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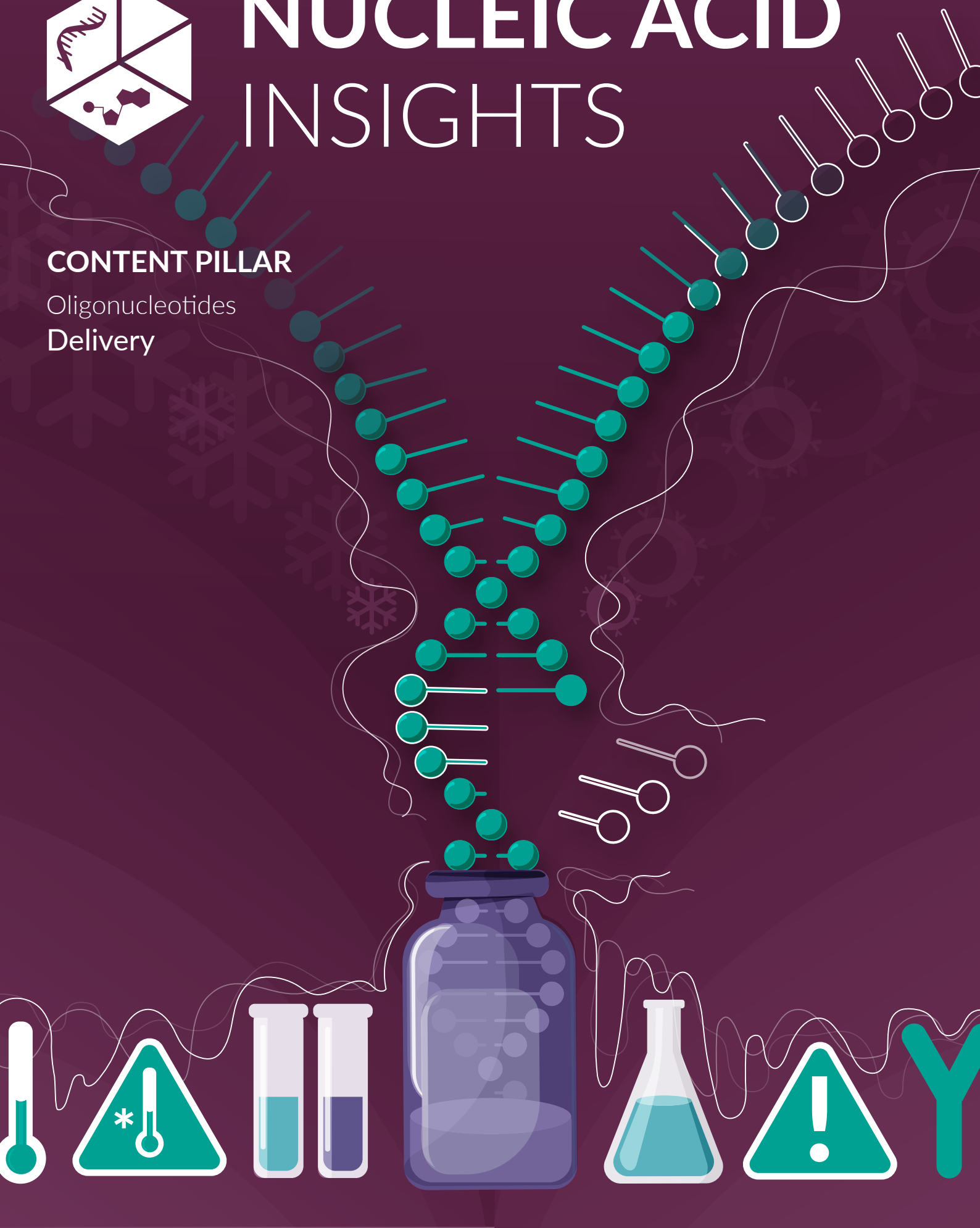
NUCLEIC ACID INSIGHTS

CONTENT PILLAR

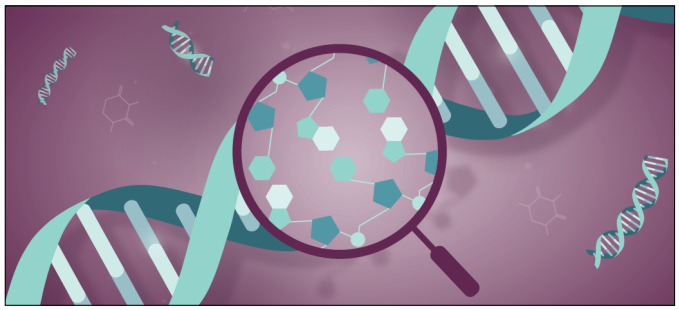
Oligonucleotides

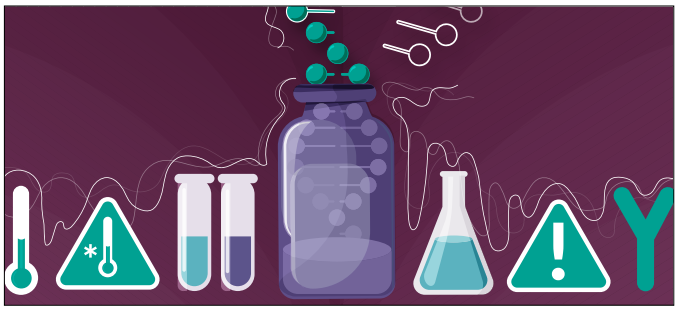
Delivery



NUCLEIC ACID INSIGHTS

CONTENTS VOLUME 2 · ISSUE 9





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DELIVERY

SPOTLIGHT

Exploring the advances and challenges in the delivery of oligonucleotide therapeutics

PANEL

"One challenge we face as a field is that it is hard to learn from our peers because oftentimes, all you see is, 'Hey, we went from delivery system X to Y,' and you see an increase in on-target delivery, but no mention of off-target effects."

Since the first US FDA approval in 1998, 22 oligonucleotide drugs have reached the market, addressing rare, genetic, and other diseases **[1]**. Today, with nearly 300 ongoing clinical trials and over 2,000 therapies in development, the field is expanding rapidly **[2,3].** Yet, significant challenges remain—foremost among them is delivery.

In this article, Kehinde Ross (Associate Professor, Liverpool John Moores University) and James Dahlman (Associate Professor, Georgia Institute of Technology) discuss the key challenges and innovations in oligonucleotide delivery—from tissue targeting, endosomal escape, and nanoparticle design to animal model translation, blood–brain barrier strategies, and equitable global access.

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Q What are the greatest challenges in the delivery of oligonucleotide therapeutics?

KR Some of the key challenges for delivering oligonucleotide therapeutics relate to targeting specific tissues beyond the liver. We have had great success over the last five to seven years with numerous oligonucleotides, especially short interfering RNAs (siRNAs), for delivery to the liver. However, when we start thinking about other tissues, although a lot of work has been done, we have not had the kind of breakthrough we saw with the launch of Patisiran for liver delivery a couple of years ago. Lipid nanoparticles (LNPs) were used to deliver Patisiran, but since then, Alnylam Pharmaceuticals has moved to using a carbohydrate group to target the liver specifically. It would be interesting to see a similar kind of molecule that can target other tissues just as specifically. In my work, I focus on the skin tissue, where there are many unmet clinical needs for inflammatory diseases, and it would be fantastic to see a molecule emerge for skin disorders.

Q What strategies can developers use to address biological barriers, such as endosomal escape and tissue-specific targeting?

KR It is crucial to have endosomal escape mechanisms that are non-toxic, so they can release enough of the oligonucleotide into the cytosol, where they can have their effect. Another key challenge relates to the design approach itself—whether it is conjugation versus bundling into nanoparticles. From a manufacturing perspective, conjugation can sometimes present challenges that might be easier to overcome if you bundle the oligonucleotide into nanoparticles.

In my laboratory, we are working on peptide nanoparticles as one approach. Although it still needs optimization, some studies have used this method before, and we are trying to build on that. Still, what will really matter is having a clear demonstration of efficacy at clinically relevant treatment levels.

JD One thing I always like to think about is the relationship between the payload itself and the delivery system. I have learned that if you change the payload, you can shift the therapeutic window while using the same delivery vehicle.

I do think the delivery system and the payload 'talk' to one another in ways we do not yet fully understand, which is another open scientific question. If you look at a peptide- or protein-based nanoparticle versus an LNP versus a conjugate, all of those interact with their payloads differently, and in very interesting ways.

Q What are the latest innovations in chemical modification to enhance the specificity of delivery?

KR For specific tissue targeting, one approach being explored is using small-molecule ligands conjugated to oligonucleotides, such as siRNAs

"From my perspective, extrahepatic delivery is very challenging. One of the key lessons I have learned during 15 years in the non-liver RNA delivery field is that it is easier to run your marathon downhill than uphill."
James Dahlman

and microRNAs. In that context, you might think about using unnatural amino acids, for instance, to improve peptide performance. This is one of the approaches we are taking in my laboratory.

Regarding lipids, there have been some interesting studies where the lipid is modified with small-molecule ligands attached to provide additional specificity for targeting certain tissues. I imagine we will see more of these kinds of approaches emerging over the next few years.

JD Regarding nanoparticles, developers usually take two approaches when they think about targeting: endogenous and active targeting. Endogenous targeting means you do not add an active targeting ligand like an antibody, a Fab region, or an aptamer. Active targeting is when you do add those ligands. The advantages and disadvantages of endogenous versus active targeting for nanoparticles are well established. With endogenous targeting, LNPs are relatively easy to manufacture, but you do not get as much specificity. In contrast, with active targeting, you are likely able to increase on-target delivery, but you may have a more challenging time in manufacturing.

Regarding conjugate systems, it is necessary to have active targeting there. The chemistry in these conjugates is very tricky—you need a conjugate that can reach the right cell type and confer other functions, such as endosomal escape. It is very challenging to build a conjugate that can do both: get to the right place and then get out of the endosome with full functionality.

Q What strategies can be employed to achieve efficient oligonucleotide delivery beyond the liver?

JD Extrahepatic delivery is a fun yet often disappointing world to live in—but when something works, it is incredibly exciting. If you look at the pipeline of various companies, such as Alnylam Pharmaceuticals, you will see dozens of different clinical programs right now, with several approvals, all using the same delivery system.

