attribute is the large pore structure, which results in reduced mass transfer resistance in comparison to other resins. This large pore structure enables linear velocity increases with minimal loss of capacity and resolution, as well as improved process productivity.

Finally, the third attribute is the small (50 µm) average particle size. This allows for reduced band broadening in packed beds, which improves the resolution of molecule separation as well as impurity removal.

mRNA PRECIPITATION POINT DETERMINATION

One of the challenges with mRNA purification is understanding and achieving good binding and recovery. This is largely dependent on the solubility of the molecule, which is dictated by the structure and size of the construct.

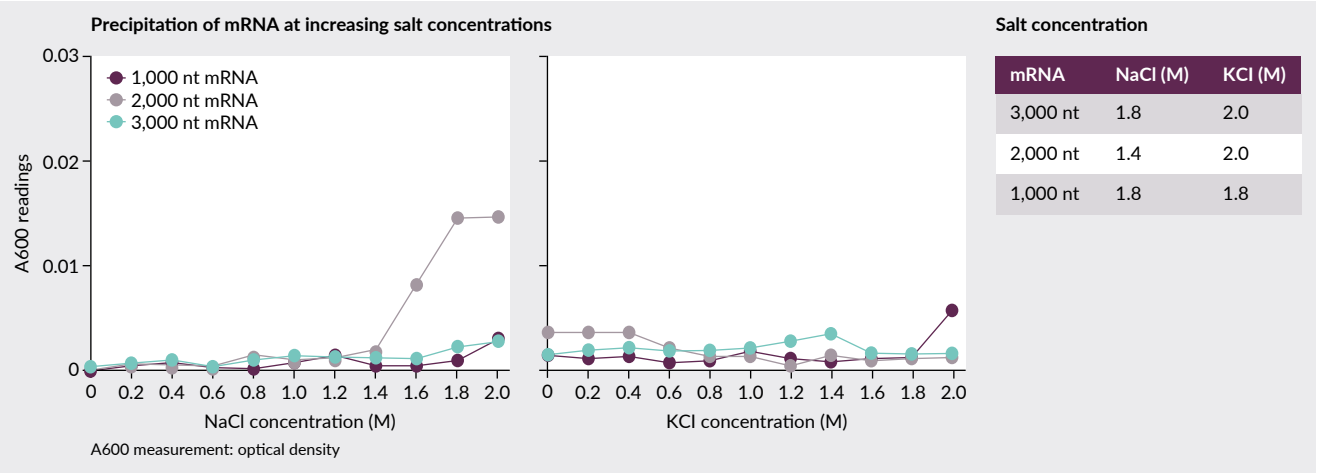
In the case study illustrated in **Figure 6**, constructs of 1,000, 2,000, and 3,000 nt were examined with the following experimental conditions:

- NaCl and KCl as salt additives
- Various salt concentrations were prepared in 10 mM Tris, 1 mM EDTA, pH 7.4
- 0.2 mg/mL mRNA solutions were prepared from the above
- 20 µL resin, 2 mg/mL mRNA load (40 µg)

Buffers containing both NaCl and KCl were tested at various concentrations. The

FIGURE 6

mRNA precipitation point determination: case study data.



Salt concentration

mRNA	NaCl (M)	KCl (M)
3,000 nt	1.8	2.0
2,000 nt	1.4	2.0
1,000 nt	1.8	1.8

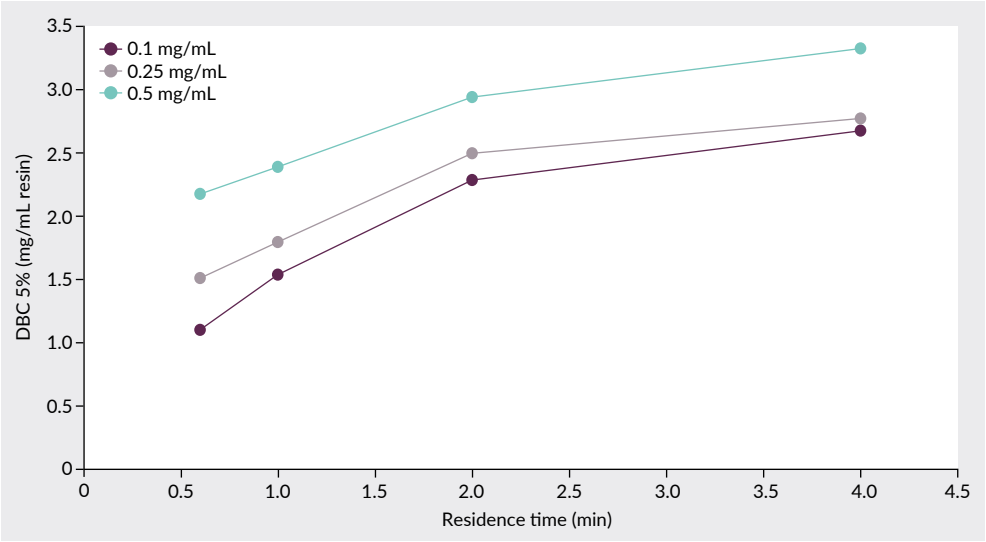
table on the right of **Figure 6** shows the salt concentrations at which precipitation began to increase. For a 2,000 nt construct, there was marked precipitation beginning at 1.4 M NaCl. These concentrations represent upper limits for further process development and optimization. The common operating window, however, is below the precipitation point (i.e., between 0.5–1 M concentration).

DYNAMIC BINDING CAPACITY DETERMINATION

The binding capacity of a capture step is an important parameter in determining how much product to load on the column. The concentration of mRNA on a mass basis is relatively low compared to monoclonal antibody or protein applications. This load concentration can impact the dynamic binding

FIGURE 7

Dynamic binding capacity determination: case study data.



capacity (DBC) because the binding is diffusion-limited. Diffusion is driven by a concentration gradient or difference between the load material and the resin. If the concentration in the load material is lower, there is a lower driving force to get the mRNA onto the resin. As an alternative explanation, if there is a lower concentration in the load material, then there is a reduced opportunity for these molecules to find a ligand to which to bind.

To determine the DBC, a breakthrough curve was generated for each of the three different load concentrations at four residence times in each case (Figure 7). The process conditions for this DBC study are based on Tris EDTA or TE saline buffers. For all three mRNA load concentrations, there was only a small increase in DBC at 5% breakthrough (BT) between 2–4 minutes' residence time, compared to that seen at ≤1 minute. In practice, this means that a process can be run at a 2-minute residence time without sacrificing capacity, which increases productivity.

The other important point to note in this data is that a higher DBC is obtained with a higher concentration of the load material, since diffusion is driven by the concentration gradient.

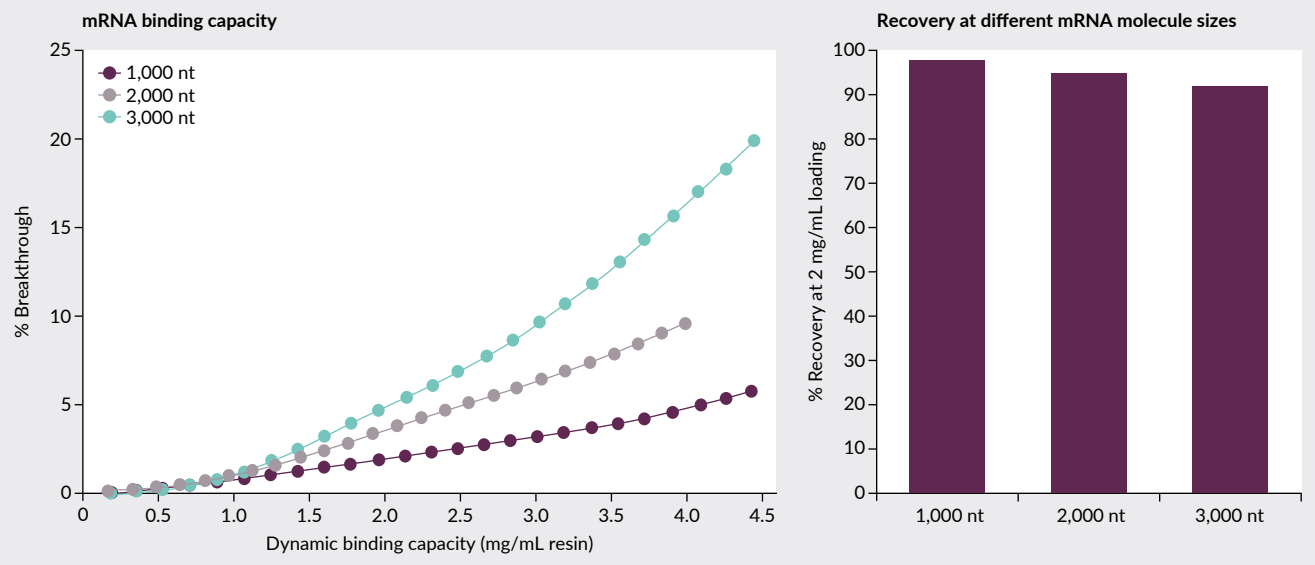
IMPACT OF MOLECULE SIZE ON BINDING CAPACITY AND RECOVERY

It is important to optimize an mRNA process for a specific molecule, especially considering binding capacity and recovery. Figure 8 examines binding capacity as a function of construct size and the corresponding recoveries. Again, 1,000, 2,000, and 3,000 nt constructs were used. For 1,000 nt mRNA, a binding capacity of 4.5 mg/mL was observed at 5% BT. However, a 4 mg/mL resin binding capacity was observed at 10% BT for a 2,000 nt mRNA. For a 3,000 nt mRNA molecule, a 3 mg/mL resin binding capacity can be obtained at 10% BT. These binding capacities were all determined at a 2-minute residence time and with 90 cm/hour column load.

Given these data, it is recommended to either reduce load density or increase residence time for larger constructs. While a >90% recovery is observed for all three mRNA molecule sizes, the key is to optimize loading densities depending on the size of the construct, and to optimize buffer conditions depending on the salt tolerance of the construct.

FIGURE 8

Impact of molecule size: case study data.



TYPICAL CHROMATOGRAM FROM A POROS OLIGO (dT)25 PRODUCTION RUN

An example chromatogram is shown in Figure 9 for the purification of a 2,000 nt mRNA from an IVT mixture with the POROS Oligo(dT) resin. The sample was loaded at 2 mg/mL resin with a final sample concentration of 0.2 mg/mL. The sample was diluted in 10 mM Tris, 0.8 M NaCl, and 1 mM EDTA at pH 7.4 binding buffer. During the sample loading, only the mRNA with the Poly(A) tail was bound to the resin, while the DNA, enzymes, and nucleotides flowed through the column. Both single-stranded mRNA and some dsRNA species bind to and elute from the POROS Oligo (dt)25 resin.

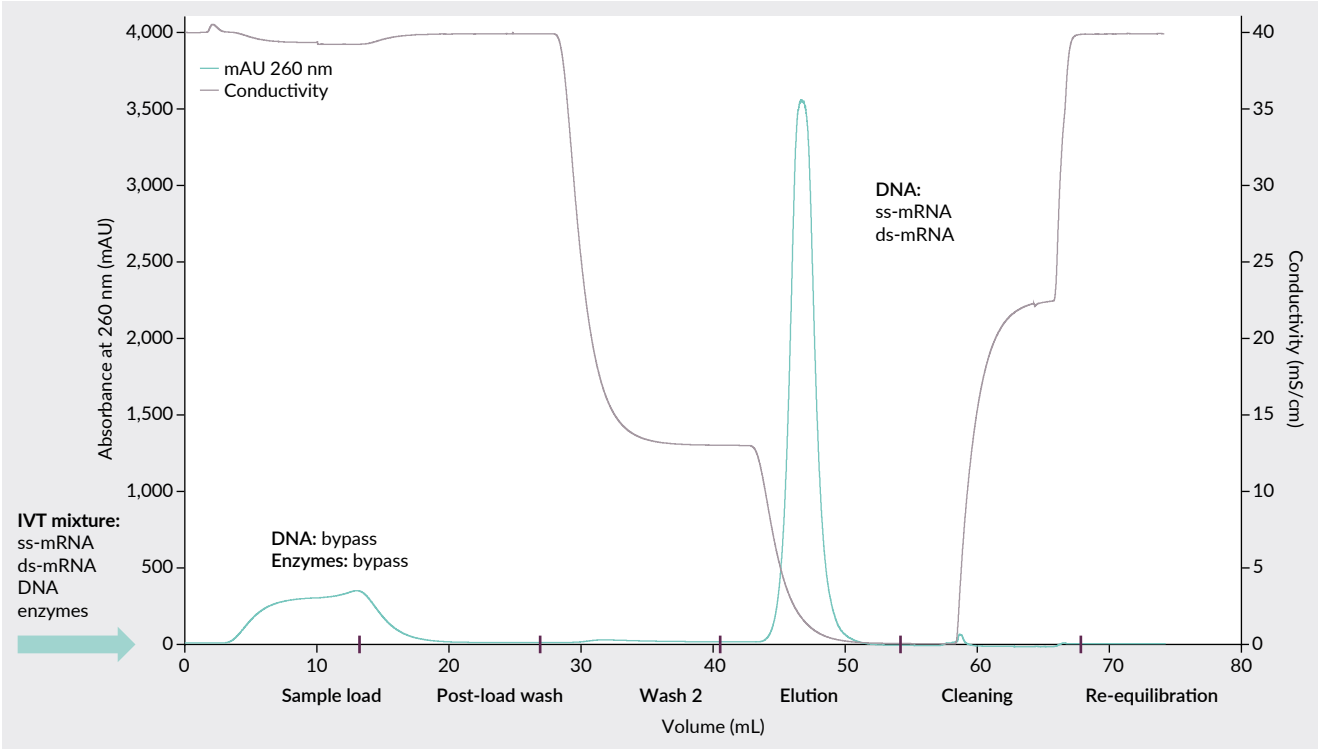
After sample loading, the column was cleaned with two washes—an equilibration buffer and a low-salt wash. Although impurities from the IVT reaction were present in the flowthrough as expected, a two-step wash is still recommended. The mRNA was eluted with RNase-free water and the column was cleaned with 0.1 M NaOH.