One robust delivery system can drive a lot of different programs, especially with conjugates. I think everyone understands that if you could replicate that in any other cell type, it would be a game-changer.

From my perspective, extrahepatic delivery is very challenging. One of the key lessons I have learned during 15 years in the non-liver RNA delivery field is that it is easier to run your marathon downhill than uphill. We try to focus on targets where we are 'running the marathon downhill'—where we can physically reach the cells—instead of 'running uphill' and having to cross multiple barriers. When I think about which cell types and tissues I am most optimistic about, it is the ones where the cells are physically accessible after a given route of administration.

"It will be interesting to see AI deployed specifically to tackle some of the oligonucleotide delivery challenges."
Kehinde Ross

KR When I think about our work on inflammatory skin disorders, such as psoriasis, eczema, or other rare genetic conditions, being able to target microRNAs, siRNAs, or antisense oligonucleotides in that context would be tremendous, once we can deal with the endosomal escape.

One of the challenges with skin is that experiments on cultured keratinocytes or fibroblasts do not necessarily translate into good data when you move to actual skin samples, reconstituted skin, or even patient-derived tissue. It is necessary to reimagine how we approach these experiments and, perhaps, start with at least *ex vivo* skin as a first step. It has been encouraging to see some studies take that approach rather than relying solely on cultured cells. It is very easy to demonstrate uptake in cultured cells, but showing uptake in the actual tissue—in this case, skin—would be much more important.

Q The accessibility of oligonucleotide therapeutics remains limited in low- and middle-income countries. What strategies are being/could be taken to improve equitable access to these treatments?

KR My thoughts relate to the need to ensure policymakers are engaged very early on with this technology and understand the benefits it can bring at scale. It will also be crucial for policymakers to see that this will benefit their health care systems in the long run. There may be initial upfront costs, but over time, the advantages to their health finances will be significant.

Public engagement is also important. We work in this space and are familiar with these drugs, but we want to ensure that the general public feels confident about the potential of these technologies to benefit them.

Q If we look five years ahead, what do you believe will be the biggest innovation or breakthrough in oligonucleotide delivery?

JD Again, if we get even a single extrahepatic tissue that is within an order of magnitude of how efficient GalNAc is, it will be a major success. This field does not need a new cell type every three years to stay interesting—a new cell type every decade is sufficient. Honestly, I think we will probably see two new cell types in the next decade. In the next five years, any new clinically relevant extrahepatic delivery will put us in a very exciting space.

KR In the age of artificial intelligence (AI), we can expect computational models, large language models, and other approaches to help develop, or at least explore, the chemical space for these kinds of medicines. We already know

Nucleic Acid Insights 2025; 2(9), 229–237 · DOI: 10.18609/nuc.2025.031

the capabilities and strengths of some of these technologies, and the impact they have on healthcare and scientific discovery. It will be interesting to see AI deployed specifically to tackle some of the oligonucleotide delivery challenges.

Q Can you discuss achieving broad delivery of ASOs to CNS tissues, assuming intrathecal delivery is acceptable?

KR The work being done to develop drugs that cross the blood-brain barrier often involves peptides, which are inherently challenging to work with. We must explore methods to get a drug across the blood-brain barrier after an intravenous injection to potentially avoid losing so much of it through the intrathecal route. However, we still need to ensure it can target specific cells and be released from the endosomes. It is possible that other molecules or alternative approaches could enable designs that achieve these outcomes in the specific cells of interest.

Q When studying delivery, how important is the choice of animal model? And are there shared insights that can be drawn across different models?

JD We have early data from our laboratory comparing about 45–50 particles administered intravenously. In mice, these particles were distributed across many different cell types and tissues. We also performed the study in non-human primates (NHPs). Unfortunately, there were substantial differences between the two species.

However, it does not mean that mice experiments are worthless. The field has traditionally used mice for experiments, which is understandable because NHPs are a lot more complex for delivery studies. When doing delivery studies, you might test, say, 20 different delivery systems in mice, pick the top one or two, and then test those in NHPs. Many of those end up failing in NHPs. The obvious conclusion then seems to be, 'Oh, it's harder in NHPs because it works in mice but fails in NHPs.'

The analogy I use is this: imagine drafting 20 top basketball players, putting them on a football team, and only one makes the football team. You might conclude, 'Football must be much harder than basketball.' But another way to think about it is that NHPs are not always harder to work with than mice—they are just different from mice.

Mice still serve a crucial role in this workflow. Firstly, we should not move willy-nilly into large animals— that is ethically concerning. Secondly, while safety in mice does not guarantee safety in NHPs, if a particle is poorly tolerated in mice, it should never move to large animals, full stop.

When we think about preclinical models—specifically mice and NHPs—we treat mice as providing required safety data and informative efficacy data, but not as fully predictive of what will happen in NHPs.

Additionally, when developers are testing non-liver delivery vehicles preclinically—whether in mice, rats, or NHPs—it is important that they report both on-target and off-target delivery from the same animals more consistently. I think that would be a really important step for the field.

One challenge we face as a field is that it is hard to learn from our peers because oftentimes, all you see is, 'Hey, we went from delivery system X to Y', and you see an increase in on-target delivery, but no mention of off-target effects. That is going to catch up with us for non-liver therapies. Preclinically, the key question is simple: 'Do you have a manufacturable compound with a therapeutic index in a non-human primate that achieves an appropriate on-to-off-target ratio?'. It is hard to answer that if we do not have on-to-off-target ratios in mice, which, I think, should be reported more often.

Q How do you view the translational value of *ex vivo* models as a bridge between cell culture and animal models, particularly in terms of cost and speed in early discovery?

JD In the cystic fibrosis (CF) community, CF biologists and geneticists have had unbelievable, world-changing success with small-molecule drugs. These drugs correct different subclasses of CF mutations, and the type of drug or mechanism you use depends on the specific mutation.

One key model in the CF community is the CFTR knockout mouse, which is important because it mimics the genetics of the disease. Interestingly, the CFTR knockout mouse does not develop the aberrant mucus phenotype at all. By contrast, the ENaC mouse does display a mucus phenotype, although the genetics behind why an ENaC knockout mouse has this phenotype does not entirely make sense. For a long time, people have used CFTR knockout mice, but in the delivery space, the question often arises: 'Would you use a CFTR knockout mouse or an ENaC knockout mouse?'

The answer is clear: use the ENaC knockout mouse—especially for nebulized delivery systems targeting the airway, not the bloodstream. The most important barrier your delivery system will face is the mucus itself. CF patient mucus can be extremely viscous, almost like a rubber ball, compared to normal healthy human mucus.

It is a case where you need to be cognizant about whether you are using an experimental system for historical reasons or because it accurately represents the conditions you want to model. CFTR knockout mice are excellent for testing small molecules, but since they do not have the mucus, they are irrelevant for optimizing a nebulized delivery system.

Q Is there an optimal particle size for LNPs to efficiently deliver oligonucleotides to the liver? It is often assumed that particles <70 nm work best—is that actually the case?

JD Firstly, a 30 nm particle versus a 300 nm particle is different. But if we are comparing, for example, 50 nm and 70 nm, or 60 nm and 80 nm, those numbers are much closer. Looking at the dynamic light scattering (DLS) spectrum, there is a lot of overlap.

Over the last few years, we looked at >10,000–15,000 particles *in vivo*. We consistently plot delivery to a specific cell type on the Y-axis and hydrodynamic diameter (measured by DLS) on the X-axis, looking for relationships—and within small ranges, we have not seen much difference.

So when I think about particle size, I am always cautious about generalized rules of thumb. Yes, there is a difference between 30 nm and 300 nm, but for small differences, such as 45 nm versus 55 nm, the vast majority—maybe 90%—is the same, and I have a hard time seeing how such minor differences in size would have a big impact on delivery.

KR I think the composition of LNPs is a much more crucial determinant of performance than size alone. Of course, if there are huge variations in size, you might see differences. But the key parameters are really the LNP composition and whether you are adding a targeting domain to improve performance.

Q What is the most surprising or unusual thing you have discovered while working with oligonucleotides and LNPs?

JD Although this is not exactly about oligonucleotides, there was a paper that came out a few weeks ago where we tested multiple particles in NHPs [4]. We structured it ethically, and there was no net loss of life, which was especially important for the large animals.

We were able to look at delivery in roughly 500,000 single cells from NHPs. We analyzed delivery across all those cells, mapped cell types using the transcriptome, and then overlaid delivery data on the transcriptome. For example, we compared cells with many particles to cells with none, controlling for cell type and other variables, and looked at differential gene expression.

Based on the results, low-density lipoprotein receptor (LDLR) correlated with high delivery. But we also found LDLR-negative cells that expressed other LDL-type receptors and still had particles. In our field, a canonical rule has been that LNPs are delivered preferentially to hepatocytes via ApoE-LDLR. That happens, but it is not the full story.

This experiment shows that using new molecular technologies to take an unbiased look at a relevant animal model can reveal many complexities. If you say, 'I will not predetermine that delivery is driven by ApoE-LDLR—just tell me what it is', you often discover the biology is more complicated.

KR Firstly, we were using a commercial nanoparticle formulation in experiments where we were loading it with our microRNA, which was supposed to kill the cells. As a control, we used the nanoparticle alone, and it seemed to kill the cells by itself. We thought, 'why is this happening?'. Then, we redesigned the experiments and ran additional response studies.

It appears that the nanoparticle itself can kill these particular cells. We do not yet know why, or whether this is specific to that nanoparticle or a more general response of these cells to any nanoparticle. Another student tried a different formulation and saw a similarly high level of unexpected cell death. This is something we plan to explore further in future work.

The other observation was with one of the siRNA controls, which also seemed to kill the cells. I ran it through an algorithm that predicts targets, and it turns out it was a microRNA with a whole host of predicted gene targets that could explain some of the effects.

All of this makes the system more complicated than we anticipated—but also more exciting, because these are new challenges and potentially new opportunities for our laboratories.

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BIOGRAPHY

Kehinde Ross is a Reader (Associate Professor) in Molecular and Cellular Biology at Liverpool John Moores University (LJMU), Liverpool, UK. He earned his PhD from the University of Sheffield where he studied cytokine signalling proteins associated with inflammatory diseases. After postdoctoral training at Newcastle University, he started independent research related to microRNA (miRNA) expression and function at LJMU. His initial focus on skin has expanded to include studies of miRNA modulation in cancer, given that dysregulation of miRNA has been linked to various aspects of the disease. His current focus is the development of interdisciplinary collaborations to support the generation of nanocarriers for miRNA-directed therapy in cancer and other conditions. Kehinde leads the Transformative Peptide Chemistry for RNA Nanotherapies Thematic Doctoral Pathway program at LJMU, one of the inaugural cohort-based PhD training schemes in the university.