RECOVERY AND IMPURITY REMOVAL

High recoveries from Oligo (dT)25 are expected, reaching 95% or higher, regardless of the purity of the starting material. Figure 10 demonstrates a 95% recovery obtained using the same load density and the same residence times for both purified and crude mRNA feed streams. This is further evidenced by the protein content in the flowthrough versus that in the elution. Despite there being a difference in the protein content of the load versus the flowthrough, the remaining protein was actually present in the cleaning in place (CIP) step, which is not shown in Figure 10.

FIGURE 9

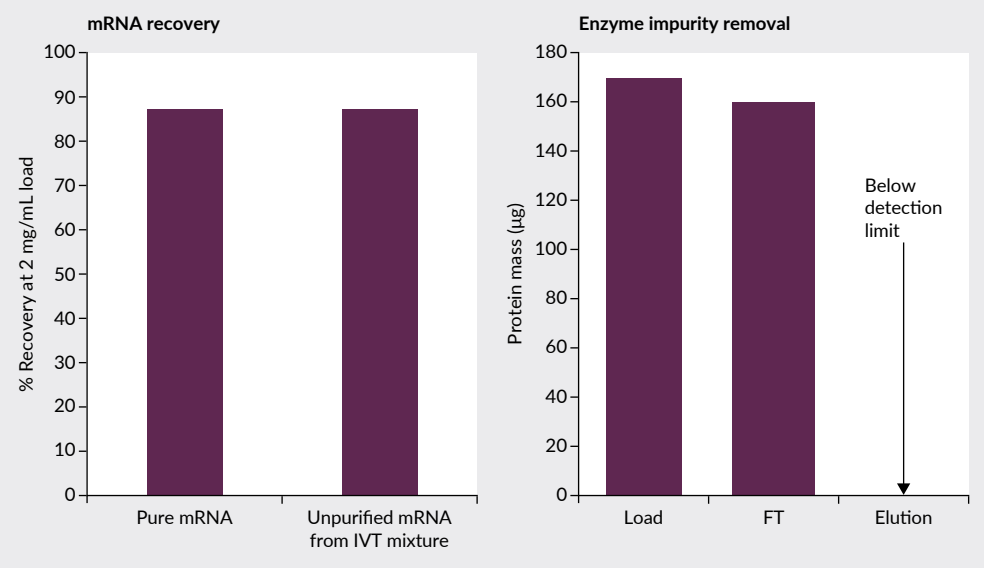
Purification of 2,000 nt mRNA from IVT mix.



IVT: *in vitro* transcription.

FIGURE 10

Recovery and impurity removal: case study data.

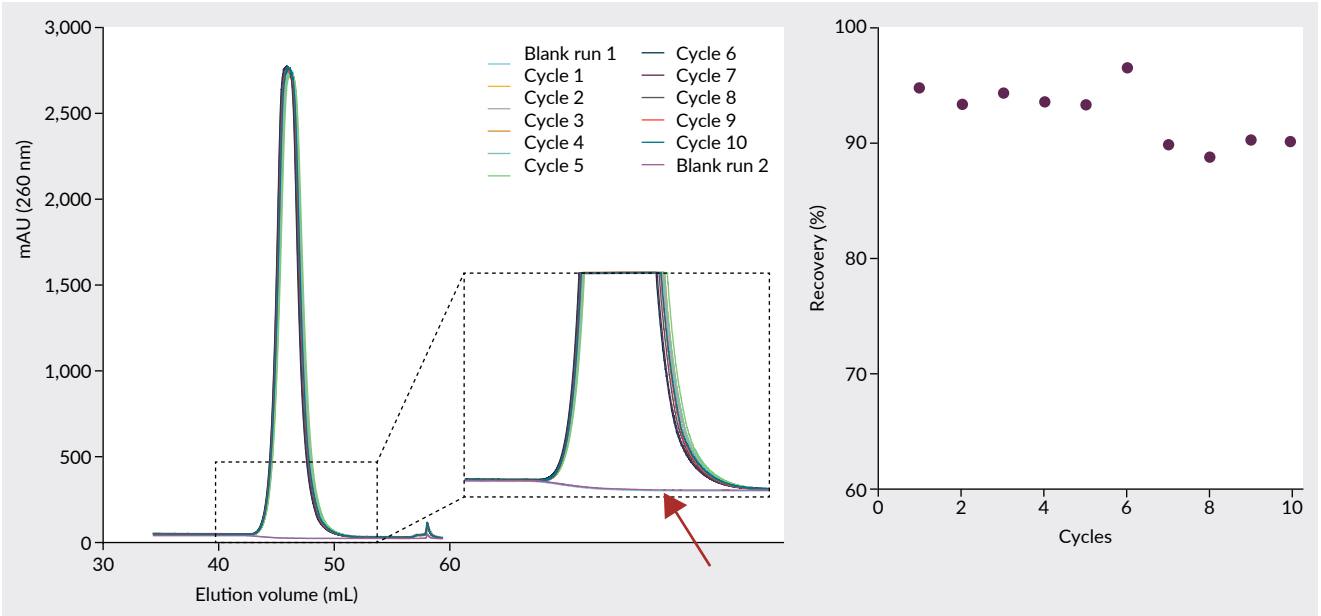


REUSE AND STABILITY

Before deciding to implement a resin in a process, it is important to determine that it is stable and functional for multiple cycles and following periods of storage in different conditions, thus ensuring cost–effectiveness. The following studies demonstrate the continued

FIGURE 11

Purification of mRNA (2,000 nt) over multiple cycles from IVT mixture.



IVT: *in vitro* transcription.

high-quality performance of the POROS Oligo(dT)25 resin in multiple cycles and various storage conditions.

Figure 11 shows the elution peak over 10 cycles for the purification of an IVT mixture. A blank buffer run was performed before and after the 10 cycles to monitor any mRNA that eluted in the final blank run. The overlay is identical to the initial run, showing that no mRNA is eluting from subsequent runs. Additionally, a >90% recovery is observed for each of the 10 cycles.

In order to supplement this reuse data, a further study was conducted. The objective of this additional study was to show that repeated uses of the resin with a molecule that binds to the Oligo (dT)25 ligand does not impact the performance. Due to limitations in the mRNA sample size, the study was designed as shown at the top of Figure 12. Specifically, the dynamic binding capacity of a 2,500 nt construct was determined on the first cycle. Then, 9 cycles were run using a dA40-mer instead of mRNA. At cycle 10, the DBC was measured again. This process was continued by loading a dA40-mer for the majority of the cycles, and every 10 cycles, the DBC was determined using the 2,500 nt mRNA construct. The box at the bottom of Figure 12 includes additional details about the two different samples used in the study.

The results of this second reuse study are presented in Figure 13. The data demonstrates consistent performance, meaning that there was no change in binding capacity for 70 cycles. All DBC values are within experimental error and assay variability limits.

Next, the cleaning stability of the POROS Oligo (dT)25 resin was tested with a variety of cleaning solutions (Figure 14). Resin samples were incubated in the following three solutions for up to 48 hours:

FIGURE 12

POROS Oligo (dT)25 reuse study: experimental summary.

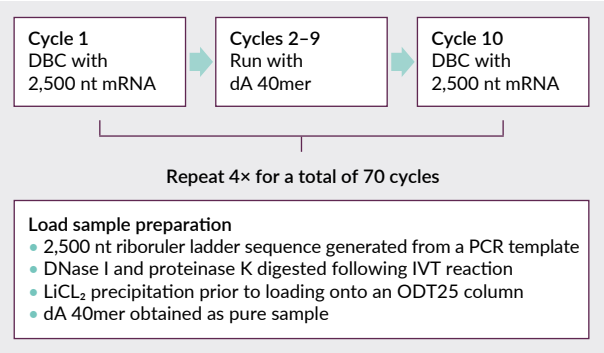
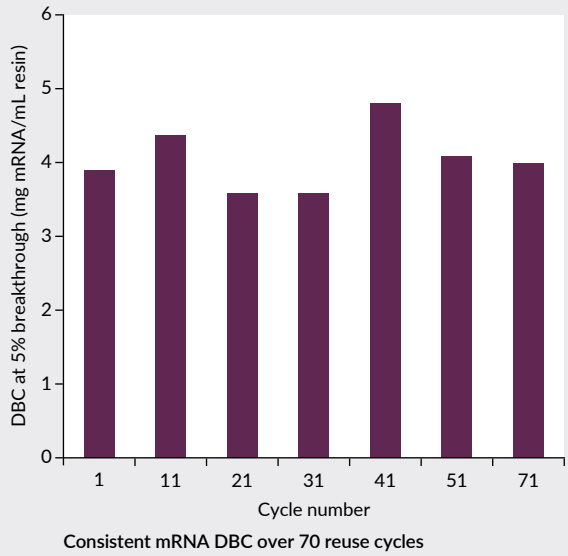


FIGURE 13

POROS Oligo (dT)25 reuse study results.



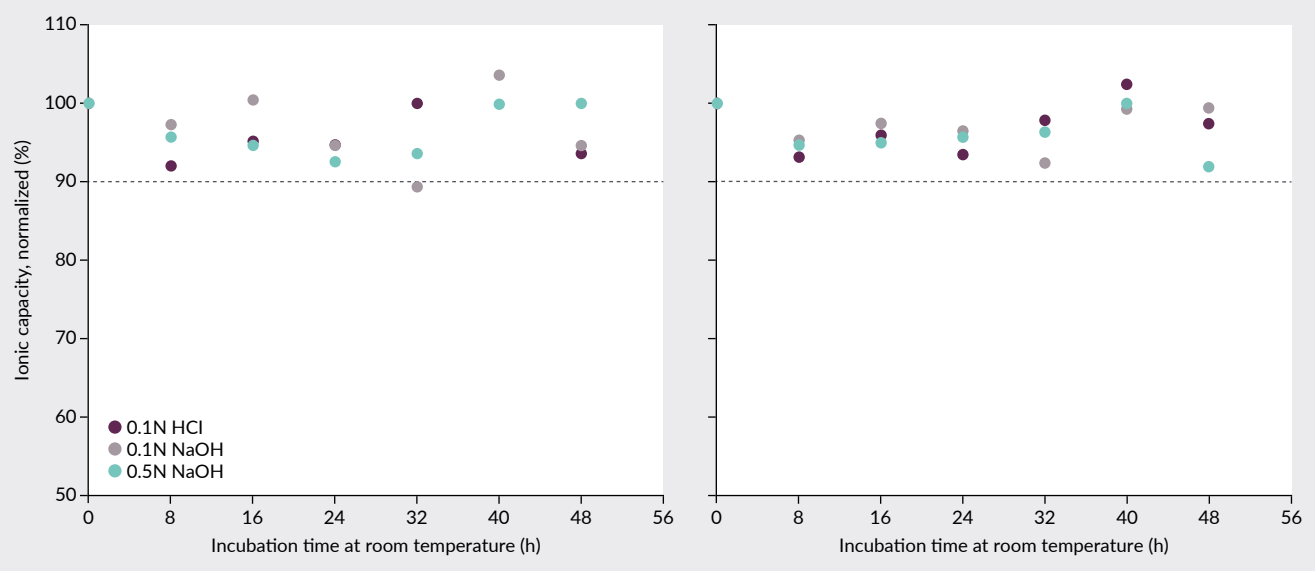
Consistent mRNA DBC over 70 reuse cycles

Column dimensions	0.5 cm D × 0.5 cm L
Resin	POROS Oligo (dT)25
Load sample A (DBC runs)	2,500 nt purified mRNA (0.25 mg/mL)
Load sample B (cycling runs)	dA40mer (0.1 mg/mL)

Step	Buffer	Res time (min)	CV
Equilibration	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5
Load A	2,500 nt purified mRNA	5.0	20
Load B	dA40mer	5.0	4
Wash 1	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5
Wash 2	10 nM Tris, 1 mM EDTA, 0.1 M NaCl pH 7.4	1.0	5
Elution	Water	1.0	5
CIP	0.1 mM NaOH	1.0	5
Re-equilibrate	10 nM Tris, 1 mM EDTA, 0.8 M NaCl pH 7.4	1.0	5

FIGURE 14

Cleaning and stability of POROS Oligo (dT)25 affinity resin utilizing a variety of cleaning solutions.



0.1 N HCl (pH at 1.1); 0.1 N NaOH (pH at 12.9); and 0.5 N NaOH (pH at 13.4). Both the resin's ionic capacity and the DBC using a 40 mer were tested after the treatments. The graphs in **Figure 14** are normalized to the time point 0 and show essentially no change in ionic capacity or dA40-mer binding capacity for the time period and conditions tested.

In most cases, end users utilize 0.–0.5 M NaOH for cleaning after every cycle and typically, the cleaning step takes 30–60 minutes. These data demonstrate the stability of the resin under such typical cleaning conditions,

FIGURE 15

Cleaning and stability of POROS Oligo (dT)25 affinity resin over 8 days.