Kehinde Ross PhD, Associate Professor, Liverpool John Moores University, Liverpool, UK

James Dahlman is Chief Scientific Officer at Readout Capital and the McCamish Early Career Professor in the Department of Biomedical Engineering at Georgia Tech and Emory School of Medicine. His lab works at the interface of chemical engineering, genomics, and gene editing by applying big data approaches to nanomedicine. The lab is known for developing DNA barcoded nanoparticles to measure how hundreds of nanoparticles deliver mRNA and siRNA in multiple cell types from a single animal *in vivo*. The lab uses these approaches to deliver RNA outside the liver.

James was a co-founder and Board Chairman of Guide Therapeutics, which was acquired by Beam Therapeutics. His trainees have become investors, started several companies, and work in some of the most cutting-edge organizations in RNA therapeutics. James received his PhD in 2015 from the Harvard-MIT HST Program, where he studied with Robert Langer, and as a post-doc, studied CRISPR-Cas9 with Feng Zhang.

James Dahlman PhD, Associate Professor, Georgia Institute of Technology, Atlanta, USA

AUTHORSHIP & CONFLICT OF INTEREST

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SPOTLIGHT

Advancing analytics in oligonucleotide therapeutics: innovations, challenges, and regulatory insights



PANEL

"Many conditions that are currently considered undruggable may soon become druggable. I believe we are entering a new era of nucleic acid medicines."

Oligonucleotide therapeutics present unique analytical obstacles due to their structural complexity, stereochemical diversity, and impurity profiles that are difficult to fully characterize with current tools.

Nucleic Acid Insights assembled a panel of leading experts, including **Afaf El-Sagheer**, (Assistant Professor, University of Southampton), **Mike Webb** (Independent Consultant, Mike Webb Pharma), and **Thomas Minshull** (Postdoctoral Researcher, University of Sheffield), to discuss the key analytical challenges facing the field, strategies for impurity characterization, and considerations for safety, comparability, and regulatory acceptance. The article also highlights future opportunities to advance the field, including the application of AI, structural biology, and cross-sector collaborative research.

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Q What are the greatest challenges in the analytics of oligonucleotide therapeutics?

AE-S One of the most important hurdles for therapeutic oligonucleotides is their purification and analysis by high-performance liquid chromatography (HPLC). These oligonucleotides are usually modified with phosphorothioates, which generates complex mixtures of stereoisomers, making both purification and characterization particularly laborious.

MW For me, the challenge is establishing a control strategy for such a complex impurity profile. We are still largely guided by standards developed for small molecules, yet oligonucleotides require analytical methods with far greater resolution and sensitivity to reliably identify, characterize, and qualify impurities for QC purposes. Our separations are complex, impurities often co-elute, and detection is resource-intensive. More advanced analytical tools need to be developed, rather than continuing to rely on those originally designed for small molecules.

I would also highlight that phosphorothioates are an emerging hot topic. A 20-mer antisense oligonucleotide that is fully phosphorothioated could theoretically generate over half a million stereoisomers, representing extraordinary analytical complexity.

Q Given the complexity of oligonucleotide structures, what strategies or tools do you find most effective for grouping and characterizing impurities, especially when dealing with truncated sequences or diastereomeric variants?

TM Ion-pair reversed-phase chromatography is one of the most versatile methods we use. It is tunable, conditions can be adjusted to separate by size or by sequence, and when combined with tandem mass spectrometry, it provides highly detailed information. This approach allows us to detect truncated species, positional isomers, and modified oligonucleotides.

For sequence-related impurities such as deamidation, phosphorothioate-to-phosphate substitutions, or the incorporation of targeting groups such as N-acetylgalactosamine, method conditions can be further optimized. Adjusting the strength of the ion-pairing agent or altering the stationary phase can help to resolve these closely related impurities. Nevertheless, chromatographic separation of such species remains technically demanding.

Additional separation dimensions can be introduced to enhance resolution. Two-dimensional liquid chromatography (LC) or coupling LC to mass spectrometry (MS) enables separation by mass while exploiting the full capabilities of tandem mass spectrometry to obtain sequence-level information on modifications or truncations, and to determine whether positional isomers are present. More recently, ion mobility has been applied as an orthogonal separation method, with some success in resolving diastereoisomers.

> "[...] if in the future we can analyze intact mRNA directly by MS, without digestion, it would represent a step-change in the field."
> Afaf El-Sagheer

Finally, for larger molecules such as mRNA, poly(A) tail length is a critical quality attribute. Here, ion-pair reversed-phase LC coupled to MS is again employed. Because the molecules are so large, digestion with a T1 enzyme is typically performed first, and the resulting fragments are separated by ion-pair reversed-phase prior to MS analysis. This provides high mass accuracy and enables discrimination between fragments differing by only a single adenine, as well as the identification of unexpected sequences. This makes it a powerful tool for characterizing oligonucleotides.

AE-S One of the major hurdles, as mentioned earlier, lies in HPLC separation. Impurities often overlap because of their structural similarity and shared charge, meaning that even when different chromatographic phases are applied, complete resolution is difficult to achieve. The greater challenge is not only separating these impurities but also quantifying them reliably. If impurities can be quantified and their levels assessed against toxicity data and regulatory expectations, we will be better placed to define what is acceptable and move the field forward.

Mass spectrometry also presents limitations. For shorter therapeutic oligonucleotides, analysis is feasible, but overlapping impurities still create difficulties. For larger modalities, such as RNAs longer than ~24–60 nucleotides, and particularly for mRNA, the complexity increases substantially. Current deconvolution methods are not sufficiently robust to provide accurate mass analysis for sequences above ~100 nucleotides, where resolution and accuracy begin to decline.

For mRNA, which may span several kilobases, the analytical burden is even greater. At present, digestion followed by MS is the only practical analytical route. However, if in the future we can analyze intact mRNA directly by MS, without digestion, it would represent a step-change in the field.

MW What we have at present are generic systems, often used by CDMOs, that are inadequate for the complexity of oligonucleotide therapeutics. Orthogonal techniques are essential, but we must be careful in how we define them. For example, ion-pair reversed-phase chromatography combined with MS is widely used, and some consider anion exchange to be orthogonal. However, both are charge-based separations, meaning they do not provide true orthogonality. What we need are approaches such as hydrophilic interaction chromatography, two-dimensional separations, or even a re-examination of electrophoresis as an additional method.

Improving the combination of MS and ultraviolet detection is feasible and already practiced, but it requires careful execution to deliver reliable data. Looking ahead, progress will depend on collaboration between academia and instrument manufacturers. This therapeutic modality is now a major focus area, and achieving the fidelity required for both product release and deeper characterization, including DoE studies, will likely require transformational innovation. At present, the analytical fidelity we need simply does not exist.

I am concerned that such progress may be pursued behind closed doors within large pharmaceutical companies. It is crucial instead that academia, industry, and instrument

developers work together through collaborative research projects. What we need are better techniques with true orthogonality, developed in partnership across the community, to achieve the step change that the field requires.

Q How can advanced analytical methods improve our understanding and monitoring of the safety and toxicity profiles of oligonucleotide therapeutics throughout their development?

MW If we are talking specifically about toxicity in terms of quality, the priority is to distinguish between the impurity profile of the material used in toxicology studies and the profile of the material later manufactured and administered. It is essential to specify individual impurities, establish their levels in the toxicology batch, and then confirm that those impurities can still be detected and quantified throughout development, even decades later at the end of the product lifecycle.

Two factors enable this continuity. The first is the availability of more advanced analytical methods, both now and in the future. The second is a regulatory framework that permits methods to evolve over time. For example, ICH Q14 defines the criteria for a successful analytical method while also allowing for improvement during development and the commercial phase. To support this, toxicology samples may need to be retained so they can be re-examined as technologies advance.

For critical therapies, such as drugs for dyslipidemia or hypertension, or treatments for spinal muscular atrophy, it is vital that improved analytical techniques can be implemented as they emerge. The research efforts we discussed earlier must be translatable into practice throughout the lifecycle of a product, so that developers can continually demonstrate safety in relation to the material originally used in toxicology.

Q When speaking about cost-effectiveness and scalability, how close are we to making analytical methods cost-efficient and scalable for routine quality or toxicity assessments?

MW To illustrate the difficulty, Ionis, which has developed the majority of conventional antisense oligonucleotides, analyzes approximately 80% of their impurities using MS. They employ a low-resolution mass spectrometer that is specifically calibrated to correct for the non-linearity of the mass spectrometric response. By comparison, if you were running a UV chromatogram, the chromatography data system would generate an almost instantaneous result, requiring only minimal user input. In contrast, the Ionis approach can take an entire day for a single chromatographic run, yet it is the method adopted not only by them but also by others in quality control environments.

My understanding is that the instrument company involved has now automated this process. It remains challenging, but automation is possible, and this is where AI also has an important role to play. These are the kinds of tools that will ultimately make impurity analysis cost-effective, since at present the limiting factor is people's time. Unlike small-molecule QC activities, oligonucleotide analysis is still highly manual.

Nucleic Acid Insights 2025; 2(9), 239–249 · DOI: 10.18609/nuc.2025.032

What is needed now are reliable software platforms and solutions from instrument manufacturers that can streamline these processes. Oligonucleotides are no longer a niche modality; they represent a growing therapeutic class. In my view, automation will be central to supporting their broader development and commercialization.

Q When manufacturing processes or materials change, which analytical quality attributes are most essential to monitor to establish comparability?

TM From an mRNA perspective, comparability depends very much on which part of the process is being modified. The most risk-averse approach would be to test extensively, but in practice, it is about developing a robust analytical testing protocol in line with ICH Q5 guidelines. For example, if a purification resin is changed, analytical methods must be in place to compare levels of truncated species or double-stranded RNA, since these can directly influence immunogenicity. Similarly, if the source of the T7 transcription enzyme is changed, then monitoring of critical quality attributes such as sequence identity, transcript size, and double-stranded RNA content becomes essential.