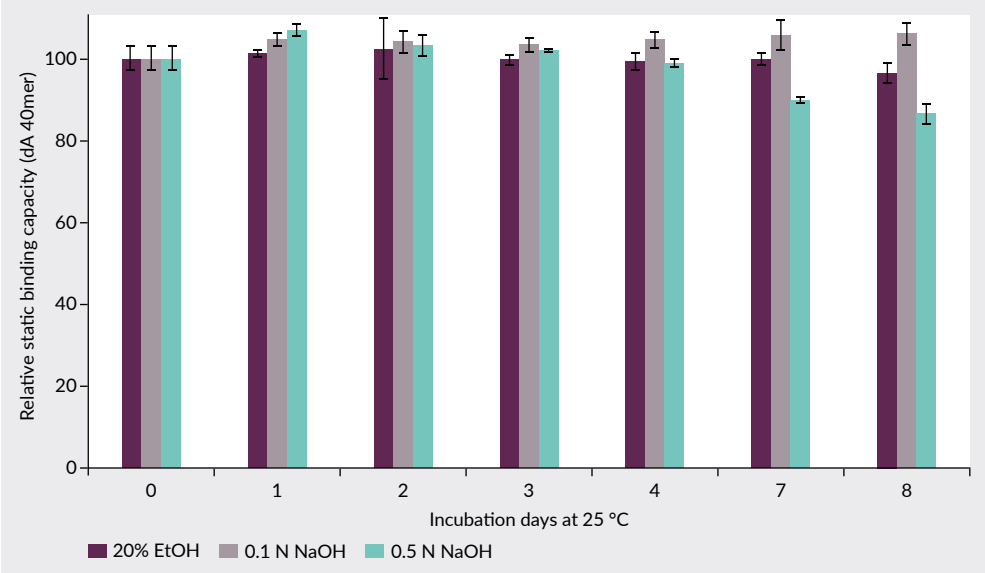
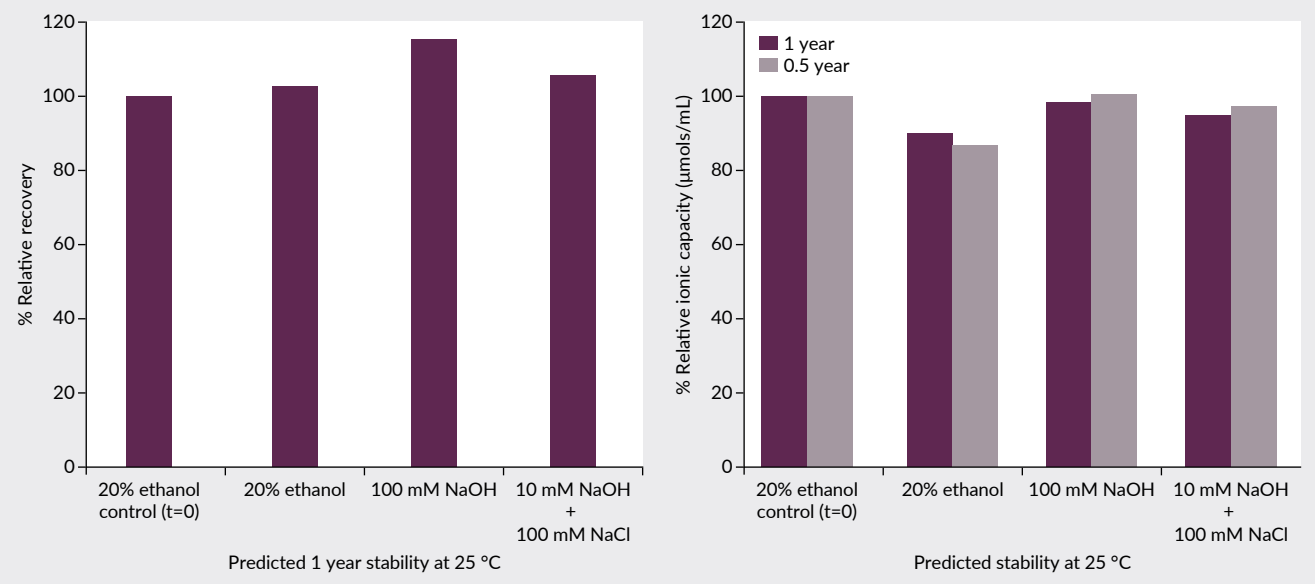


FIGURE 16

Long-term predicted stability in various storage solutions.



and can be extrapolated to the equivalent of approximately 96 cleaning cycles.

The study was then expanded to test the stability and performance of the resin after cleaning and storage in NaOH compared to ethanol over a longer period of time (up to 8 days). This particular study was important for demonstrating simplicity of use where employing ethanol cleaning and storage are challenges. The data in **Figure 15** shows that the resin has good caustic stability and can be cleaned and sanitized using alkaline conditions as an alternative to ethanol. Although a decrease was observed with 0.5 M NaOH, the number of sanitization cycles where no drop in static binding capacity was observed exceeded 200 cycles (assuming 48 × 30 min sanitization cycles per day). A higher cycle count could be achieved by using either a lower concentration of hydroxide or shorter cleaning steps.

To further investigate the resin's stability and performance after storage for longer periods of time, an accelerated stability study was performed **(Figure 16)**. High-temperature accelerated studies for a duration equivalent to 1 year of storage were performed at 25 °C. On the left of **Figure 16**, a 4,000 nt construct was bound and eluted from resin that was incubated in one of each of the following storage solutions:

- 20% ethanol
- 100 mM NaOH
- 10 mM NaOH plus 100 mM NaCl

Compared to 20% ethanol storage at t=0, mRNA recovery was consistent across all three conditions. The graph on the right shows that similar performance based on ionic capacity was observed in the three different storage conditions for both the equivalent of 6 months and 1 year at 25 °C.

These data support the use of POROS Oligo (dT)25 affinity resin for alternative storage conditions.

Q&A

Chantelle Gaskin

Q Do you need to use heat to elute the RNA?

CG: No, you do not need heat to use the Oligo (dT). In some cases, though, an end user will opt to use heat for disruption of higher order structures and things of that nature. All of the studies discussed above were carried out at ambient room temperature, but Oligo (dT) is stable up to 65 °C.

Q Is the Oligo (dT) resin only available as loose resin, or do you have columns as well?

CG: We do have columns. We are stocked with 0.2 mL, 1 mL, and 5 mL pre-pack columns, as well as RoboColumns. In addition, we also just launched the 96-well plate formats for screening. The one thing that we do not carry is the analytical size columns, so if you are interested in that, you should reach out to your local technical sales specialist.

Q What sizes of RNA can be purified? Is there a construct size limit?

CG: There is no limit. We have tested various sizes in-house, including the 1,000–4,000 nt constructs I discussed today. We also have customers who have tested this resin with much larger constructs such as self-amplifying (sa)RNA constructs, for example. Though you can obtain high recoveries in these cases, we do recommend opting for a higher residence time that will help with binding capacity. Furthermore, as with any process, optimization is usually required, so I also recommend reaching out to your local field application scientist. They should be able to help with determining process conditions for those larger constructs.

Q Does the length of the poly(A) tail impact the capacity of the resin and the final purity?

CG: No, it does not. By far, the largest constructs we have seen have around a 100–120 mer poly(A) tail, and with that, we have seen consistent results specifically in terms of capacity and purity. The resin only has a 25 mer Poly(dT) ligand, which is enough to bind the construct. We have also seen some shorter tail constructs that perform decently with this resin.

Q Will POROS Oligo (dT)25 remove truncated mRNA from IVT reactions for longer constructs?

CG: If the poly(A) tail is damaged or missing, it will not bind. This would ensure the removal of truncated mRNA.

Q Is proteinase K treatment necessary?

CG: Proteinase K is not needed when using the POROS Oligo (dT) resin.

Q Does POROS Oligo (dT) remove double-stranded mRNA?

CG: The POROS Oligo (dT) will not remove double-stranded mRNA. It will bind the poly(A) tail, so some forms of double-stranded mRNA will bind and co-elute from the resin. There are some preliminary data showing that either hydrophobic interaction chromatography or anion exchange chromatography could be used as a polish step for removing double-stranded mRNA, but most customers tend to optimize the IVT reaction to reduce the production of double-stranded RNA instead.

Q Is POROS Oligo (dT) compliant with CGMP, and is it scalable?

CG: This resin is fully scalable, up to commercial scale. As far as CGMP goes, we do have regulatory support files that you can request from either your local technical sales representative or field application scientist. They can provide you with the documentation needed to support your regulatory process.

Q What is a typical lifetime for mRNA purification with the column?

CG: We have data showing little significant decrease in resin performance for >70 cycles. It is likely that you can reach a higher number of cycles than that, however, in the event that a sufficient cleaning protocol is established.

BIOGRAPHY

CHANTELLE GASKIN is a Field Applications Staff Scientist, specializing in protein and viral vector purification and downstream process development. She held leadership positions at Applied Genetic Technology Corporation and Brammer Bio, prior to joining the Thermo Fisher Scientific Bioproduction Division, Alachua, FL, USA in 2020. With over 10 years of experience in gene therapy, Chantelle has accumulated comprehensive knowledge of standard industry practices and regulatory standards, applying this knowledge to advance development of therapies for a variety of indications including ocular, CNS and systemic disease. Chantelle holds an MSc in Chemistry from University of Florida, Gainesville, FL, USA and a BSc in Chemistry from Smith College, Northampton, MA, USA.

AFFILIATION

Chantelle Gaskin
Staff Scientist, Field Applications,
Thermo Fisher Scientific,
Alachua, FL, USA

AUTHORSHIP & CONFLICT OF INTEREST

Disclaimer: Pharmaceutical grade reagent. For manufacturing and laboratory use only.

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author is a Thermo Fisher Scientific employee and stock holder.

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was based on a webinar, which can be found here.

Webinar conducted: Jun 13, 2024; **Revised manuscript received:** Aug 12, 2024;

Publication date: Sep 4, 2024.

ThermoFisher SCIENTIFIC

Curated content to boost your mRNA purification process

Your destination for mRNA insights

Access to expert industry insights can help you streamline your mRNA process development and manufacturing.

That's why our virtual Chromatography Learning Lab offers a broad collection of mRNA webinars, articles, eBooks, infographics, and other digital resources.

Here, you can discover expert approaches to topics such as:

- Vaccine manufacturing approaches
- Addressing large-scale purification challenges
- Scalable purification methods
- Optimizing mRNA purification conditions

View resources here

Discover more at
thermofisher.com/chromatography-learning-lab

thermo scientific

EXT5821 0923

NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES:
PROCESSING AND ANALYTICS

SPOTLIGHT

INTERVIEW

Advancing treatment of genetic diseases through engineered tRNA therapeutics

Engineered transfer RNA (tRNA) therapeutics carry the potential to provide disease-modifying treatments for patients with genetic 'Stop Codon' Disease. **David McCall**, Senior Editor, BioInsights, speaks to **William Kiesman**, CTO, Alltrna, about his company's pioneering work in the tRNA field, which is driven by recent advancements in machine learning (ML).

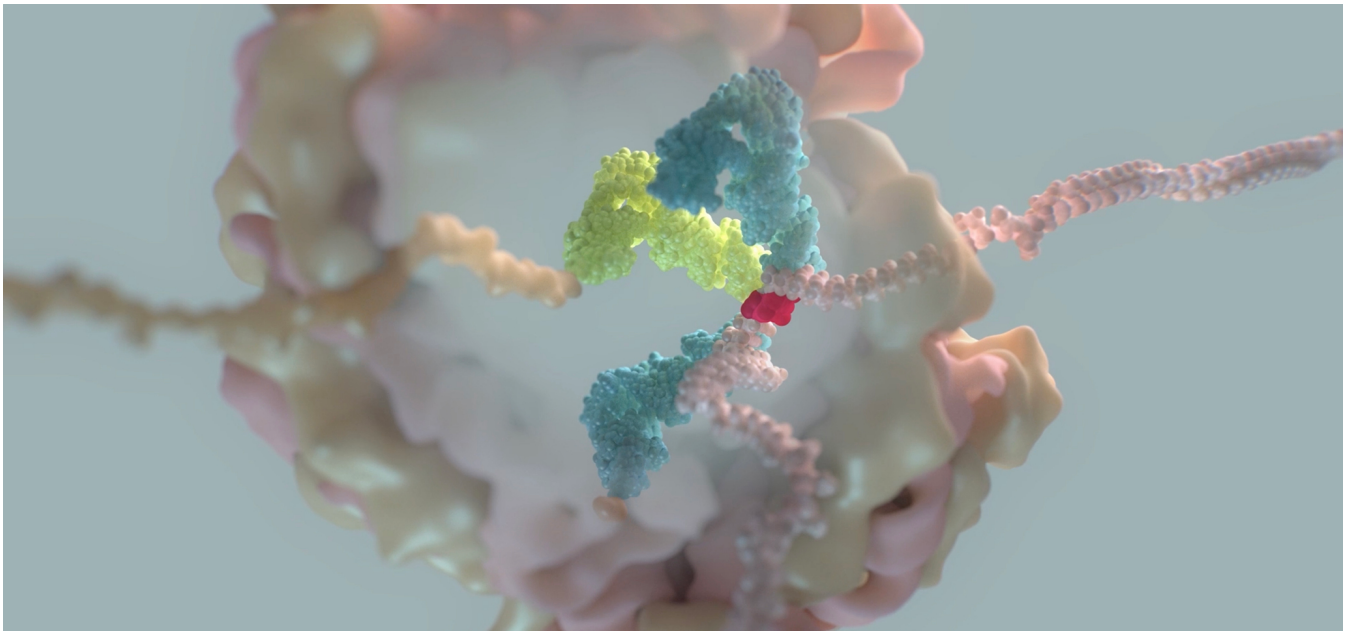
Nucleic Acid Insights 2024; 1(8), 293–299

DOI: 10.18609/nai.2024.036

Q What are you working on right now?

WK: I have been in the industry since the late 1990s, working on many exciting projects over the years. In October 2021, I joined Alltrna, a company focusing on transfer RNA (tRNA) therapeutic development, which is where I am now.

At the genesis of Alltrna, the founders were exploring whether tRNAs have functions beyond just shuttling amino acids into the growing protein chain. The key research question in the beginning was whether tRNA could be used as a therapeutic during mRNA translation. One



of the early findings that launched the company was that tRNAs can be engineered to address premature termination codons and thereby prevent the incorrect production of proteins.

tRNAs are short molecules, approximately 70–90 nucleotides long, but with very complex biology. They undergo many modifications in the cell and are involved in complex signaling pathways. tRNAs have a very compact, 3D structure, usually resembling the letter 'R' or an inverted 'L' shape. Due to the number of nucleotides and variations, there are around 10^{34} possible tRNA structures when considering the sequence and chemical modifications at each position—a number that exceeds the total number of atoms in the universe. This degree of complexity provides an opportunity to explore different engineering targets. Currently, we are working on building first-in-class and best-in-class tRNA therapeutics to treat genetic diseases [1].

Q Having spent many years in the oligonucleotide research space, can you reflect on your journey and share your high-level commentary on the current status of the oligonucleotide therapeutics sector?

WK: I started my career in medicinal chemistry, focusing on small molecules, but over the years, I transitioned into development and manufacturing. In 2012, while working at a previous company, I was lucky to be involved in the development of nusinersen (SPINRAZA®), an antisense oligonucleotide (ASO) targeting the *SMN1* gene for spinal muscular atrophy (SMA), which was a groundbreaking treatment at the time.

Fast forward to today and there has been an explosion in the number of clinical- and commercial-stage oligonucleotides, with ASOs and siRNAs leading the way. Additionally, the range of target indications has broadened considerably, now spanning everything from ultra-rare diseases to much more common conditions. The events of 2020 further accelerated this progress,

"I see the expansion of oligonucleotide-based therapeutics to treat a larger number of diseases as the next big wave of innovation."

with mRNA vaccines for COVID-19 showing that safe RNA therapies could be delivered to millions of patients using lipid nanoparticles (LNPs). This breakthrough has opened up even more opportunities for RNA-based treatments.