By contrast, if the modification occurs further downstream, for example, replacing a tangential flow filtration membrane in the final formulation step, then the focus shifts. Sequence identity may be less relevant, while parameters such as final pH and the removal of process-related impurities become more important. In lipid nanoparticle formulations, for instance, monitoring and minimizing residual ethanol is particularly critical to ensure both safety and regulatory compliance.

AE-S The primary tools we currently have for comparability are MS and HPLC. These techniques allow us to reproduce and verify results; for example, if a certain level of toxicity is observed, the same analytical approach can be applied again to confirm whether the impurity profile is consistent. Reliable comparability depends on these methods.

However, both HPLC and MS require significant optimization to meet the needs of oligonucleotide development. This will only be achieved through closer collaboration between industry and academia. Traditional funding bodies are unlikely to prioritize incremental improvements to analytical platforms, so pharmaceutical companies must take a more active role in supporting this research, for example, by funding PhD studentships and postdoctoral projects. The best path forward is to strengthen MS and HPLC as comparability tools through sustained collaborative effort.

MW One advantage we have with oligonucleotides, compared with small-molecule processes, is scalability. Scale-down models can be applied successfully, and performance at a small scale is generally predictive of larger-scale outcomes. Unlike other manufacturing platforms, we are not constrained by issues such as mass transfer, heating and cooling rates, or stirring rates. This greatly assists in characterizing the impact of process changes and supports the effective use of analytical tools for comparability. Importantly, this approach allows us to avoid, as far as possible, the need for bioequivalence studies or bioassays, since characterization data can provide the evidence required.

Looking forward, we need to strengthen collaboration across the field to define, as an industry, what we regard as appropriate comparability assessments. This will require more open publication and sharing of best practices. With our collective experience, we are in a strong position to set those expectations.

There are lessons to be drawn from other areas of manufacturing. For example, continuous processing and biotransformation in chemical manufacturing, areas that remained unchanged for decades, have recently undergone rapid, large-scale adoption, supported by novel catalysis. By reflecting on how those advances were implemented for existing drugs and processes, we can inform how the oligonucleotide community develops and applies comparability strategies in the future.

Q How open are regulators to newer or non-traditional analytical methods, especially when it comes to demonstrating comparability after manufacturing changes or characterizing complex impurities?

MW In my experience, it is unhelpful to generalize about how regulatory authorities worldwide will respond to new analytical methods. Past responses are not a reliable predictor of future outcomes, particularly in areas where guidelines may not yet exist. What matters is demonstrating that the approach is scientifically sound and that it establishes the quality of the material being analyzed. That provides the strongest basis for regulatory acceptance. Simply including a new method in a submission without a clear justification is unlikely to succeed.

When introducing innovation, early engagement with regulators is essential. If a completely new analytical platform is being proposed, interaction should occur as early as possible, beginning with scientific advice. The key is not just to ask for approval but to present the scientific rationale: explain what you plan to do, why it is necessary, and how it will benefit patients, supported by evidence of equivalence or suitability.

Confidence in these discussions comes from scientific rigor. You must be certain that your chosen approach is correct and able to demonstrate that confidence to regulators. That, in my view, is the most effective way to gain acceptance of new or non-traditional analytical methods.

Q If we look five years ahead, what do you believe will be the biggest innovation or breakthrough in oligonucleotide analytics?

AE-S With the support of AI, I believe that MS software will advance significantly over the next five years. Improved algorithms will enhance our ability to analyze oligonucleotides of any length, including mRNA, and to identify impurities at much lower levels than is currently possible.

At present, MS typically cannot detect impurities below 5%. In our own laboratory work, when samples are deliberately spiked with impurities, they remain undetectable until present at relatively high levels. The real breakthrough will be methods that are both fully quantitative and able to detect impurities at much lower abundance. Achieving this level of sensitivity will represent a qualitative advance in oligonucleotide analytics.

TM As a structural biologist at heart, I would be most excited to see advances in structural biology applied to oligonucleotides. Structural insights are fundamental to understanding stability and biological function, yet the tools we have today remain limited compared with those available for proteins.

In the protein field, breakthroughs such as AlphaFold have transformed our ability to predict and interpret structure. Developing equivalent approaches for oligonucleotides, whether for short oligos, aptamers, or mRNAs, would be equally transformative. Progress in this area would provide critical information for both fundamental science and therapeutic development.

MW When I began molecular modeling 30 years ago, the progress we have since seen in protein structure prediction, culminating in the advances enabled by AI, would have been unimaginable. A similar transformation is needed for oligonucleotide analytics. In particular, AI could help solve the complex problem of impurity characterization. Other industries, such as petrochemicals and fine chemicals, already apply advanced two-dimensional LC techniques to highly complex mixtures, and the data volumes generated demand AI-driven interpretation.

Another pressing obstacle is the enormous number of diastereomers produced by phosphorothioate-modified oligonucleotides, potentially half a million for a 20-mer. While daunting, this problem is solvable, and progress will need to come from three directions. Firstly, we must understand the clinical significance of these stereoisomers: do they matter for efficacy or safety, and if so, to what extent? Secondly, we need deeper insight into chemistry, particularly the relationship between synthesis conditions, purification strategies, and the distribution of stereoisomers. Thirdly, advanced analytical methods such as ion mobility, high-field phosphorus nuclear magnetic resonance, and pattern recognition techniques must be applied to characterize these species more effectively.

Addressing this issue will require collaboration and leadership from the industry to agree on what constitutes appropriate characterization. At present, there is still uncertainty about correlation versus causation and about the levels of analysis required. Nevertheless, with the techniques already available today, I believe that in the next five years we can begin to answer these questions from both analytical and synthetic perspectives.

Ultimately, definitive insight into the clinical significance of stereochemistry will require large-scale trials, but the foundation can and should be laid now.

Q How can collaborations between academia, industry, and regulatory agencies accelerate the development and adoption of more robust analytical methods for oligonucleotides?

TM Collaboration between academia, industry, and regulatory agencies is essential to advancing oligonucleotide analytics. Open communication across these groups is the only way to address challenges of this scale.

From my own experience, having worked in both academia and industry and participated in numerous joint partnerships, I have seen firsthand how productive these collaborations can be. Initiatives such as industrial studentships are particularly valuable. When we face questions as complex as how to address the half a million possible stereoisomers

> "Collaboration also requires a serious, long-term commitment to infrastructure and funding."
> Mike Webb

in a single phosphorothioate-modified oligonucleotide, we need the best minds, the right expertise, and sufficient resources brought together.

Ultimately, there is no alternative to collaboration. The most effective way forward is to create more opportunities for stakeholders to work together directly and to establish practical mechanisms for doing so.

AE-S Collaboration is essential because nucleic acid therapeutics are still a relatively new field, and regulatory expectations remain mixed between frameworks established for small molecules and those for biologics. Impurities in oligonucleotides differ fundamentally from those in small molecules: they are not always directly linked to toxicity, but they still need to be characterized and understood within a distinct profile.

Industry-academia collaboration is particularly important for advancing the science of analytics. Academic projects allow for years of focused investigation into a single analytical question, and if multiple groups work in parallel, progress can be accelerated across the field. However, this requires the industry to recognize that solving analytical challenges cannot be done in isolation and to commit resources accordingly.

These collaborations must also be cross-disciplinary. Chemistry, physics, computational methods, AI, and biology all need to contribute to building a more comprehensive analytical toolkit. What we need is a broad analytical consortium to anticipate the future and address the obstacles we expect to face in five to ten years' time.

Finally, stronger analytical science will help shape regulation. The more evidence we can generate to show that impurities are well understood, quantified, and contextualized within biological and toxicological frameworks, the more regulators will be convinced. Connecting analytics with biology and toxicology is the only way to solve these long-term limitations.

MW Collaboration also requires a serious, long-term commitment to infrastructure and funding. In the UK, we diluted much of our strength in chemistry and analytical science 20–30 years ago, including centers of excellence that once led in oligonucleotide manufacturing. Encouragingly, that capacity is now being rebuilt through initiatives such as the Nucleic Acid Therapy Accelerator and the Medicines Manufacturing Innovation Centre.

We are creating academic, manufacturing, and therapeutic centers of excellence, but these efforts must be reinforced through sustained investment. What is needed is not small-scale projects but well-funded, coordinated consortia, on the scale of the former European Innovative Medicines Initiative, which invested billions into advancing new technologies. Comparable levels of funding, from both research authorities and industry, are essential if we are to accelerate analytical development.

Equally important is training the next generation of scientists. Centers of excellence must be supported by a pipeline of skilled chemists and analysts who are passionate about

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this field. While I have described the UK context, the same principle applies globally: collaboration must be serious, properly resourced, and built to deliver lasting impact, rather than relying on small groups working in isolation.

Q Beyond traditional chemical characterization, do you see a role for emerging technologies such as AI-driven data analysis in advancing oligonucleotide analytics?

AE-S AI will have a major impact on oligonucleotide analytics. In particular, AI-driven algorithms will transform the analysis of mass spectrometry data and could also enhance techniques such as capillary electrophoresis.

One of the greatest current challenges is deconvolution, especially for long RNAs such as mRNA. At present, we can generate signals from these molecules, but the analysis is extremely difficult. Improved AI algorithms could enable us to resolve and interpret these complex spectra, making it possible to analyze long RNA or DNA sequences in ways that are not currently achievable.

MW Oligonucleotides are complex molecules with complex separations, so it is natural to ask how AI can contribute. My one caution is that AI can sometimes appear as a 'black box', and the critical issue will be how the models are trained, validated, and verified. That concern aside, we are generating increasingly complex datasets, and AI will be essential in helping us to interpret them and extract meaningful insights.

TM Both academia and industry are already generating vast amounts of data, and AI depends on access to big data to reach its full potential. The key will be finding ways to share data more effectively, so that these algorithms can be trained on diverse and representative datasets. That, in my view, will be the most important factor in driving the AI revolution in oligonucleotide analytics.

Q What would you say are the key take-home messages that summarize the current state of the oligonucleotide analytics?

AE-S I am very optimistic about the future. This is the time for nucleic acid therapeutics. We are still at the beginning, but biology will continue to raise important questions, and as those are answered, new therapeutic targets will emerge. Many conditions that are currently considered undruggable may soon become druggable. I believe we are entering a new era of nucleic acid medicines.

TM The analytical field for oligonucleotides has advanced rapidly over the past five years. Techniques such as ion-pair reversed-phase chromatography, MS, and ion mobility have become indispensable, but there is still much more to do. That is a positive thing; it is what makes science exciting. These challenges drive us to innovate and to push the boundaries of what is possible.

MW Rather than saying we are at a tipping point, I would emphasize that we are already moving forward. With therapies such as inclisiran and candidates for conditions like hepatitis B, oligonucleotides are expanding beyond rare diseases into broader areas, including complex indications such as lung fibrosis. If we can address diseases that currently have no effective therapies, oligonucleotides will become an increasingly important modality for public health.

This expansion will attract more attention, resources, and talent into the field. With the right collaborations and funding, oligonucleotide analytics will take its place alongside small molecules, biologics, and cell and gene therapies as a core therapeutic platform. Analytical challenges should be seen as opportunities: the more minds and technologies devoted to solving them, the faster the field will progress into a truly exciting space.

BIOGRAPHY

Afaf El-Sagheed studied her PhD in Chemistry at Southampton University, Southampton, UK, with Professor John Mellor then moved back to Egypt to become a Lecturer then Professor at Suez University. She was a Research Fellow at Southampton University from 2006–2013 followed by a Senior Research Fellow at Oxford University from 2013–2022 working with Professor Tom Brown. She is currently an Assistant Professor at Southampton University working in the area of therapeutic oligonucleotides and modified mRNA and their targeted delivery. Afaf has expertise in therapeutic oligonucleotides synthesis, HPLC purification and characterisation. Afaf also has long term expertise in sequencing, biophysical, biochemical and cell studies. She has pioneered click chemistry and other synthetic methods to assemble novel, long and modified biocompatible DNA/RNA constructs for custom gene synthesis/editing and their applications in biology and medicine. Some of the reagents she developed are commercially available from Glen Research and the work was highlighted on National DNA Day and in research outreach.

She has more than 190 Peer-reviewed publications including high impact journals such as (*Science*, *Nature Chemistry*, *Nature Structural & Molecular Biology*, *Nature Communications*, *JACS*, *PNAS*, *Angewandte Chemie*), patents and book chapters with H-index 48 and more than 7800 citations (Google Scholar). She was invited to give talks in UK, many countries in Europe, USA and China.

Afaf El-Sagheer PhD, Professor, University of Southampton, Southampton, UK

Mike Webb has over 40 years of experience of CMC development of API from late discovery to file and launch and beyond. In his former role as Vice President of API Chemistry and Analysis UK for GSK, he led a department of 150 scientists. Had has been closely involved in eight world-wide marketing submissions. Mike also has a publication record in Analytical Sciences including papers, book chapters, podium presentations at international meetings and has edited three books.

More recently, Mike has taken a specific interest in the chemistry, analysis, manufacturing and registration of therapeutic oligonucleotides. After leaving GSK in 2016 he has been consulting on CMC matters mainly pertaining to oligonucleotide development, analysis and manufacturing with both biotech and big pharma companies. He focusses on covering therapeutical product and process understanding and associated manufacturing, analysis, regulatory and quality issues.

Mike Webb PhD, Independent Consultant, Mike Webb Pharma, Hertfordshire UK

Thomas Minshull is a Postdoctoral Researcher at the University of Sheffield, working with Professor Mark Dickman and Dr Zoltan Kis. With a PhD from the University of Sheffield, Sheffield, UK, his early research used mass spectrometry to investigate post-translational modifications of histone proteins in the context of sepsis. After completing his PhD, Tom spent over five years in industry as a Senior Scientist at Porton Biopharma and Immunocore, developing analytical methods for a wide range of biopharmaceuticals, from vaccines to anti-cancer therapeutics. He then returned to academia at the University of Leeds, where he managed a mass spectrometry facility and developed biophysical and structural proteomic techniques to understand how RNA impacts protein amyloid formation. His current work at Sheffield focuses on leveraging this broad expertise to develop new analytical tools to characterise mRNA for biotherapeutic applications

Thomas Minshull PhD, Postdoctoral Researcher, University of Sheffield, Sheffiled, UK

AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

INNOVATOR INSIGHT

Accelerating UF/DF of nucleic acids with real-time monitoring and automated process control

Nigel Herbert

The implementation of variable pathlength spectroscopy in ultrafiltration and diafiltration processes addresses critical limitations in nucleic acid manufacturing through real-time concentration monitoring and automated process control. This article presents real-world case studies demonstrating significant time savings and achieving less than 5% deviation from target concentration endpoints through integrated tangential flow filtration systems.

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ANALYTICAL CHALLENGES IN NUCLEIC ACID UF/DF PROCESSING

Ultrafiltration and diafiltration (UF/DF) processes serve essential roles in nucleic acid purification, concentration, and formulation throughout therapeutic manufacturing workflows. Although these unit operations effectively remove impurities and achieve target product specifications, current analytical approaches present significant limitations that can compromise process control and manufacturing efficiency **(Figure 1)**.

Reliance on mass balance calculations represents a fundamental constraint in traditional UF/DF operations. These processes require precise scale calibration, accurate hold-up volume determination, and carefully timed manual operator interventions to achieve target endpoints. Concentration measurements typically only occur at process initiation and completion, providing limited insight into real-time process dynamics or product behavior during critical purification steps.

Analytical complexity increases substantially when processing nucleic acids due to their exceptionally high optical densities. Even minimal sample quantities can saturate conventional UV-Vis detectors, necessitating extensive dilution protocols that introduce measurement errors ranging from 5–20%. Traditional analytical methods, including UV spectroscopy, RiboGreen assays, and PCR, require multiple sample preparation steps involving dilution, buffer correction, and extensive manipulation that fragment the overall process and contribute to increased variability. For mRNA therapeutics, additional complexity arises

following *in vitro* transcription, where impurities such as enzymes, residual nucleotide triphosphates, and double-stranded RNA byproducts require removal through purification steps including chromatography and tangential flow filtration (TFF).

VARIABLE PATHLENGTH SPECTROSCOPY ELIMINATES SAMPLE DILUTION REQUIREMENTS

Variable pathlength spectroscopy offers an analytical method to overcome the fundamental constraints of traditional UV-Vis analysis by inverting the Beer-Lambert relationship **(Figure 2).** This technique maintains a constant sample concentration while systematically varying the pathlength distance between light source and detector, eliminating the need for sample dilution in high-concentration applications.

Variable pathlength spectroscopy employs precise micron-level pathlength control to accommodate samples that would otherwise saturate conventional analytical systems. During measurement,

FIGURE 1

Downstream ultrafiltration and diafiltration challenges in nucleic acid processing.

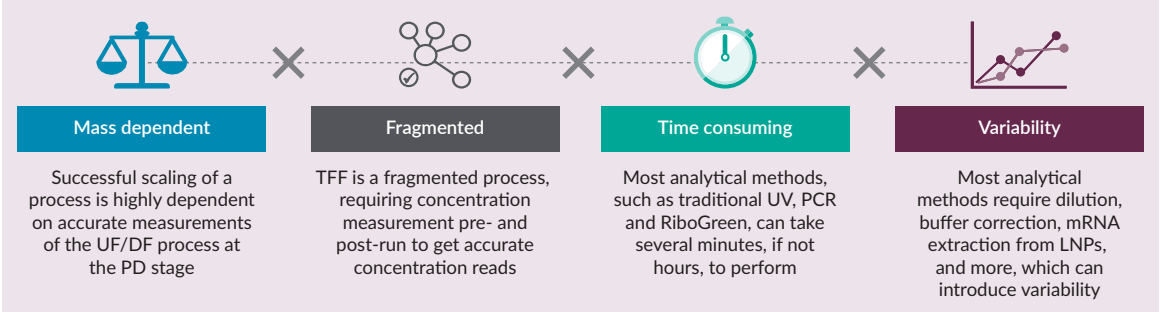
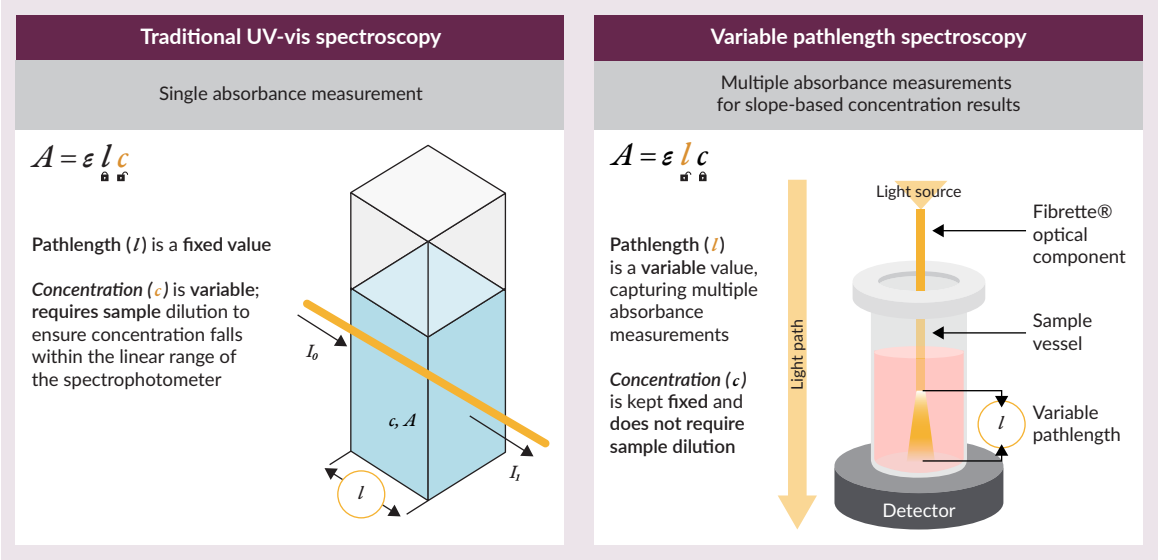


FIGURE 2

Comparison of traditional fixed-pathlength and variable pathlength spectroscopic approaches.



a search algorithm collects absorbance readings at different pathlengths to identify optimal measurement conditions. The system subsequently acquires 5–10 data points across a defined pathlength range to establish a linear slope relationship that directly correlates to sample concentration.

Each measurement generates an associated R^2 correlation coefficient that serves as a quality indicator for compliance with Beer's Law. This technique reduces the complexity of traditional UV analysis while enabling direct quantification of highly concentrated samples, including oligonucleotides at concentrations up to 80 mg/mL with optical densities approaching 2,000 OD units per mL.