I see the expansion of oligonucleotide-based therapeutics to treat a larger number of diseases as the next big wave of innovation. There is a huge upside not only in creating highly programmable therapeutics but also in targeting diseases with no current treatments, where small molecules or proteins cannot be effectively administered.

Q Can you go into more depth on Alltrna's R&D approach and pipeline? What differentiates it?

WK: When tRNAs perform their function, they interact with multiple other molecules in the cell—from modification enzymes to synthetases that charge the tRNA with the amino acid, and the ribosome with its associated machinery that reads the mRNA message. Each one of these interactions is a structure-activity relationship target, which allows us to work across numerous aspects simultaneously and optimize them. It is a big opportunity as well as a big challenge, but we have developed several powerful technologies and techniques to synthesize and modify our tRNAs, quantify them, deliver them into cells, and test their activity. Due to the large variety of tRNA sequences, we recognized the need for high-throughput screening and automation to manage the process. We have implemented these systems to facilitate our design cycle, allowing us to interrogate targets, create many compounds, test them, and then refine the process further.

Regarding our pipeline, we are currently developing several solutions for patients. However, before diving into these solutions, it is important to understand the role that tRNA plays in protein production in human cells.

When genes are expressed as mRNA, the sequence consists of triplets of nucleotides called codons. tRNA molecules read these codons and add the corresponding amino acids to the growing polypeptide chain, forming a protein. When the ribosome reaches a termination codon, which tRNA does not recognize, the protein production is stopped and the complete, full-length protein is released.

In certain diseases, mutations in the mRNA code create premature termination, or 'stop', codons (PTCs) within the gene. When the ribosome encounters these PTCs, the tRNA is unable to read them, leading to early termination in protein production which creates either a truncated or absent protein, both of which can cause disease. At Alltrna, we have designed tRNAs to recognize and read these PTCs at any position in the gene, inserting the appropriate

amino acid. This in turn allows the ribosome to produce a full-length protein, which is then released normally, and the ribosome can continue its work in this catalytic process.

In essence, our R&D pipeline is focused on targeting diseases caused by PTCs by installing the correct amino acids at the right positions to produce full-length proteins. There are about 6,000 genetic diseases, and approximately 10% of patients with these conditions have a PTC causing them. Therefore, there are around 30 million people worldwide with Stop Codon Disease in total.

Q What does tRNA bring to the table in terms of its capabilities and advantages versus other therapeutic modalities, specifically?

WK: Firstly, we can precisely produce the right protein at levels intended by nature, which is unique to our tRNA-based approach. For example, unlike protein supplements, which often introduce excessive amounts of protein, we restore protein production to what the cell normally needs. Essentially, our approach relies on cells' normal regulatory systems, which is a big advantage over other modalities.

Another unique aspect of tRNAs is their universal ability to repair PTC mutations at any position within a gene. Unlike targeted genetic modifications that repair specific genes or specific points within a gene, our approach can potentially fix any gene with a PTC anywhere along its length with a single engineered tRNA.

Lastly, what sets our approach apart from other gene editing modalities is that we do not make permanent changes to the genome. Instead, we simply correct the translation process by reading through and addressing the mutations. This means we do not face the same concerns around long-term incorrect gene editing because we are not editing the genetic code itself.

One of my colleagues presented at the American Society of Gene and Cell Therapy 2024 Annual Meeting, showcasing our technology's ability to address various mutations across 25 disease models, 14 different genes, and 7 different mutation sites, successfully restoring protein production in all cases.

We have demonstrated our approach in numerous cell models and are now taking our first tRNA medicine into the clinic. We are initially focusing on some 400 rare genetic liver diseases caused by PTCs, which we have identified through clinical literature and practice.

Q Can you expand on the machine learning (ML) capabilities that underpin Alltrna's approach, and the benefits and considerations they present?

WK: The vast size and diversity of the data sets we manage—often involving millions of possibilities—is beyond the capacity of the human brain to readily process. To address this

"...by optimizing for sequence and chemical modifications using our platform, we can substantially improve the activity of our engineered tRNAs over endogenous molecules."

fact, we use ML-guided design. Our proprietary ML algorithms and systems help us identify specific sequence and chemical modification patterns, optimizing our tRNAs for both activity and selectivity.

By combining optimized sequences with ML-derived insights, we have been able to identify the specific positions that require chemical modifications. This approach of leveraging large data sets and computational expertise has enabled us to expand the potential of tRNAs. We have shown that by optimizing for sequence and chemical modifications using our platform, we can substantially improve the activity of our engineered tRNAs over endogenous molecules.

One of the elements that makes our team and the platform so efficient is the combination of human creativity and intuition from traditional medicinal chemists with the insights generated by our ML computational team. Essentially, we get the benefit of both worlds by leveraging the expertise and experience of chemists and the power of ML to find patterns in large data sets that could be missed during manual analysis.

Q What will be some of the specific challenges in working with tRNA oligonucleotide therapeutics as your product candidates advance towards and into the clinic, including on the process and product development side? And what steps is Alltrna taking to prepare to address them?

WK: At Alltrna, we chemically synthesize tRNAs and can incorporate specific chemical modifications at any position anywhere in the sequence. While this feature is very powerful, it also involves considerations of appropriate yield, purity, folding, and stability to ensure that the tRNAs hit the mark of excellence required for clinical- and commercial-stage therapeutics. This challenge is exacerbated by the fact that we are pioneering this field. For example, when we first started, there was no pre-existing framework for the production of chemically modified tRNAs.

Another challenge relates to the complex biochemistry of tRNAs. For example, the methods used to synthesize siRNAs and similar molecules proved not to be as effective for tRNAs. Due to the length and complex folding of tRNAs, we needed to learn the specific requirements for their synthesis and characterization. We have since developed synthesis processes that enable us to scale production to meet the clinical demands of our early programs and provide materials for preclinical toxicology studies. We have also developed proprietary analytical techniques to understand the impurity profiles of our compounds and identify factors to watch out for during

the synthesis and downstream purification steps. This groundwork will turn into quality control and lot release specifications. Having previously managed a large pipeline of ASOs, I am looking forward to applying the lessons learned in this area to revolutionize tRNA therapeutics.

Q Can you share your vision for the future of tRNA—and of oligonucleotides in general—in therapeutic application?

WK: Our vision is to develop a single tRNA therapeutic that can universally treat Stop Codon Disease and enhance outcomes for millions of patients by offering a disease-modifying medicine rather than just palliative care.

Regarding oligonucleotides in general, I believe the potential of these therapeutics to address the needs of underserved patient populations is unmatched by any other modality. Oligonucleotide-based therapeutics initially focused on small indications, but they are increasingly expanding into larger diseases by patient population, and I am certain this trend will continue.

However, in order to ultimately succeed, there are a couple of things to consider. Firstly, while the chemical synthesis methods we currently use meet today's market needs, we must improve our approach and make it more ecologically sustainable by including less chemical-intensive, more enzymatic processes to reduce waste. Secondly, I think the ligation of smaller oligonucleotides to make bigger, more complex systems will become more and more important to meet growing demand for treatments in the future.

Thirdly, one of the biggest challenges in this industry is the *in vivo* delivery of oligonucleotides to cells. While dosing at particular levels can be managed, the therapeutics might not be efficiently taken up by cells. To improve cellular uptake, we are initially working with clinically validated LNPs, while other researchers in the field are utilizing ligands or other methods. Advancing these delivery technologies will enhance the potency of our molecules and improve both safety and convenience for patients.

Q What are some key goals and priorities for your work over the foreseeable future?

WK: I came to Alltrna to work on creating a novel modality by turning biologically active tRNA into a therapeutic. Having been in the industry for years and meeting patients and families suffering from rare genetic diseases, I am fundamentally driven by the goal of helping improve their lives.

REFERENCE

1. Anastassiadis T, Köhrer C. Ushering in the era of tRNA medicines. *J. Biol. Chem.* 2023; 299(10), 105246.

BIOGRAPHY

WILLIAM KIESMAN, Chief Technology Officer of Alltrna, Cambridge, MA, USA, has more than two decades of experience in the design, development, and manufacturing of small molecule and oligonucleotide therapeutics. Prior to joining Alltrna, he served as the Vice President and Head of Oligonucleotide and Small Molecule Development at Biogen. During his 24 years with Biogen, he was responsible for a wide range of activities from medicinal chemistry research and automated parallel synthesis to building both the chemical development and the end-to-end oligonucleotide development and manufacturing teams. Will and his teams have supported dozens of small molecule and oligonucleotide clinical programs and played pivotal roles in the successful worldwide regulatory CMC approvals of Tecfidera® (dimethyl fumarate) in multiple sclerosis and Spinraza® (nusinersen) for spinal muscular atrophy. Will earned his BS and PhD in Chemistry from the University of Connecticut, Storrs-Mansfield, CT, USA and pursued his postdoctoral research at Duke University, Durham, NC, USA. He is co-author on more than 40 publications and patents and has contributed to five book chapters.

AFFILIATION

William Kiesman PhD
Chief Technology Officer,
Alltrna,
Cambridge, MA, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author is an Alltrna employee and stock holder.

Funding declaration: The author received nxo financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Interview conducted: Jul 23, 2024; **Revised manuscript received:** Aug 23, 2024;

Publication date: Sep 24, 2024.

NUCLEIC ACID INSIGHTS

pDNA: NEXT-GENERATION TECHNOLOGIES

SPOTLIGHT

INNOVATOR INSIGHT

Precision in production: optimizing monitoring and quality control for high-value plasmids

Oleksandr Karpenko

Gene therapy production processes are time-consuming and challenging, and many hurdles in the industry—such as challenges around ensuring regulatory compliance, and lack of industry standardization—are hard to directly influence. However, challenges related to process efficiency can be tackled more easily. This includes analytics, which directly influences product quality, and can save precious time and resources. Traditional UV spectroscopy, commonly used for bioprocess analytics, has limitations such as lengthy assay times, labor-intensive procedures, and susceptibility to errors. Variable pathlength technology, also known as slope spectroscopy, addresses these issues by adjusting pathlengths to maintain a constant concentration and eliminate the need for dilutions. A case study on using the SoloVPE® system to determine plasmid DNA purity ratios will be explored, along with other variable pathlength technology applications across various stages of gene therapy, including fermentation, downstream processing, chromatography, mRNA purity measurements, and AAV titer analysis.

Nucleic Acid Insights 2024; 1(8), 249–262

DOI: 10.18609/nai.2024.032

KEY CHALLENGES WITH TRADITIONAL UV SPECTROSCOPY FOR GENE THERAPY ANALYTICS

UV-based measurements hold a considerable share of today's bioprocess analytics due to their simplicity, accuracy, and conventionality. For example, UV spectroscopy is commonly used when manufacturing plasmid DNA (pDNA), an integral component of many gene therapy products.

However, while being a relatively simple method, UV spectroscopy also brings a lot of challenges. Firstly, traditional UV spectroscopy requires a long assay time, ranging from 30 min to 3 h, depending on the number of

samples being tested. Additionally, this process is labor-intensive, as it typically involves sample preparation, dilutions, standard curves, and manual calculations.

Secondly, sending samples to the analytics laboratory for UV spectroscopy analysis also takes time, often delaying the receipt of results. This waiting time can also be extended by a backlog of samples in QC, varying shifts, or sample batch schedules, all of which create bottlenecks that must be addressed before making decisions for the next step in product development or manufacturing.

Thirdly, UV analytics may be complicated, involving manual dilutions and calculations, and therefore requires additional personnel training, which increases the time needed for validation. These manual steps can delay the implementation process, adding both time and stress to the overall workflow.

Finally, sample dilutions can often introduce errors, as UV sensors can only provide reliable signals up to a certain product concentration before saturating. Dilution errors, which can vary significantly depending on whether the process is volumetric or gravimetric, can compound and accumulate, ranging from 5 to 20%. The volumetric approach is simpler but riskier, relying on an analyst's skills and additional training, which may lead to human error. The gravimetric approach is more accurate but requires extra equipment, regular maintenance, and further training, making it more time-consuming. Regardless of the chosen approach, dilution errors will inevitably be introduced into the method, which is undesirable.

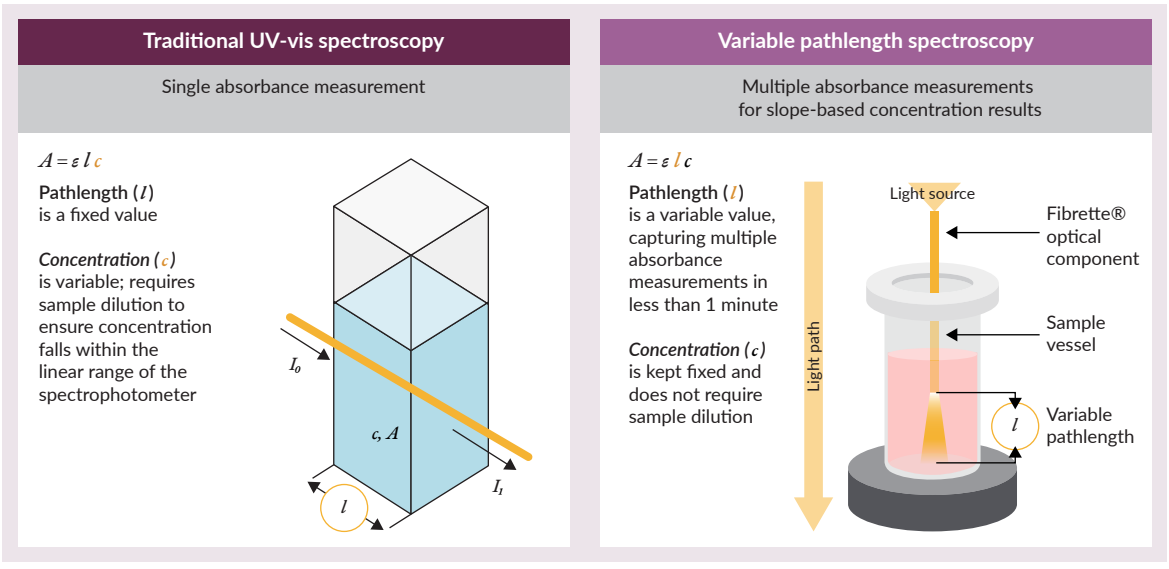
TRADITIONAL UV SPECTROSCOPY VERSUS VARIABLE PATHLENGTH SPECTROSCOPY

Traditional UV spectroscopy is based on Beer-Lambert's law, which states that absorbance equals the extinction coefficient multiplied by pathlength and concentration (Figure 1). In all conventional UV devices, whether benchtop or in-line sensors, a fixed pathlength is used, (e.g. 10 mm for a standard cuvette). Given that UV sensors have a limited reading range for concentration, exceeding this range results in no usable data. Therefore, the only solution is to dilute the sample.

Variable pathlength technology (VPT) also relies on Beer-Lambert's law but differs from

FIGURE 1

Traditional UV spectroscopy versus variable pathlength spectroscopy.



conventional methods by allowing the pathlength to adapt to the product concentration, thus keeping the concentration constant. By adjusting the pathlength—the distance light travels through the sample—the concentration value is fixed, which removes the need for sample dilution. As a result, VPT significantly increases process efficiency, analysis robustness and reduces cycle times by providing instant analytical results, which enhances the process understanding with real-time insights.

VPT FOR AT-LINE AND IN-LINE ANALYTICS

VPT can be implemented in both at-line (sample-based) and in-line (built in into a process) analytical systems. These technologies can be adopted across a wide range of process steps in pDNA production, including tangential flow filtration, chromatography, fill and finish, and upstream processes like bacterial fermentation.

At-line VPT spectrophotometers such as SoloVPE® and in-line systems like FlowVPX® are designed to be used in various applications, including analysis of proteins, nucleic acids, oligonucleotides, antibody-drug conjugates, and viral titer measurements.

SoloVPE system for at-line concentration monitoring

SoloVPE is a sample-based at-line VPT system. During data collection, the system moves the fibrette to set the zero pathlength, then adjusts it to find the optimal pathlength for a one absorbance unit reading using a software algorithm. The algorithm is automatically adapted to the sample's concentration, and the software defines the linear regression slope and calculates concentration using Beer-Lambert's law if the extinction coefficient is provided. The system scans from small to large pathlengths to find the one that achieves 1 AU. The software then adjusts pathlengths based on absorbance changes, collecting 5–10 absorbance data points at different pathlengths to define the slope value of the linear regression and in turn the concentration in a sample.

This innovative technology allows working on a large concentration range—from 5 µm to 15 mm, which provides 3,000 choices of pathlengths to establish true linearity within Beer Lamberts Law. The process is fully automated and requires no operator adjustments.

Compared with traditional UV sample-based instruments, SoloVPE offers a streamlined two-step analytical method that takes approximately 2 min, as opposed to the traditional seven-step method which can take between 30 and 70 min, depending on conditions. Furthermore, the SoloVPE system eliminates the need for estimations, dilutions, or manual calculations, resulting in a more accurate, reproducible, and high-quality analysis. This slope-based technique simplifies validation processes and facilitates smoother inter- and intra-method transfers. While still UV-based, it offers at-line process analytics concentration measurements by utilizing slope spectroscopy.

FlowVPX system for in-line analytics

Variable pathlength extension technology (VPE technology) is also adaptable to in-line analytics. For example, FlowVPX is a GMP-compliant in-line system that can be incorporated directly into the production process for real-time concentration monitoring.

It offers the same benefits of VPT such as high accuracy and no need for baseline correction, while also providing real-time data acquisition for enhanced process efficiency. Besides, the in-line VPE technology provides a broad dynamic range, allowing measurement of concentrations from 0.1 to 300 mg/ml. This feature shortens development time by providing deeper process understanding, reducing the risks by increasing the process control and bringing real-time insights.

CASE STUDY: UTILIZING SLOPE SPECTROSCOPY TO DETERMINE PDNA PURITY RATIOS IN HUMAN GENE THERAPY PRODUCTS

Introduction to limitations of traditional nucleic acid analytics

There are many challenges and issues associated with traditional nucleic acid analytics. First, there are limitations regarding different light absorptions at 260 nm and 280 nm. Nucleic acids absorb light at both wavelengths, but the amount of light absorbed at each wavelength differs, requiring distinct extinction coefficients and R-values for different sequences. This introduces several challenges, such as the need to perform separate dilutions for each wavelength and prepare the samples for each measurement. Additionally, DNA purity is assessed using the ratio of absorbance at 260 nm to 280 nm, known as the R-value, with a number between 1.8 and 2.0 indicating purity. The necessity for multiple measurements and calculations in this method not only increases the potential for errors but also makes the process time-consuming. Furthermore, traditional methods are often limited by the need for a low sample volume, which can be a significant restriction.

Study objectives

This study, in collaboration with Pfizer, was carried out to determine the pDNA purity ratios in human gene therapy products using slope spectroscopy, namely SoloVPE, in place of traditional nucleic acid analytics.

The primary objectives of this study were to demonstrate that the SoloVPE system is capable of accurately measuring plasmid concentrations and determining the purity ratio, specifically the R-values associated with 260 nm and 280 nm measurements. The aim was to evaluate the impact of dilution on the measurement process and to compare the reliability and accuracy of the SoloVPE method with traditional spectrophotometry techniques.

TABLE 1

25 protein and DNA levels (%) measured using SoloVPE

Level	% protein	% DNA
1	100.0	0.0
2	97.5	2.5
3	95.0	5.0
4	92.5	7.5
5	90.0	10.0
6	85.0	15.0
7	80.8	20.0
8	75.0	25.0
9	70.0	30.0
10	65.0	35.0
11	60.0	40.0
12	55.0	45.0
13	50.0	50.0
14	45.0	55.0
15	40.0	60.0
16	35.0	65.0
17	30.0	60.0
18	25.0	75.0
19	20.0	80.0
20	15.0	85.0
21	10.0	90.0
22	7.5	92.5
23	5.0	95.0
24	2.5	97.5
25	0.0	100.0

Methodology

Before the analysis, system suitability tests were performed, and the concentrations of stock solutions of plasmids and insulin were measured, which had to be strictly 1.0 mg/ml. The appropriate extension coefficient for plasmids and insulin was applied. Spectral scans of stock solutions were also collected to define the wavelengths of interest and to double-check if the right wavelengths were being measured.

Next, in order to assess purity levels for the mixture of DNA (pCI-neo vector from LakePharma) and protein (insulin solution from Sigma-Aldrich), the R-values were measured. The R-value is typically in the range of

1.8 to 2.0, though it can vary depending on the specific molecule and application.

Afterward, 25 different purity levels for a mixture of DNA and protein were defined, and corresponding solutions for each level were prepared. These ratios ranged from 100% protein and 0% DNA to 100% DNA and 0% impurities (Table 1). Triplicate measurements were taken at each purity level to obtain the 260/280 nm absorbance ratio and slope values. The SoloVPE software automatically calculated the R-values for each measurement. To compare the experimental R-values with theoretical values, theoretical purity ratios were calculated for each solution and compared with the R-values obtained from SoloVPE (Table 2).

Results

Based on the measurements, the observed purity ratio results closely matched the theoretical values (Table 2). To provide a graphical representation, the percentile differences were plotted on a chart against a ±5% tolerance scale. If values that exceed the typical variation of 1% are consistently observed, it could indicate impurity levels of ~15%. For a 1.0 mg/mL plasmid, this would indicate protein contamination of 150 µg/mL, which is only slightly higher than the sensitivity threshold of colorimetric techniques. However, the chart reveals that all differences fall within 2%, with most differences under

TABLE 2

pDNA purity study results, including theoretical purity ratios (left column), experimental purity ratios (middle column), and their differences (right column).

Level	Theoretical purity ratio	Observed purity ratio	% difference
1	0.62590	1.62723	0.21
2	0.87087	0.90315	-0.22
3	1.05311	1.06122	0.74
4	1.18483	1.17076	-1.14
5	1.28451	1.28847	0.30
6	1.42528	1.42358	-0.11
7	1.52996	1.52481	0.31
8	1.58798	1.58959	0.10
9	1.63927	1.65568	0.96
10	1.67930	1.67489	-0.25
11	1.71134	1.69203	-1.090
12	1.73770	1.71515	-1.25
13	1.75964	1.73533	-1.33
14	1.77821	1.77064	-0.41
15	1.79418	1.81956	1.36
16	1.80804	1.77874	-1.56
17	1.82026	1.80414	-0.85
18	1.83094	1.81184	-1.00
19	1.84046	1.82975	-0.56
20	1.84902	1.85457	0.29
21	1.85672	1.84667	-0.52
22	1.86028	1.88378	1.22
23	1.86365	1.85282	-0.56
24	1.86692	1.84941	-0.90
25	1.87000	1.87147	0.08

pDNA: plamid DNA.

1.5%, indicating accurate measurements using SoloVPE system (Figure 2).

Discussion

In traditional spectrophotometry techniques, samples must be diluted for each wavelength and measurements must be performed separately, applying different extension coefficients for 260 nm and 280 nm. Additionally, the R-value must be calculated manually. In contrast, the SoloVPE system streamlines this process by automatically handling the measurements at both 260 nm

FIGURE 2

The theoretical and observed R-values and error rates (%).

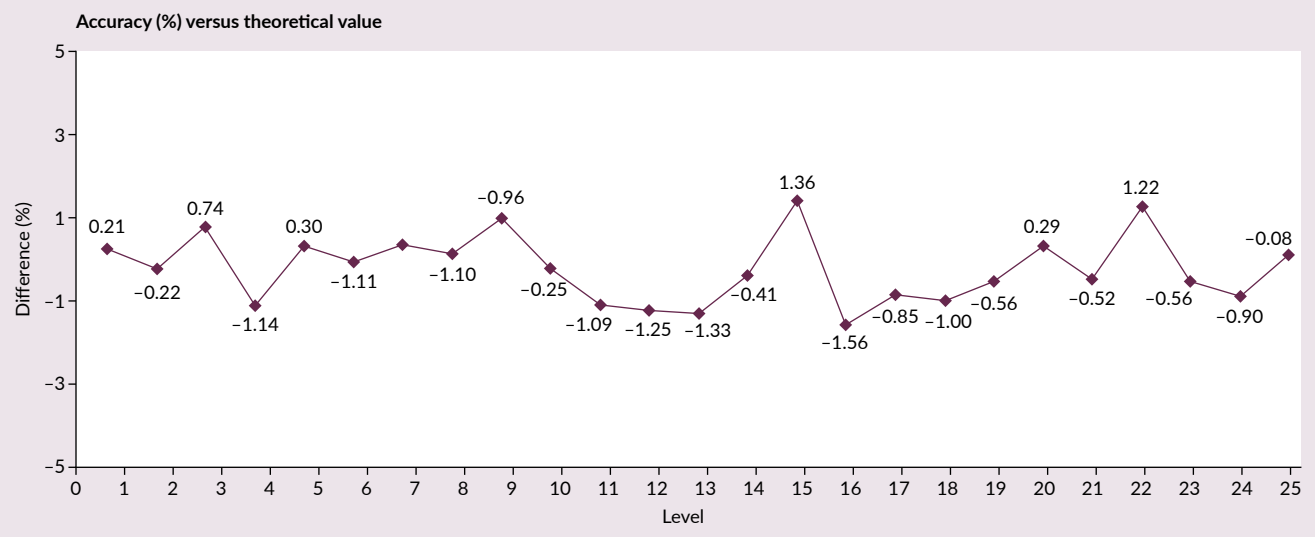
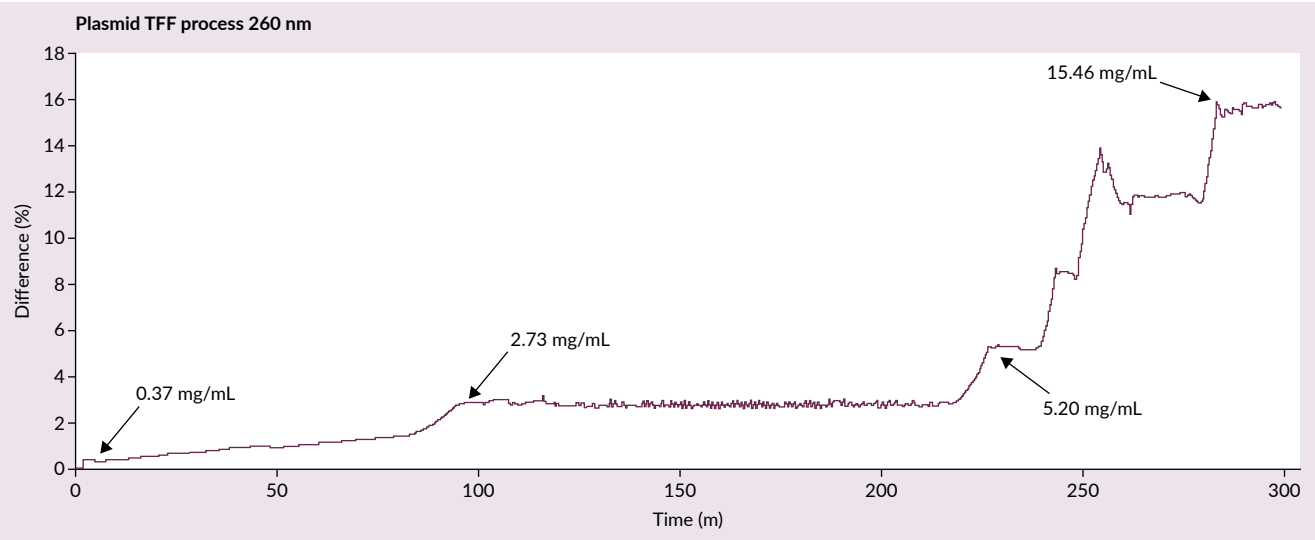


FIGURE 3

In-line measurement in real-time with the during UF/DF step for pDNA in C/D/C mode.



pDNA: plasmid DNA, UF/DF: ultrafiltration/diafiltration.

and 280 nm, performing the necessary calculations, and providing the purity ratios (R-values) directly.

The slope spectroscopy technique used by SoloVPE addresses challenges associated with sample volume, dilution, and inconsistent wavelength readings. The SoloVPE System and its dedicated software enable simultaneous measurements at 260 nm and 280 nm, with results automatically calculated and reported. This system proves to be a pivotal analytical tool for determining plasmid DNA purity, expediting sample testing, providing immediate feedback, and expanding possibilities for meeting medical needs.

Conclusion

The plasmid purity measurements in this study exhibited small, consistent variations. The case study demonstrates that the scope spectroscopy-based SoloVPE system demonstrates significantly improved sensitivity compared to traditional spectrophotometers.