REAL-TIME PROCESS MONITORING ENABLES AUTOMATED ENDPOINT CONTROL

Practical implementation of variable pathlength spectroscopy in manufacturing environments enables continuous process monitoring unavailable with traditional analytical approaches. FlowVPX® systems utilize a modular architecture comprising three primary components: a precision pathlength control mechanism, a process-contact flow cell, and an integrated detector assembly.

Flow cell design enables continuous sample flow while facilitating optical measurement through embedded fiber optic components. Measurement frequency of approximately 12 readings per minute, with 5-second acquisition intervals, provides sufficient temporal resolution for real-time process monitoring and control.

Integration with existing TFF platforms occurs through strategic placement on feed lines prior to main circulation pumps. This positioning ensures representative concentration measurements while minimizing interference from pump pulsations. Automated endpoint management based on concentration criteria rather than traditional mass balance calculations eliminates guesswork and manual intervention required in conventional processes. Process completion requires both target concentration achievement and R^2 values ≥0.999 to ensure measurement reliability.

Automated endpoint management eliminates the variability associated with manual operator interventions and mass balance calculations, providing consistent process control based on direct analytical measurements rather than indirect weight-based estimations.

FLEXIBLE CONFIGURATIONS SUPPORT RESEARCH THROUGH PRODUCTION APPLICATIONS

This platform addresses diverse analytical requirements across different stages of development and manufacturing. Cary 60 UV-Vis spectrophotometers enable multiwavelength measurement across broad spectral ranges, supporting research applications that require comprehensive analytical characterization and method development flexibility. Beams LED systems provide focused single-wavelength measurement capabilities, meeting production environment needs where specific wavelengths have been established and validated for particular applications.

Such configurational flexibility eliminates the need for multiple analytical platforms across the development-to-production pipeline, enabling consistent analytical approaches and seamless technology transfer as processes scale from laboratory through commercial manufacturing. Available systems span laboratory KrosFlo® platforms through production-scale RS systems, maintaining consistent analytical performance during scale-up while accommodating different operational requirements from flexible development applications to fully automated GMP production.

AUTOMATED DNA PROCESSING ELIMINATES OFFLINE SAMPLING

Validation studies evaluated the KrosFlo KR2i system with integrated FlowVPX monitoring for crude double-stranded DNA processing **(Figure 3)**. The protocol employed concentrate-diafiltrate-concentrate steps targeting initial concentration of 0.16 mg/mL and final concentration of 0.7 mg/mL using 100 kDa flat sheet cassettes.

FlowVPX measurements correlated well with NanoDrop results at initial (0.08 mg/mL for both methods) and final (0.70 versus 0.77 mg/mL) concentration

FIGURE 3

DNA concentration monitoring during ultrafiltration and diafiltration processing with FlowVPX® and NanoDrop comparison.

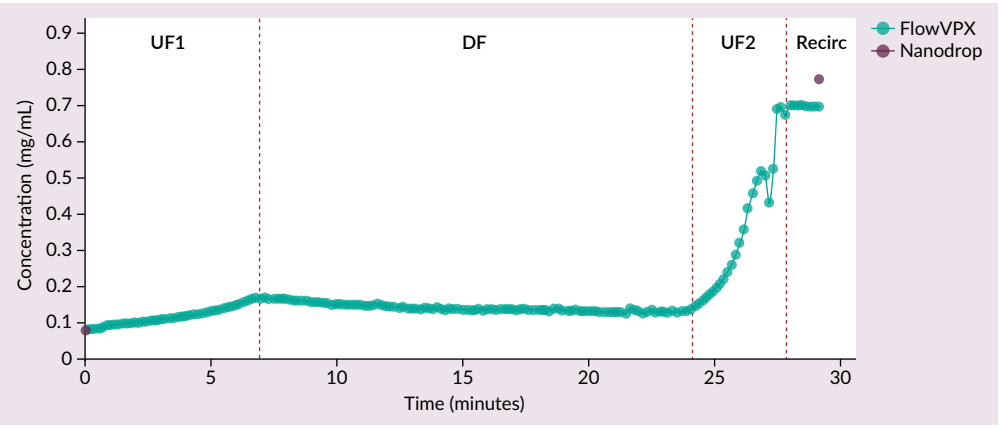
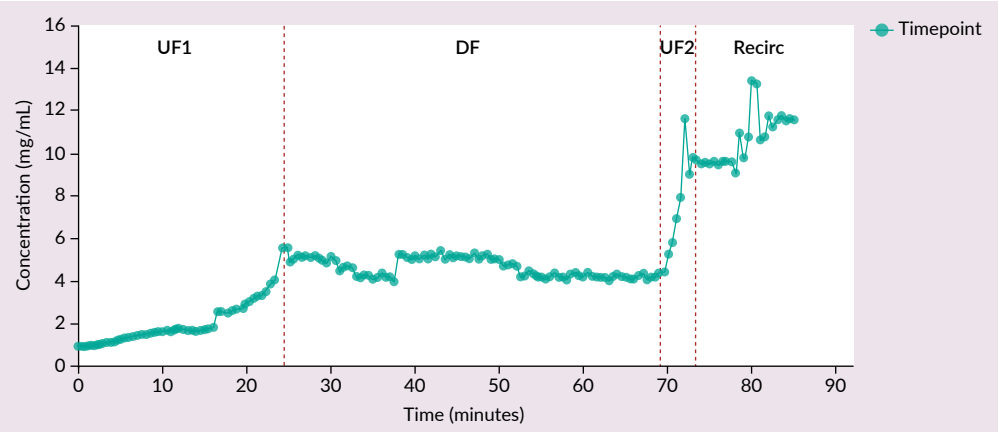


FIGURE 4

mRNA concentration monitoring during UF/DF processing showing FlowVPX and NanoDrop correlation across process steps.



Bioprocess step	FlowVPX® System (mg/mL)	NanoDrop System (mg/mL)
Initial	0.96	0.97
Final	11.57	11.60
Recovery	2.3	2.27

Nucleic Acid Insights 2025; 2(9), 199–209· DOI: 10.18609/nuc.2025.028

points. NanoDrop measurements required dilution factors ranging from 10–100-fold to achieve readings within the instrument's linear detection range (typically <2.0 absorbance units at 260 nm). Continuous monitoring revealed impurity removal during diafiltration through gradual concentration decreases, providing process insights unavailable with offline methods. The study demonstrated automated endpoint control based on concentration rather than mass calculations, eliminating manual sampling requirements and reducing processing time.

CASE STUDY: REAL-TIME MONITORING REVEALS PROCESS OPTIMIZATION OPPORTUNITIES

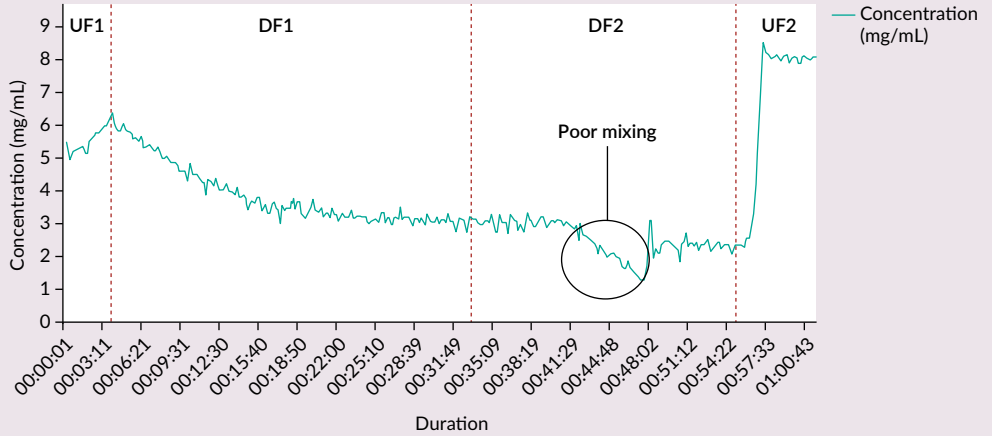
Two mRNA case studies validated FlowVPX performance across different processing conditions. Initial studies using the KrosFlo FS-15 system showed strong correlation between FlowVPX and NanoDrop measurements at initial (0.96 versus 0.97 mg/mL), final (11.57 versus 11.60 mg/mL), and recovery (2.3 versus 2.27 mg/mL) timepoints **(Figure 4)**.

Real-time monitoring revealed membrane polarization effects. Membrane polarization occurs when solutes accumulate at the membrane surface due to convective transport exceeding back-diffusion rates, creating a concentration boundary layer that reduces permeation efficiency. Real-time monitoring enables immediate detection of this phenomenon through characteristic concentration profile changes, allowing corrective measures to restore optimal processing conditions. Recirculation steps recovered the polarized material, demonstrating the value of continuous concentration monitoring for process optimization.

A second mRNA study highlighted mixing performance assessment through real-time monitoring. Concentration instabilities during diafiltration indicated inadequate mixing, enabling immediate process correction during operation rather than detection only at final analysis **(Figure 5)**. The concentrate-diafiltrate-diafiltrate-concentrate mode performs initial sample concentration,

FIGURE 5

mRNA concentration profile during concentrate-diafiltrate-diafiltrate-concentrate processing demonstrating mixing performance detection.



Bioprocess step	FlowVPX® System (mg/mL)	SoloVPE System (mg/mL)
Final	10.2	10.6

FIGURE 6

Precision and accuracy validation of encapsulated mRNA concentration measurement across multiple instruments, analysts, and days.

Multiple SoloVPEs

Multiple analysts

Multiple reads

Multiple days

Day	Instrument	Analyst	Rep 1 (mg/mL)	Rep 2 (mg/mL)	Rep 3 (mg/mL)	Mean concentration (mg/mL)	%RSD
1	1	1	0.95245	0.94678	0.94627	0.9485	0.36%
		2	0.96969	0.96353	0.96518	0.96613	0.33%
	2	1	0.97705	0.96699	0.99298	0.97901	1.34%
		2	0.97658	0.95837	0.97384	0.9696	1.01%
Day 1: Mean and precision						**0.96581**	**1.41%**
2	1	1	1.03739	1.04023	1.05891	1.04551	1.12%
		2	1.05177	1.0501	1.04446	1.04877	0.37%
	2	1	1.00343	1.0143	1.01013	1.00929	0.54%
		2	1.01699	1.01295	1.01412	1.01469	0.21%
Day 2: Mean and precision						**1.029565**	**1.88%**

Method	Concentration (mg/mL)	Mean concentration (mg/mL)	%RSD
SoldVPE	1.01502	**1.01204**	0.58%
	1.00529		
	1.01579		
RiboGreen	0.99	**0.99**	0.71%
	0.98		
	0.99		

followed by two sequential diafiltration steps for buffer exchange and impurity removal, and concludes with final concentration to target specifications. These studies demonstrate how real-time monitoring enables immediate identification and correction of process issues that would otherwise go undetected until completion.