OTHER GENE THERAPY APPLICATIONS USING VPT

Apart from measuring pDNA purity ratio in human gene therapy products, there are various other areas where VPT could be applied, including pDNA downstream processing monitoring, fermentation analysis, chromatography, mRNA purity ratio measurements, and AAV capsid and genome titer analysis among others.

In-line VPE technology in downstream pDNA processing

VPT could be used to increase the efficiency of the downstream pDNA processing with in-line concentration monitoring and control. , Both SoloVPE and FlowVPX could be utilized to produce real-time concentration measurements during the ultrafiltration/diafiltration (UF/DF) step, demonstrated by the experimental data illustrated in Figure 3.

During the study, the initial concentration of pDNA was less than 0.5 mg/ml, then increased to about 2.70 mg/ml, and further concentrated to over 15 mg/ml during diafiltration. Parallel measurements with SoloVPE demonstrated excellent comparability between at-line and in-line methods. FlowVPX yielded a final concentration of 15.46 mg/ml, compared to 15,00 mg/ml from SoloVPE, highlighting the high accuracy and reliability of the in-line monitoring system (Figure 3).

VPT for monitoring *E. coli* fermentation

VPT can also be used to monitor and control *E. coli* fermentation, a critical step in plasmid production. High-quality pDNA production requires a closely monitored and controlled process to determine the optimal time for induction and harvest. However, standard UV-Vis spectrophotometer measurements require sample dilution and subtraction of media components, which is time-consuming and labor-intensive.

In collaboration with the Biofactory Competence Center in Switzerland, a method to measure OD600 and monitor bacterial growth utilizing both SoloVPE and FlowVPX was developed, and it was discovered that FlowVPX offers greater benefits. By connecting FlowVPX to a bioreactor, bacterial growth can be constantly monitored in real time, building the growth curve without the errors and time wasted in offline methods. This continuous monitoring is especially advantageous for collecting data overnight when analysts are unavailable.

Results show that *E. coli* cell growth curves can be monitored using both at-line and in-line VPT systems with high accuracy and repeatability, without needing dilution or baseline correction. The data is comparable to standard spectrophotometer OD600 measurements (Figure 4), but the FlowVPX system's cycle time of around 30 s allows for two measurements per minute, offering real-time analytics, and doesn't demand sampling, dilution and blanking.

In-line VPE analytics for optimizing chromatography

VPT can also be utilized to optimize capture and polishing chromatography steps, which are crucial components of downstream processing across various modalities including pDNA. By integrating FlowVPX into the chromatography setup, it is possible not only to monitor the process but also to provide key chromatography metrics such as loading mass, breakthrough time, DBC, and elution mass by using fewer input parameters and performing simple calculations.

FIGURE 4

In-line measurement in real-time with the FlowVPX and the off-line measurements with the standard spectrophotometer for the sampling time points.

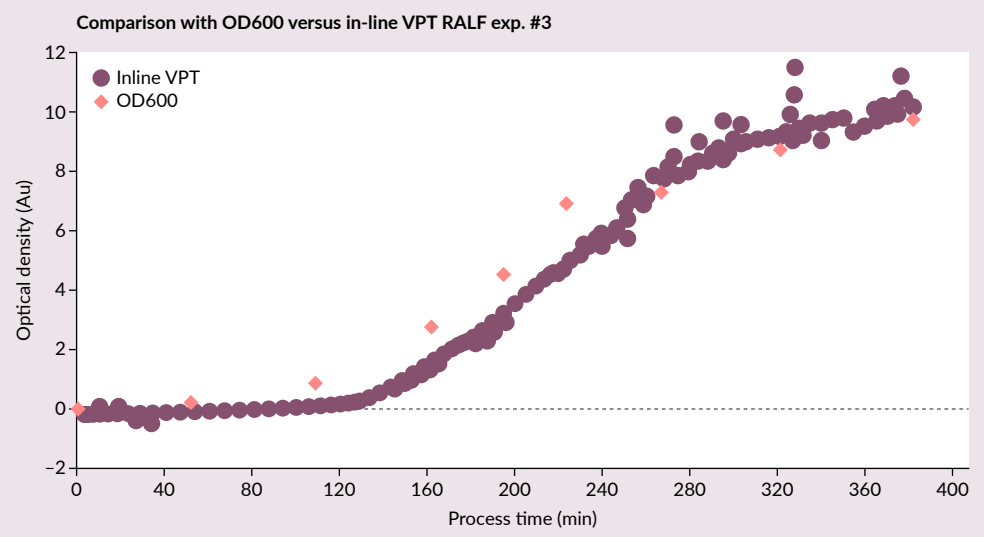


FIGURE 5

Harvested cell culture material flown through the FlowVPX unit to get a baseline signal during chromatography.

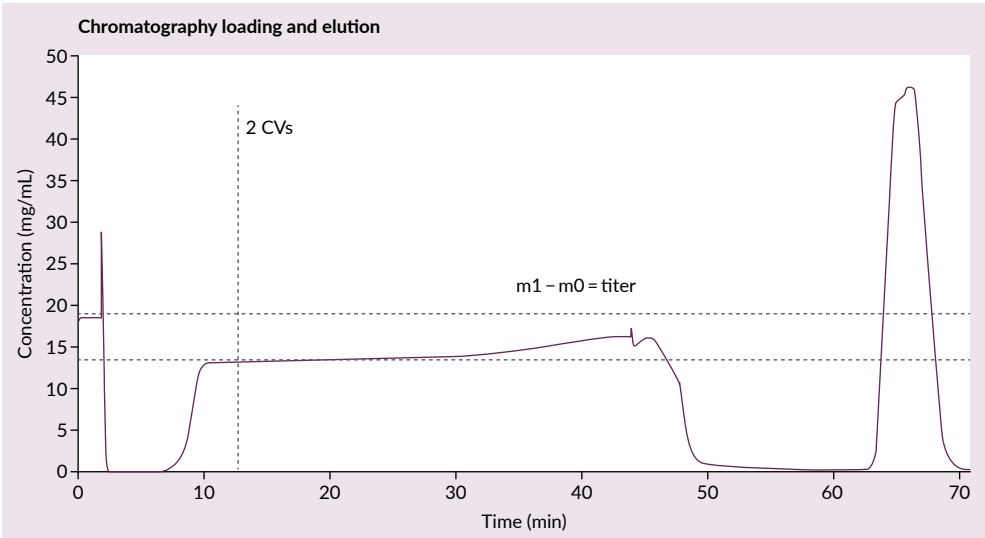
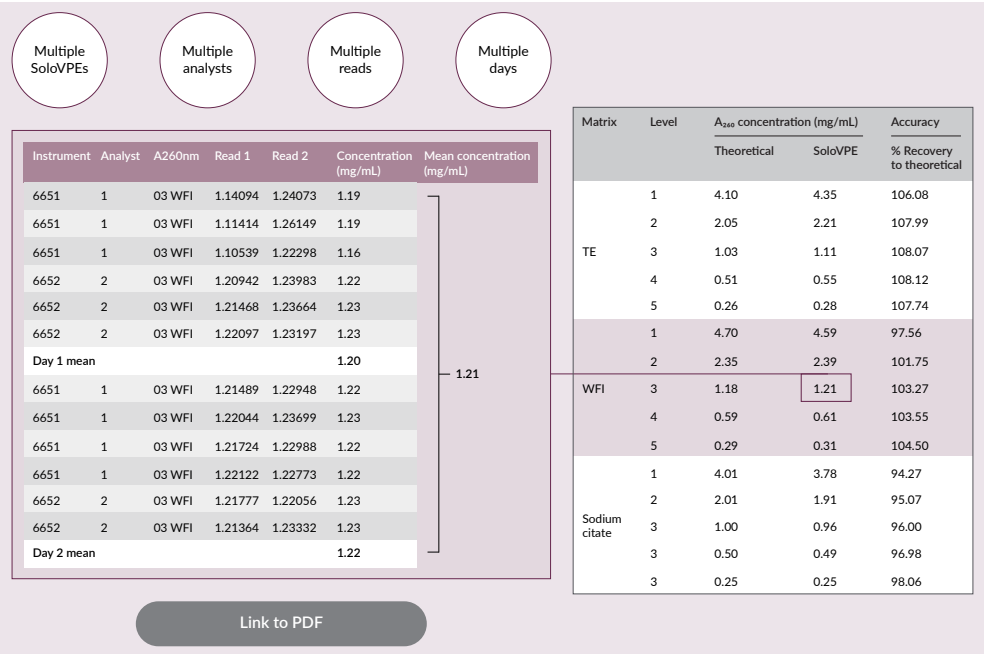


FIGURE 6

Capabilities of the SoloVPE technology as a platform analytical method for mRNA purity ratio measurements. VPE: variable pathlength extension.



Instrument	Analyst	A260nm	Read 1	Read 2	Concentration (mg/mL)	Mean concentration (mg/mL)
6651	1	03 WFI	1.14094	1.24073	1.19	
6651	1	03 WFI	1.11414	1.26149	1.19	
6651	1	03 WFI	1.10539	1.22298	1.16	
6652	2	03 WFI	1.20942	1.23983	1.22	
6652	2	03 WFI	1.21468	1.23664	1.23	
6652	2	03 WFI	1.22097	1.23197	1.23	
Day 1 mean					1.20	
6651	1	03 WFI	1.21489	1.22948	1.22	1.21
6651	1	03 WFI	1.22044	1.23699	1.23	
6651	1	03 WFI	1.21724	1.22988	1.22	
6651	1	03 WFI	1.22122	1.22773	1.22	
6652	2	03 WFI	1.21777	1.22056	1.23	
6652	2	03 WFI	1.21364	1.23332	1.23	
Day 2 mean					1.22	

Matrix	Level	A_{260} concentration (mg/mL)		Accuracy
		Theoretical	SoloVPE	% Recovery to theoretical
TE	1	4.10	4.35	106.08
	2	2.05	2.21	107.99
	3	1.03	1.11	108.07
	4	0.51	0.55	108.12
	5	0.26	0.28	107.74
WFI	1	4.70	4.59	97.56
	2	2.35	2.39	101.75
	3	1.18	1.21	103.27
	4	0.59	0.61	103.55
	5	0.29	0.31	104.50
Sodium citate	1	4.01	3.78	94.27
	2	2.01	1.91	95.07
	3	1.00	0.96	96.00
	3	0.50	0.49	96.98
	3	0.25	0.25	98.06

For example, in the process shown in Figure 5, harvested cell culture material was flown through the FlowVPX unit to establish a baseline signal of approximately 18 mg/ml. As the sample flows through the chromatography column, it is possible to wait until the signal stabilizes, and then measure the difference to obtain the titer in real-time. This titer value, multiplied by the time and flow rate, allows converting the concentration chromatogram into a mass plot, offering insights into the mass loaded onto the column.

This approach is particularly beneficial for optimizing continuous processing or multi-column processes, as it helps to understand and adjust them based on process parameters and product types. At-line VPT for measuring mRNA purity ratio.

In addition, VPT-based systems could be used to measure mRNA purity ratio. In an experiment carried out to compare the theoretical method with the SoloVPE system for mRNA purity validation, it was shown that SoloVPE technology is a highly robust platform for analytical methods.

Accuracy and repeatability tests were performed using five levels with three results per level, as outlined in Figure 6. A single mRNA molecule was formulated in three representative sample matrices, and method comparability was included to bridge from Aldevron's cuvette-based assay.

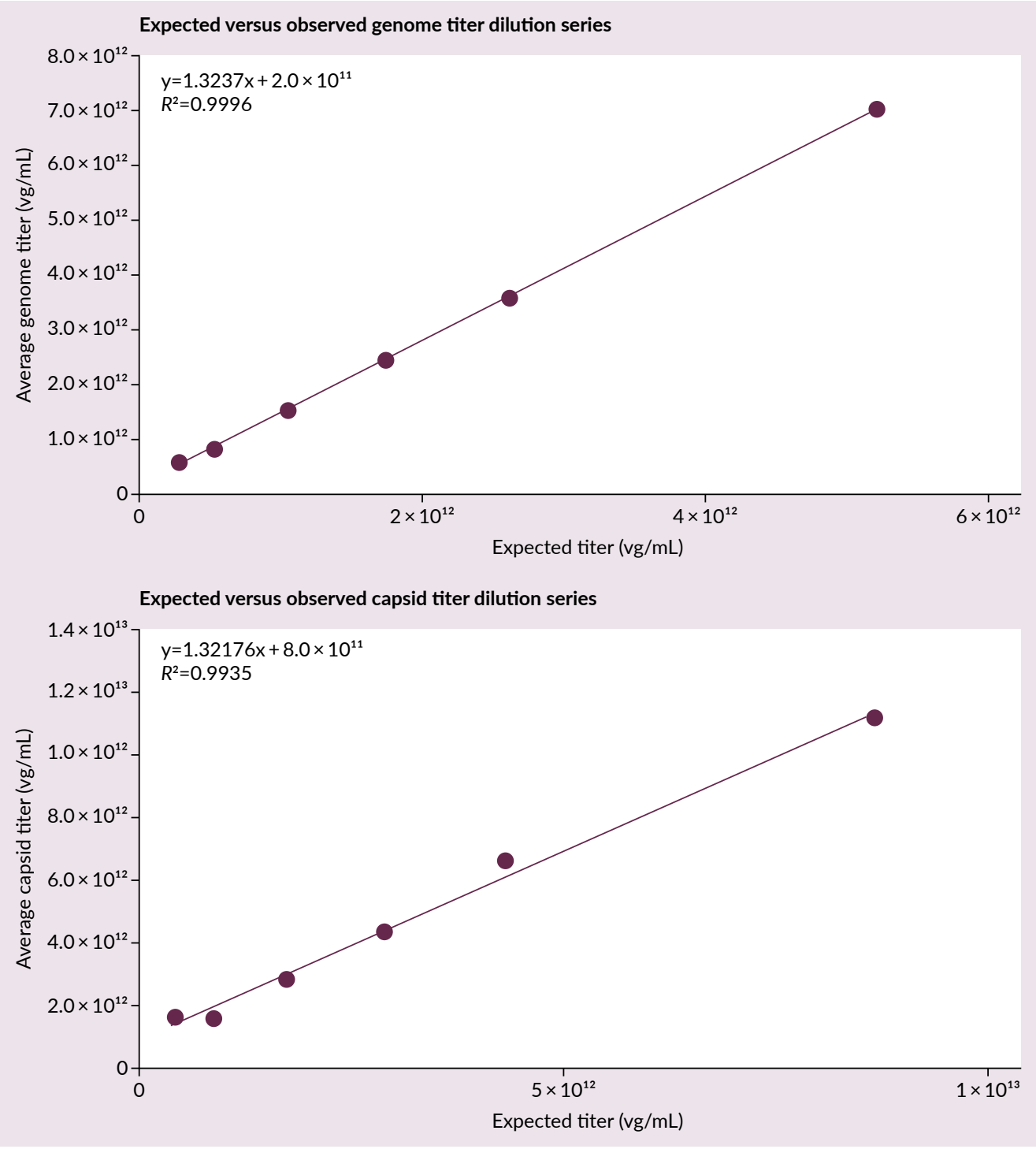
The results demonstrated that the key validation parameters such as specificity, intermediate precision, repeatability, linearity, and accuracy met the necessary standards for validation. The observed purity ratios from the SoloVPE system closely matched the theoretical ratios, verifying the SoloVPE system's efficiency for this application.

At-line VPT for AAV capsid and genome titer: linearity

The slope spectroscopy method also allows precise determination of the AAV genome and capsid titers, demonstrated by the experiments outlined in Figure 7. This process is less error-prone and less dependent on personal training, and it can also define the

FIGURE 7

Linearity between expected & observed genome and capsid titer dilution series of AAV.



empty-full ratio, a process typically heavy on calculations.

SUMMARY

Utilizing VPT for at-line and in-line concentration measurements can offer significant advantages by increasing efficiency, reducing cycling time and risk, and providing real-time process insights at every stage of the nucleic acid manufacturing process, including fermentation, harvesting, downstream processing, formulation, fill and finish, chromatography, and filtration. Adopting a unified analytical platform with VPT may offer benefits, such as a broad concentration range without the need for baseline correction, robust and accurate measurements, and no dilution required. Each result produced by VPT systems is based on multiple measurements, providing an R^2 value for every outcome, ensuring reliable and precise data.

Q&A

Oleksandr Karpenko

Q Are the Flow Cells for the FlowVPX autoclavable?

OK: We have recently performed an extensive study on autoclaving Flow Cells and discovered that they can be autoclaved up to 15 times and are applicable to cleaning in place.

Q Can VPT be used for other molecules like siRNA, oligonucleotides, and larger molecules such as mRNA?

OK: For all the mentioned molecules, the methods are already established for both SoloVPE and FlowVPX. In general, all substances or materials that can be detected in traditional UV spectrophotometers are also detectable by SoloVPE and FlowVPX. Both devices work based on Beer-Lambert's law like conventional UV analytical devices.

Q Can the *E. coli* growth process be automated using the OD600 real-time reading?

OK: FlowVPX can be connected to the reactor to measure OD600 and provide the growth curve in real-time during bacterial fermentation. By integrating ViPER software for FlowVPX into the software that controls the bioreactor, one can not only monitor the process but also control it. For example, it allows users to automatically add some excipients, such as feed portions or trace elements into the bioreactor when the growth curve reaches a certain value.

Q Can the SoloVPE method be used for releasing GMP-finished drug products?

OK: Both SoloVPE and FlowVPX are completely GMP-compliant, including 21 CFR Part 11 conformity, meaning they could be used for release testing. FlowVPX could be used also for real-time release testing.

Q How can Repligen support the instrument, computer, and software qualification?

OK: After purchasing the device, our technical support specialist provides the complete Installation Qualification/Operational Qualification (IQOQ) procedure, performing all the necessary tests to ensure the device is working properly. There is also an option for continued process verification. Repligen can also provide preventive maintenance for the devices. Finally, there is an additional service of computer software validation upon the customer's request.

BIOGRAPHY

OLEKSANDR KARPENKO serves as a Bioprocess Analytics Field Application Scientist at Repligen Corporation, Waltham, MA, USA where his primary focus is ensuring that our CTech Analytical solutions meet customers' expectations and analytical needs. Collaborating closely with bioprocess teams, Oleksandr provides scientific guidance, supports implementation, validation, and conducts training sessions on CTech™ Analytical Solutions. His expertise lies in developing and optimizing customized methods to ensure optimal usage of analytical instruments and overall customer process success.

Before joining Repligen, Oleksandr held leadership positions at both BioCape GmbH and Biotensidon GmbH. At BioCape, he led a team in synthesizing, characterizing, and scaling bio-based surfactants. Earlier, at Biotensidon, he began as a scientist, focusing on fermentation process optimization, before advancing to Head of Bioprocess Development, overseeing the design, optimization, and scaling of bioprocesses for high-value biosurfactants and raw materials.

Oleksandr holds a PhD from Kyiv Polytechnic Institute, Kyiv, Ukraine and an MSc in Biotechnology from Lviv Polytechnic National University, Lviv, Ukraine. His skills include expertise in spectroscopy, process development, downstream processing, bacterial fermentation, continuous improvement, and statistical analysis.

Oleksandr's wealth of experience has shaped him into an exceptionally knowledgeable partner who tirelessly ensures his customers achieve optimal analytical results while operating efficiently.

AFFILIATION

Oleksandr Karpenko
Bioprocess Analytics Field Application Scientist,
Repligen Corporation,
Waltham, MA, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: Karpenko O is an employee of Repligen.
Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is based on a transcript of a webinar, which can be found here.
Webinar held: Jun 6, 2024; **Revised manuscript received:** Aug 5, 2024;
Publication date: Oct 2, 2024.

CTech™ SoloVPE® Variable Pathlength Spectroscopy

CHANGE YOUR PLATFORM, NOT YOUR SAMPLE

The CTech™ SoloVPE® System leverages the power and flexibility of variable pathlength technology to create a rapid, robust, and repeatable, non-disruptive concentration measurement method for proteins, plasmid DNA, and nucleic acid modalities, with results in minutes, not hours.

- **Preserve sample integrity:** Direct sample measurement with no sample prep/dilution required maintains precious sample integrity for additional testing.
- **Highly sensitive:** Wide range of concentrations provides accurate measurement regardless of sample concentration.
- **High repeatability and reproducibility:** Slope-based measurement confirmed with R^2 of ≥0.999 and ≤2% CV ensures system-to-system consistency, enabling easy transfer of methods.
- **Compliance:** Automated data analysis software supports 21 CFR Part 11 and EU Annex 11 for GMP environments.

Upgrade your platform for improved accuracy

repligen.com/solovpe

repligen.com

REPLIGEN

NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: LNPs

SPOTLIGHT

INTERVIEW

Advancing delivery of RNA therapeutics with cationizable xenopeptides

In this interview **Róisín McGuigan**, Editor, *Nucleic Acid Insights*, speaks to **Ernst Wagner**, Chair of Pharmaceutical Biotechnology, Department of Pharmacy and Center of Nanoscience at LMU Munich, exploring advances in non-viral nucleic acid delivery systems and focusing on polycationic carriers and novel formulations including lipo-xenopeptides.

Nucleic Acid Insights 2024; 1(8), 285–291

DOI: 10.18609/nai.2024.035

Q What are you working on right now?

EW: I have been working in the nucleic acids field since 1988, mostly focusing on polycationic carriers and receptor targeting. In 1994, we conducted the first worldwide clinical study using polycationic carriers for gene therapy in humans, which was a pioneering effort at the time.

More recently, we have developed a novel class of cationic carriers based on chemical evolution. These carriers are called lipo-xenopeptides, but you also could call them 'molecular

chameleons' due to their ability to evolve throughout the delivery process: they are neutral in the bloodstream but get positively charged in the endosome. Currently, we are exploring this novel class of polycation carriers for various nucleic acids, as we believe that different nucleic acids require specific formulations. These different formulations of polycation carriers include lipid nanoparticles (LNPs), polyplexes, and conjugates. Our current work also involves comparing these carriers for small nucleic acids like siRNA and large structures like CRISPR-Cas9 mRNA.

Q How would you frame the current state of the art and key challenges in nucleic acid therapeutic delivery?

EW: There are around 30 gene therapies and a similar number of RNA therapies on the market, with many more in the pipeline. However, gene therapies and cell therapies, based on viral or non-viral vectors, still face significant challenges and limitations.

Firstly, when it comes to non-viral vectors, achieving high potency is a big issue. Viral vectors are usually far more potent than non-viral vectors at the nanoparticle level, and you need only a few viruses to infect the cell. On the other hand, non-viral transfections require 100,000 or more particles per cell—this illustrates that there are still improvements to be made.

Secondly, there are challenges associated with tissue targeting. Synthetic non-viral vectors such as LNPs only work in hepatocytes and local tissues after vaccination, and there are siRNA conjugates used in clinical products that also work primarily in hepatocytes. There are a lot of chemically modified molecules that work in various organs, but not at high efficacy. The challenge is figuring out how to target tissues outside the liver effectively.

Finally, there are also challenges caused by long development times. Every new compound requires in-depth development before it can be used. If you have systems like LNPs or siRNA conjugates, which took decades to develop, it still takes a lot of time to translate them into clinical products because of GMP and regulatory considerations. Innovation is hampered by the important but lengthy process of demonstrating product safety in clinical settings. Further, for 10 different indications, you might need 10 different approaches, rather than using identical LNPs for all, significantly adding to the challenge.

Q Focusing on LNPs, where do you see progress being made in addressing key challenges such as stability, liver targeting, and endosomal escape?

EW: The development of LNPs has been significantly sped up by the COVID-19 pandemic. Of course, Pieter Cullis, a pioneer in this field since the early 1990s, laid much groundwork. However, it took a long time to get them into applications. For example, the

"...current ongoing studies that focus on generating new ionizable lipids are expected to find versions that offer even greater stability."

siRNA-based drug patisiran was ready just before the pandemic, and since then we have seen an incredible development in LNPs.

Starting with stability concerns, they first arose because the product had to be released in an emergency and there was not enough time to develop the final formulations. However, the stability of these RNA-based products can be enhanced via approaches including storage buffers, specific formulation, and lyophilization, which makes me optimistic. For COVID-19 vaccines, stability relies on mRNA's inherent stability and chemical modifications designed to further enhance it. LNPs can also enhance stability through the inclusion of cationizable lipids or components. These elements are important in LNPs, which are responsible not only for wrapping the nucleic acid inside the LNP, but also for packaging and protecting it. On top of that, current ongoing studies that focus on generating new ionizable lipids are expected to find versions that offer even greater stability.

Turning to the hurdles associated with targeting cells and tissues outside of the liver, this is something the field is working to solve. One solution, as seen in the COVID-19 vaccines, is local intramuscular application. This primarily results in expression outside the liver, but is not specific. Another potential approach involves utilizing different ionizable lipids. A series of developments have shown that the ionizable lipid component of LNPs has multiple functions other than being responsible for the packaging of the nucleic acids; it also helps with tissue targeting and endosomal escape. Our recent studies show that novel ionizable compounds called lipo-xenopeptides have more than one charge, which enables LNPs to more potently disrupt endosome membranes, allowing siRNA to escape and target hepatocytes.

Q Can you expand more on your own activities in this area?

EW: It is important to understand that chemical retargeting is not a novel concept, but it rather shows that the existing work that people have developed is robust and reproducible, even for introducing new ionizable compounds.

The idea of biological targeting has been that nanoparticles can be surface-modified to target receptors. We started using this approach 35 years ago with transferrin, a natural protein that is taken up by its natural receptor, enabling entry into those cells. The other example is siRNA-GalNAc conjugates like givosiran, which targets hepatocytes by binding to asialoglycoprotein receptors. This method is usually called biological targeting, which is similar to how viruses work—receptors and ligands on their surfaces bind to cellular receptors.

"Our novel cations ... become active by protonation in the acidic endosome, which helps to achieve at least a five-fold improvement in endosomal escape over traditional LNPs."

The novel discoveries made by people like James Dahlman and Dan Siegwart involve physicochemically modifying the nanoparticle on the surface to make it a bit more positively charged, or adding new chemical components. As a result, these particles can bind to different blood proteins, forming a protein corona, which acts as a ligand for receptors. A classic example is LNPs containing cholesterol and phosphatidylcholine binding apolipoprotein E to target the low-density lipoprotein receptor of the liver. If a different compound is used, then other proteins will bind, directing LNPs to different cell types.

In our case, we found that xenopeptide-modified LNPs target liver endothelial cells instead of hepatocytes. Standard siRNA LNPs can enter hepatocytes and silence the blood coagulation factor VII, which is produced by hepatocytes. This has been a common test system for LNP development. Our formulation does not silence factor VII but instead targets liver endothelial cells that express blood coagulation factor VIII, a protein deficient in conditions like hemophilia. Based on mouse studies, our lipo-xenopeptides can silence the factor VIII gene. This is not in itself useful as you need this factor, but we can use this knowledge to understand what else you could potentially modify in the endothelial cells of the liver.

Moreover, researchers also found formulations that enhance LNP delivery to tumors. Other researchers have also made great efforts in chemically modifying LNP surfaces with natural receptor ligands or antibodies, which enables the targeting of hematopoietic stem cells, bone marrow, or T cells. This strategy could also be used—for example, by using antibodies against specific targets and leveraging surface chemistry to guide LNPs to the right cells in the blood, and subsequently into tissues. Some researchers have successfully targeted lung tissue using cationic molecules within LNPs. This approach, known as selective organ targeting, facilitates LNP delivery to the lungs using positively charged particles that bind to specific proteins. Examples like these highlight the successes and challenges of LNPs *in vivo*.

Then there is endosomal escape, which has been a known issue for many years. Even potent LNPs are estimated to only deliver 2–3% of nanoparticles out of the endosome into the cytosol, according to Anders Wittrup and Judy Lieberman. Generally, viral transfection is more efficient for nucleic acid delivery, considering only a few viral particles are sufficient to infect a cell. In contrast, the classical dose used for non-viral transfection might be, for example, 100 ng per cell culture, equating to 100,000 particles per cell. For vaccines, a typical dose may contain 50 μg of mRNA, which is about 30 million nanoparticles. This excessive dose is not toxic, but it highlights that viruses are far more effective. One of the reasons for this disparity is the fact that most non-viral lipid particles accumulate in the endosomes, where they are degraded and kicked out of the cell again.