SCATTER CORRECTION ENABLES COMPLEX FORMULATION ANALYSIS

Validation of lipid nanoparticle-encapsulated mRNA analysis demonstrated measurement capability despite light scattering from lipid components. A dual logarithmic scatter correction algorithm enables accurate mRNA concentration determination in complex formulations.

Precision studies across multiple analysts, instruments, and days yielded values consistently below 2% for both intra-day and inter-day measurements **(Figure 6)**. Cross-validation studies confirmed method transferability with relative standard deviations below 2% across different analytical platforms and operators. The dual logarithmic scatter correction algorithm maintained measurement accuracy within 2.2% of reference methods while reducing analysis time from 45–60 minutes to under 5 minutes. Accuracy assessment showed 2.2% error compared to RiboGreen reference methods, while completing measurements

in minutes compared to RiboGreen's 45–60 minute workflow. Linearity assessment across five concentration levels produced R^2 values >0.999.

SUMMARY

Variable pathlength spectroscopy addresses fundamental analytical limitations in nucleic acid ultrafiltration and diafiltration processing through real-time, high-precision concentration monitoring without sample dilution requirements. Integration with TFF platforms enables automated process control based on direct concentration measurements rather than mass balance calculations.

Validation studies demonstrate strong correlation with traditional analytical methods, while providing enhanced process insight and control capabilities. Scalable platform architecture supports consistent analytical performance from development through commercial manufacturing scales.

Case studies confirm this technique's capacity to improve process consistency, reduce manufacturing timelines, and enhance product quality through real-time process optimization. As nucleic acid therapeutic manufacturing continues to expand, these analytical capabilities address critical process control requirements for maintaining product quality and manufacturing efficiency.

Q&A

Addressing implementation and technical considerations with **Nigel Herbert**

Q What is the highest nucleic acid concentration the FlowVPX® has measured?

NH FlowVPX systems have successfully analyzed 80 mg/mL oligonucleotide concentrations during chromatography processes, representing some of the highest concentrations measured to date. mRNA samples up to 12 mg/mL have also been accurately quantified, demonstrating the system's capability to handle highly concentrated nucleic acid samples that would saturate traditional analytical systems.

Q Which real-time process management (RPM) system is recommended for development-scale applications requiring GMP software?

NH KrosFlo® RS 10 RPM systems represent a suitable option for development-scale applications requiring GMP compliance. This system supports processing volumes from 15 ml to 10 L with compatibility for both hollow fiber and flat

sheet cassettes. Multiple pump configurations including centrifugal, diaphragm, and peristaltic options provide operational flexibility, while compatibility with both Cary 60 and Beams light sources enables applications across different nucleic acid modalities.

Q How are RPM systems qualified for GMP applications?

NH Repligen field service engineers provide installation and qualification services for integrated RPM systems. Qualification procedures encompass both the TFF system and FlowVPX components individually, followed by integrated system testing to validate overall performance. This approach ensures regulatory compliance and operational readiness for GMP manufacturing environments.

Q What cleaning and maintenance procedures are required for the flow cells?

NH Single-use flow cells are designed for disposal after each processing run, eliminating cleaning requirements while ensuring sterility for each application. Stainless steel flow cells support cleaning-in-place protocols using 0.5–2 M sodium hydroxide solutions. Recommended cleaning procedures involve initial sodium hydroxide flushing, 30-minute recirculation, and final rinse with deionized water or appropriate buffer to remove cleaning agents.

Q What are the minimum processing volumes for integrated systems?

NH KrosFlo KR2i systems can accommodate minimal processing volumes, potentially as low as 2–3 mL using the smallest tubing configurations. Integration of FlowVPX systems adds approximately 1 mL of hold-up volume when using the 3 mm flow cell, resulting in minimum processing volumes of approximately 4–5 mL for fully integrated systems.

Q What has been the customer feedback on variable pathlength spectroscopy for mRNA applications?

NH Customer feedback has been favorable, with particular appreciation for the capability to measure high concentrations and optical densities in real-time. This technology has enabled customers to both monitor and control TFF processes while providing process development data regarding buffer selection, membrane chemistry optimization, and process parameter effects on product quality.

Q How accurate are FlowVPX measurements?

NH System accuracy depends on the quality of reference standards used for calibration, requiring validation through appropriate analytical protocols. FlowVPX systems ensure measurement linearity through continuous R^2 monitoring with each reading. Comparative studies with at-line SoloVPE systems typically show agreement within 5%, providing confidence in measurement reliability and consistency.

Q How is diafiltration buffer addition controlled?

NH Buffer addition during diafiltration operates through automated weight-based control systems rather than concentration-dependent algorithms. As permeate removal occurs, equivalent volumes of diafiltration buffer are automatically added to maintain constant reservoir levels. This process utilizes auxiliary pumps and is measured in diavolumes—the number of processing volumes of diafiltration buffer that have passed through the system, calculated as the cumulative volume of permeate collected divided by the working volume. Alternative endpoint controls such as conductivity monitoring can be integrated through additional sensor systems when process requirements dictate ionic strength or buffer exchange monitoring.

BIOGRAPHY

Nigel Herbert has a BSc degree in Biomaterials Engineering and a minor in Chemistry from Alfred University, Alfred, NY, USA. He brings a wealth of skills to his role as North America Bioanalytics Manager at Repligen. Since joining our team in 2019, Nigel has been on the forefront of advancing our customers' analytical methods. He specializes in UV-Vis spectroscopy, analytical processes, and method validation, offering invaluable support to ensure the highest standards of precision and reliability. One of Nigel's remarkable achievements is his collaborative spirit, resulting in numerous published application notes. Through close partnerships with our customers, he has made significant strides in solv-ing complex challenges and driving innovation in the biotechnology field. Nigel Herbert's unwavering commitment to enhancing your analytical capabilities exemplifies Repligen's dedication to your success.

Nigel Herbert, North America Bioanalytics Manager, Process Analytics, Repligen, Bridgewater, NJ, USA

AUTHORSHIP & CONFLICT OF INTEREST

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REPLIGEN

TAKE CONTROL OF YOUR UF/DF PROCESS

The new PAT-driven KrosFlo® KR2i RPM™ System combines the power of automation with real-time concentration data, so you can take control of your UF/DF process. Using variable pathlength technology, the system continuously measures concentration and controls the process to deliver the desired concentration end points, every time.

- **Strengthen your process control** with high accuracy and reproducible results
- **Increase process efficiency** by reducing cycling time and increasing yield throughput
- **Reduce process risk** by ensuring accurate concentration throughout the TFF process

Take Control

repligen.com/kr2i-rpm

REPLIGEN

Adenylator DNA/RNA 5′-Adenylation Kit: clean, efficient enzymatic adenylation of DNA and RNA

Lucas Onder, Senior Scientist, R&D, CELLSCRIPT™

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Adenylation of 5′-monophosphorylated oligonucleotides is a critical enzymatic step in the preparation of sequencing libraries and adapter-linked constructs, particularly in small RNA sequencing (small RNA-seq) and native elongating transcript sequencing (NET-seq) workflows. This modification enables subsequent ligation steps by generating a pre-adenylated intermediate, which is required for efficient and directional ligation. Traditionally, this is achieved using thermostable RNA ligases such as Mth RNA Ligase, which requires an elevated reaction temperature of 65 °C and can produce unwanted ligation side products, such as concatemers or circularized oligos.

The Adenylator™ DNA/RNA 5′-Adenylation Kit offers a novel alternative by utilizing a non-ligating, high-efficiency enzyme that specifically catalyzes the 5′-adenylation of both DNA and RNA oligonucleotides under mild conditions (37 °C) and without formation of circularized oligo and concatemerization byproducts. This poster evaluates the enzymatic activity and substrate specificity of the Adenylator Enzyme and provides a direct performance comparison with Mth RNA Ligase under standardized assay conditions.

COMPARING ADENYLATOR ENZYME WITH Mth RNA LIGASE

Adenylator Enzyme was compared with Mth RNA Ligase, a widely used enzyme for adenylating 5′-monophosphorylated DNA oligonucleotides (Figure 1). Adenylation reactions were performed using 300 picomole input of 5′-monophosphate DNA oligonucleotides (22-nt DNA) and incubated at 37 °C for one hour. For benchmarking, parallel reactions were carried out using Mth RNA Ligase under its standard protocol, including incubation at 65 °C for one hour. After incubations, all reactions were quenched with loading dye for direct analysis via PAGE. Products were resolved on 20% acrylamide, 8 M urea, 1×TBE gels, and with a band shift indicative of successful 5′-adenylation. SYBR®-Gold staining was used for detection.

In the Mth RNA Ligase reaction (Figure 1, Lane 5), a downward nucleotide shift (blue arrow) reveals an artifact caused by oligonucleotide self-circularization, while DNA oligonucleotide concatemers (red arrows) formed due to the enzyme's non-specific ligase activity. These side products decrease ligation efficiency and introduce contaminants into downstream workflows. In contrast, Adenylator Enzyme avoids these issues entirely due to its high specificity and lack of ligase activity (Figure 1, Lane 3).

EVALUATING LIGATION OF ADENYLATED OLIGONUCLEOTIDES

In another experiment, the compatibility of Adenylator Enzyme-modified oligonucleotides for downstream ligation and library preparation using T4 RNA Ligase 2, truncated KQ (NEB) was tested. An unadenylated DNA and 5′-OH RNA mixture treated with the T4 RNA Ligase 2 truncated KQ failed to ligate (Figure 2, Lane 4). In contrast, Adenylator Enzyme-treated DNA oligonucleotide and 5′-OH RNA together with the T4 RNA Ligase 2 truncated KQ resulted in a novel 50-nt ligation product (Figure 2, Lane 5, green arrow). These data demonstrate the ability of Adenylator Enzyme to efficiently generate ligation-ready DNA oligos.