Our novel cations could help overcome these hurdles. They become active by protonation in the acidic endosome, which helps to achieve at least a five-fold improvement in endosomal

escape over traditional LNPs. In our best polyplex cases, we have observed mRNA activity with amounts as little as 3 pg, equating to only two nanoparticles per cell, similar to viruses. While not every cell expresses mRNA with such low quantities, there is still a signal, meaning that endosomal escape can be significantly improved.

Q What are your chief goals and priorities for your work moving forward?

EW: Currently we are working on LNPs and also developing novel cationic complexes. Some new xenopeptide carriers can be used within LNPs as cationic lipid compounds to encapsulate nucleic acids, as a kind of lipidic bag, others can be used to complex nucleic acids into soluble nanoparticles termed polyplexes. But what distinguishes cationic polyplexes from LNPs, and what are their potential future applications?

Polyplexes have an advantage over LNPs in terms of nucleic acid compaction. LNPs contain lipids with a single positive charge, making them neutral once bound to nucleic acids and only protonated during particle formation. Unlike polycations in our cells that compact DNA tightly, LNPs lack this compaction ability. Polyplexes, with multiple positive charges, are ideal for large nucleic acids like DNA, forming stable nanoparticles.

Polyplexes and LNPs also differ in stability. For small nucleic acids like siRNA, polyplexes are sufficiently stable in cell culture but are unstable in the blood. In contrast, siRNA LNPs are stable *in vivo* due to hydrophobic lipidic cooperativity. Further, while polyplexes require a coating for targeting and larger polycations can be toxic, LNPs use natural and artificial lipids for better compatibility. Developing new polycation systems involves proving their efficacy, safety, and biocompatibility, and some can be made biodegradable to prevent persistence in the body.

Overall, LNPs are highly flexible for different nucleic acid cargos, whereas polyplexes work well for larger ones. Each carrier type has its advantages, but the main challenge for both is tissue targeting. In our new system, we incorporate molecules that selectively target receptors expressed on tumor tissues, aiming to deliver therapeutics into solid tumors. This approach works as a proof of concept but needs more improvements.

When it comes to endosomal escape, we found that our chameleon-like molecular systems could solve this issue, but interestingly, we also discovered that maximum endosomal escape might cause cellular damage. Therefore, achieving an optimal, mild, and non-inflammatory level of endosomal escape is our next step.

Our main focus in the near future remains on dynamic, polycationizable systems and rather than simple cationic lipids. Since starting in around 1990, I realized that targeting, shielding, compaction, and endosomal escape complicate molecule conjugation. Inspired by natural evolution, my lab moved towards sequence-defined artificial structures, similar to peptides made via solid-phase synthesis, creating precise xenopeptides that evade the immune system.

Xenopeptides, while complex, offer extreme precision and can be evolved chemically. For instance, starting with 100 structures, we identify few lead structures that we further optimize

via small libraries of ~50. Some xenopeptides work well for DNA but not for siRNA or mRNA, though they succeed with siRNA when formulated with LNPs. This approach demonstrates evolutionary principles where nature determines what is best. You must have a screening system that avoids biases toward a predetermined molecular structure. The challenges lie in applying this screening and evolution in living systems like mice or even primates, which is something researchers like James Dahlman are actively working on. This could also be an opportunity to include artificial intelligence and machine learning to make predictions on what should be the best structure.

FURTHER READING

1. Yazdi M, Pöhmerer J, Hasanzadeh Kafshgari M, *et al. In vivo* endothelial cell gene silencing by sirna-lnps tuned with lipoamino bundle chemical and ligand targeting. *Small* 2024; e2400643.
2. Cheng Q, Wei T, Farbiak L, Johnson LT, Dilliard SA, Siegwart DJ. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. *Nat. Nanotechnol.* 2020; 15(4), 313–320.
3. Wittrup A, Ai A, Liu X, *et al.* Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat. Biotechnol.* 2015; 33(8), 870–876.
4. Thalmayr S, Grau M, Peng L, *et al.* Molecular chameleon carriers for nucleic acid delivery: the sweet spot between lipoplexes and polyplexes. *Adv. Mater.* 2023; 35(25), e2211105.
5. Berger S, Lächelt U, Wagner E. Dynamic carriers for therapeutic RNA delivery. *Proc. Natl Acad. Sci. USA* 2024; 121(11), e2307799120.
6. Dobrowolski C, Paunovska K, Schrader Echeverri E, *et al.* Nanoparticle single-cell multiomic readouts reveal that cell heterogeneity influences lipid nanoparticle-mediated messenger RNA delivery. *Nat. Nanotechnol.* 2022; 17(8), 871–879.

BIOGRAPHY

ERNST WAGNER is Chair of Pharmaceutical Biotechnology, Department of Pharmacy and Center of Nanoscience at LMU Munich, Munich, Germany since 2001. After a PhD in Chemistry (1985, TU Vienna, Vienna, Austria) he was postdoc at ETH Zurich (1985–1987) working on origin-of-life chemistry of nucleic acids, Group Leader at IMP Vienna (1988–1995) exploring receptor-mediated gene delivery and artificial virus-like gene transfer systems, translating gene transfer at Boehringer Ingelheim Austria as Director Cancer Vaccines (1992–2001) into GMP production of gene-modified cancer vaccines (worldwide first polymer-based gene therapy trial in 1994). In 1996, he was also FC Donders Professor for Biopharmaceutical Sciences, Utrecht University. Ernst is an Academician of the European Academy of Sciences, a member of the College of Fellows of Controlled Release Society (CRS), an Honorary Professor at Sichuan University, a Board Member of the German Society for Gene Therapy, a Member of the Nonviral Therapeutic Delivery Committee, America Society of Gene and Cell Therapy (ASGCT). He has authored 518 publications, with 53,227 citations and an h-index of 114 (GS).

AFFILIATION

Ernst Wagner
Chair of Pharmaceutical Biotechnology,
Department of Pharmacy and Center of Nanoscience,
LMU Munich,
Munich, Germany

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

Funding declaration: The author has received funding from the European Union's Horizon 2020 research and innovation programme, the German Research Foundation (DFG) and the German BMBF Cluster for Future.

Article source: Invited.

Interview conducted: Jun 10, 2024; **Revised manuscript received:** Jul 23, 2024;

Publication date: Sep 24, 2024.

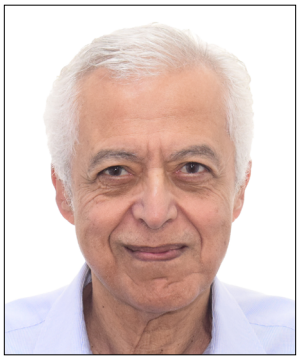
NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: LNPs

SPOTLIGHT

Potential use of lipid nanoparticles in non-vaccine therapeutics

Nagy Habib
Imperial College London and Dawn Therapeutics



"...lipid delivery systems are showing great promise in a number of areas, from cancer to rare diseases, as the technology field continues to evolve beyond vaccine applications."

VIEWPOINT

Nucleic Acid Insights 2024; 1(8), 309–312

DOI: 10.18609/nai.2024.038

On October 7, 2024, **David McCall**, Senior Editor, BioInsights, spoke to **Nagy Habib**, Professor of Surgery, Imperial College London; and Founder, Dawn Therapeutics, about current and future applications of lipid nanoparticles in non-vaccine therapeutic applications. This Viewpoint article was written based on that conversation.

As a Professor of Surgery, I operate on patients with liver and pancreatic cancer. 80% of patients with pancreatic cancer are already at an advanced stage of disease by the time I see them. The focus of my own lab's research is on solving this problem.

For the past decade, I have also been working on small activating (sa)RNA therapeutics, in a bid to develop oligonucleotides that upregulate tumor suppressor gene expression. A crucial aspect of this work is considering how best to deliver these therapeutics, as they cannot possibly succeed if one cannot take them to the right place within the patient. In this regard, lipid delivery systems are showing great promise in a number of areas, from cancer to rare genetic diseases, as the technology field continues to evolve beyond vaccine applications.

POTENTIAL APPLICATIONS OF LNPs FOR BOTH BROAD AND SPECIFIC DELIVERY

The mRNA-LNP vaccines developed for the COVID-19 pandemic were injected into muscle in order to have the vaccine stay in situ and cause maximum inflammation to stimulate the immune system. For therapeutics, however, you want to have the opposite effect. You want to deliver them, in a much more targeted way, taking the drug to the desired specific cells or tissue. You also need it be as stealthy as you can so that the immune system doesn't react, thus opening up the possibility for multiple administrations.

Therefore, when you are working with LNPs, you have the option to send them everywhere in the body, or to a very specific location. At Dawn Therapeutics, we have developed a LNP that is linked to a transferrin receptor RNA aptamer. Transferrin receptors are present in every cell in the body, which means that when you inject the therapeutic, you will get widespread delivery. For a lot of applications that is exactly what you don't want—it is highly undesirable for toxic treatments such as chemotherapy. But for rare genetic diseases that affect almost every cell, it is an ideal delivery system. For example, there are over 7,000 rare genetic diseases where a single gene is missing or not functioning.

For more specific delivery, several opportunities have emerged in the so-called 'dark genome', which has recently shown a lot of promise. When we talk about our human genome, we are usually talking about 'coding' genome that codes for a protein, which is only about 2% of the overall genome—the other 98% 'non-coding' genome does not code protein. At the time when it was discovered, it was dubbed the dark genome after the dark matter in space, alluding to the mystery surrounding it. But this year, the dark genome seems to be taking center stage. Three major deals happened in September 2024 in the field—Eli Lilly agreed to pay US$1 billion to HAYA Therapeutics for their knowledge on the dark genome, and Bayer and others did parallel deals.

Why did we see that sudden rush to explore the dark genome further? Previously, the information we had on the dark genome was mainly similar to microRNA, which can be non-specific and can result in off-target effects. However, it is now emerging that long non-coding RNAs (lncRNA) are truly specific. They can be extremely specific to the organ, tissue, cell, and even the status of the cell (i.e., if there is cancer or inflammation present). A non-specific delivery system would therefore work well in tandem, with lncRNA providing the specificity. This is very exciting, and a break from the past reluctance in drug discovery to touch the non-coding genome.

On the other hand, you can have a very specific LNP delivery system. We have developed an LNP that will only go to the cartilage and bone, and we believe it can have a number of therapeutic applications. Looking again to the rare disease space, there are at least 50 rare genetic diseases where the joints are affected. It could have a fantastic effect in conditions like mucopolysaccharidosis (MSPI) I (also

known as Hurler disease) or MSP III, and so on. Many patients with more common diseases could also benefit, for example, in conditions like osteoporosis.

BARRIERS TO DEPLOYING LNPs IN THE THERAPEUTIC SETTING

The LNP space is not without its challenges, but promising progress is being made in tackling many of these issues. Firstly, in drug development one of the biggest challenges you face is de-risking your product, as investors and pharma companies alike are risk averse. With billions of COVID-19 vaccine doses produced and administered worldwide, there is no drug that has been given to more people worldwide than mRNA-LNPs. This de-risks your product before you even begin, as your cargo is then the only element introducing risk.

Another area to address is developing lipids that can escape the endosome and get to the cytoplasm. A number of companies are currently fighting in court over who discovered LNP technology that can be liberated from the cell—but whoever discovered it first, it will likely be in the public domain in a few years regardless, and then everyone will be able to use it. There are also now many other lipids that have been developed that can escape the endosome to the cytoplasm. In my view, the most important focus here is developing these liberated non-antigenic LNPs and finding a formula to manufacture them as cheaply as possible, so the maximum number of patients can benefit.

Another challenge the field is working on is cold chain transportation and storage, and how to extend the temperature range so that these products can travel and be stored more easily. All of these remaining issues are now being addressed, one by one, and I am confident that they will be resolved, allowing the LNPs currently being used in vaccines to be used next in therapies for both rare and common diseases.

NANOPARTICLE VERSUS VIRAL DELIVERY

A final point to consider is the unique advantages that LNP-based delivery can offer over other delivery platforms. Gene therapies utilizing viral-based delivery systems are developing quickly, and there have been some 25 products approved by the FDA in the last three or four years. However, they are very expensive. If you are using lentivirus, the final product can cost anywhere up to US$4 million. But who can afford to buy them? The UK NHS, for example, cannot justify authorizing a drug that expensive when there are millions of patients on the waiting list. Other European countries like Germany and France face the same issue. We are now seeing great drugs that cannot be used because of their price, even in richer countries like the USA.

If we take the viral transduction method used for CAR T-cell therapy as an example, this was a great development for patients, and it works very well for blood cancers. But if we look at the last five years, only 5% of the patients who could have taken these drugs in the US have received them, which means the other 95% didn't get this treatment. Possibly they couldn't afford it. You can have a fantastic lentivirus- or AAV-driven gene therapy, but only a small number of patients will get to benefit from it. It is imperative to reduce the costs of goods for the field to progress.

I foresee that people will use LNPs to get around these issues, as they offer a number of advantages over viral options. You can administer therapies intravenously versus the complex process of removing cells from the body in order to engineer them *ex vivo*, as is done in autologous CAR-T therapy. Apart from extra cost associated with the mobilization of the cells outside the body, patients are at high risk to developing life-long immune suppression as well as sterility. That is why the *in vivo* approach of LNPs is much more desirable.

The radical decrease in cost is imperative. We should aim to take two zeros out of the cost

of these therapies. Instead of US$1 million, it should be US$10,000. Instead of US$4 million, it should be US$40,000. This makes it possible for patients around the world to be treated. And of course, the more you sell, the more profit you make. At the moment, nobody is winning. Companies cannot sell the product because nobody can buy it, and patients are not benefiting.

I predict huge mileage in the next few years for LNP-based approaches, and I am excited to see where the field goes next.

BIOGRAPHY

NAGY HABIB is Professor of Hepatobiliary Surgery at the Imperial College London, London, UK at the Hammersmith Hospital campus. He is a leading surgeon, translational researcher in liver cancer and its treatment, and a serial entrepreneur. He co-founded Apterna Tx that is developing siRNA therapy to improve liver function and more recently Dawn Tx for a novel gene therapy in MPS1H and liver cancer.

AFFILIATIONS

Nagy Habib
Professor of Surgery,
Imperial College London,
London, UK
and
Founder,
Dawn Therapeutics,
London, UK

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author is the CEO of and a shareholder in DawnTx. He also has a patent filed in DawnTx for the development of Tfr-LNP to cross the blood–brain barrier.

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Revised manuscript received: Oct 15, 2024; **Publication date:** Oct 22, 2024.