SUMMARY

These data demonstrate that the Adenylator DNA/RNA 5′-Adenylation Kit provides a superior alternative to traditional adenylation methods. Compared to Mth RNA Ligase, the Adenylator kit offers cleaner reaction profiles with no side products, milder reaction conditions (37 °C versus 65 °C), improved capability for downstream ligation workflows, and enhanced exonuclease resistance via 5′- capping.

These advantages translate into higher reliability, reduced reagent waste, and greater efficiency in molecular biology workflows. The absence of unwanted ligation artifacts simplifies downstream processing and improves the fidelity of applications such as small RNA-seq and synthetic biology constructs.

In conclusion, the Adenylator DNA/RNA 5′-Adenylation Kit provides a robust method to generate 5′-adenylated DNA or RNA oligonucleotides. Compared to Mth RNA Ligase, Adenylator enables superior workflow control and reaction cleanliness, making it highly suitable for sensitive applications such as small RNA library construction.

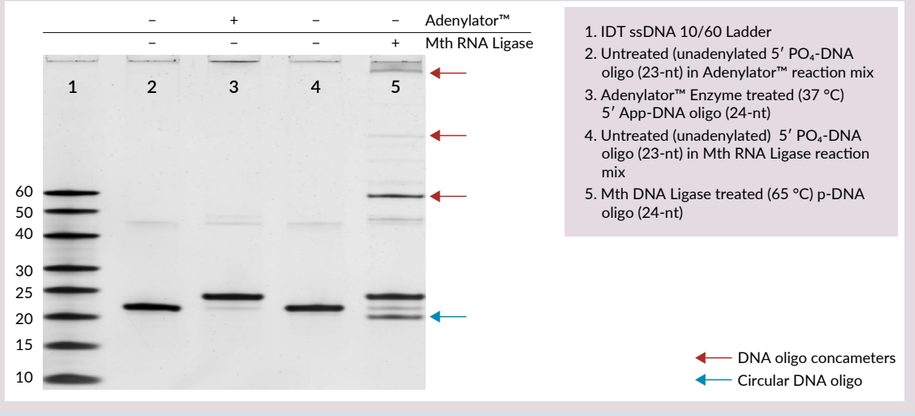

Figure 1 (far left). Comparison of Adenylator™ Enzyme and Mth RNA Ligase for DNA adenylation.

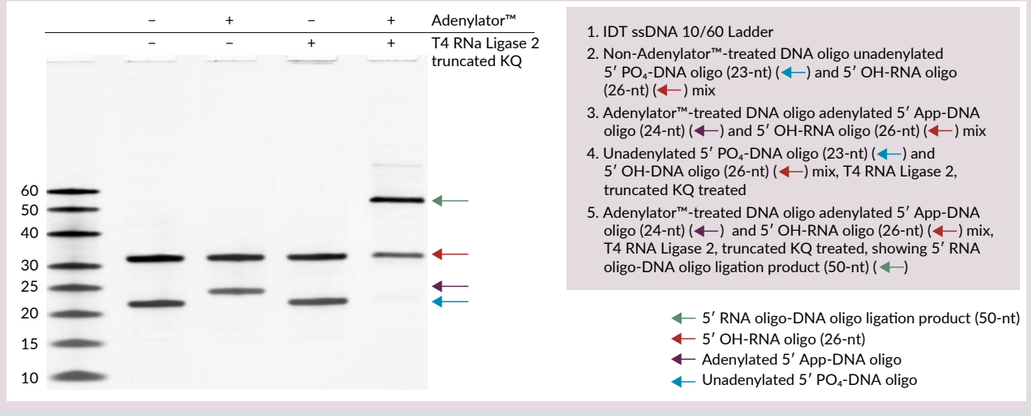

Figure 2 (left). Ligation of Adenylator™ Enzyme-adenylated oligonucleotides.

Lucas Onder PhD is a Senior R&D Scientist at CELLSCRIPT™ where he develops next-generation molecular biology tools. Prior to joining CELLSCRIPT™, he worked as an Amplitude Operations Specialist for Thermo Fisher Scientific supporting high-throughput COVID-19 diagnostics. Lucas earned his PhD in Genetics from the University of Wisconsin–Madison, Madison, WI, USA where he studied transcriptional quality control and nutrient stress response in *E. coli*.

NUCLEIC ACID INSIGHTS

INDUSTRY INSIGHTS

Industry insights, October 2025

Jokūbas Leikauskas

This month has seen a number of exciting discoveries and noteworthy advancements in the nucleic acid therapeutics space. Along with our recent infographic—Oligotherapeutics: status quo and future outlook [1]—which discusses the current landscape and future directions in oligonucleotide-based therapies, our new *Nucleic Acid Insights* Industry Insights series will present the latest updates and developments from across the nucleic acids space, including new collaborations accelerating RNA-based drug development, innovative manufacturing platforms, progress in clinical trials for rare and cardiovascular diseases, emerging AI and gene-editing tools, and regulatory updates supporting novel nucleic acid therapies.

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CLINICAL TRIALS AND RESEARCH

CRISPR Therapeutics shows promise for one-time treatment of alpha-1 antitrypsin deficiency [2]

CRISPR Therapeutics has recently presented new preclinical data for CTX460™, an investigational *in vivo* gene-editing therapy targeting alpha-1 antitrypsin deficiency (AATD). Utilizing the company's proprietary SyNTase gene-editing platform, CTX460 demonstrated efficient correction of the Z allele mutation in AATD models. In the NSG-PiZ mouse model, a single dose of CTX460™ led to sustained expression of functional AAT protein in the liver, with no observed off-target effects. These results support the potential of CTX460 as a one-time treatment option for AATD.

Argo Biopharma to start Phase II trial of siRNA therapy for rare blood disorder [3]

Argo Biopharma has received IND approval from China's National Medical Products Administration for BW-40202, an investigational small interfering RNA (siRNA) therapy targeting complement factor B (CFB). The Phase II clinical trial is set to begin in January

2026. BW-40202 aims to treat paroxysmal nocturnal hemoglobinuria and other complement-mediated diseases by suppressing CFB expression in the liver, thereby inhibiting complement alternative pathway activity. Preclinical studies have shown that BW-40202 significantly reduces serum CFB protein levels with long-lasting effects and a favorable safety profile.

REGULATORY CHANGES AND UPDATES

Ionis receives breakthrough therapy designation for ION582 in Angelman syndrome [4]

Ionis Pharmaceuticals has received the US FDA Breakthrough Therapy designation for its investigational antisense oligonucleotide (ASO) medicine, ION582, in the treatment of Angelman syndrome (AS). AS is a rare neurological disorder characterized by severe developmental delays, motor dysfunction, and seizures. ION582 is designed to inhibit the expression of the UBE3A antisense transcript (UBE3A-ATS), thereby reactivating the expression of the UBE3A gene in neurons.

Ribo's siRNA therapeutic receives orphan drug designation for hepatitis delta virus infection [5]

Ribo Life Science has received Orphan Drug Designation from the EMA for RBD1016, an investigational siRNA therapeutic targeting hepatitis delta virus (HDV) infection. HDV is a severe form of viral hepatitis that occurs only in individuals co-infected with hepatitis B virus, accelerating liver disease progression. RBD1016 is designed to selectively silence key viral factors involved in HDV infection. The efficacy of RBD1016 is currently being assessed in global Phase II clinical development, aiming to provide a new treatment option for patients affected by this rare disease.

Olezarsen is approved in the EU for familial chylomicronemia syndrome [6]

Ionis Pharmaceuticals and Sobi announced that ASO therapeutic TRYNGOLZA® (olezarsen) has been approved in the EU as an adjunct to diet for the treatment of genetically confirmed familial chylomicronemia syndrome in adults. The approval follows a positive opinion from the Committee for Medicinal Products for Human Use and is based on data from the Phase 3 Balance study. The study demonstrated that 80 mg of olezarsen significantly reduced fasting triglyceride levels at six months, with sustained effects through 12 months, and substantially decreased acute pancreatitis events over 12 months.

Corsera Health combines RNAi and AI to advance cardiovascular disease prevention [7]

Corsera Health has recently developed Klotho Health, an AI-powered tool to assess lifetime cardiovascular risk and guide preventive measures [7]. Additionally, Corsera is advancing

a preventive RNAi medicine targeting PCSK9 to lower LDL cholesterol and angiotensinogen to reduce blood pressure, with plans to enter clinical trials by the end of 2025. The company has raised over $50 million from founders and insiders to support these initiatives.

TOOLS AND TECHNOLOGIES

Novel AI-powered CRISPR tool to accelerate gene-editing research [8]

Stanford Medicine researchers have developed CRISPR-GPT, an AI tool designed to streamline gene-editing experiments. Acting as a "copilot", CRISPR-GPT assists researchers in designing experiments, analyzing data, and troubleshooting. By automating much of the experimental design and refinement process, the tool aims to expedite the development of gene therapies. In a demonstration, a student used CRISPR-GPT to successfully turn off genes in lung cancer cells on the first attempt, a task that typically requires extensive trial and error. The goal is to reduce the time needed to develop new drugs from years to months.

bYoRNA raises €1.5 million to advance yeast-based mRNA manufacturing platform [9]

bYoRNA, a French biotech company, has secured €1.5 million in pre-seed funding to advance its innovative yeast-based platform for producing therapeutic mRNA. This *in vivo* fermentation approach utilizes recombinant yeast cells to generate long, stable, and naturally matured mRNA molecules, offering a cost-effective and scalable alternative to traditional in vitro transcription methods. The technology aims to enhance the purity and reduce the immunogenicity of mRNA, potentially lowering production costs by up to 100 times. bYoRNA plans to apply this platform to the development of mRNA-based vaccines and therapies for oncology, gene editing, and infectious diseases.

COLLABORATIONS AND PARTNERSHIPS

Arrowhead and Novartis partner on siRNA therapy targeting neurodegenerative disorders [10]

Arrowhead Pharmaceuticals has struck a global licensing and collaboration agreement with Novartis for ARO-SNCA, its preclinical siRNA therapy targeting alpha-synuclein for synucleinopathies (e.g., Parkinson's disease). Novartis will obtain exclusive worldwide rights to research, develop, manufacture, and commercialize ARO-SNCA, and the parties will also apply Arrowhead's TRiM™ (Targeted RNAi Molecule) platform to additional collaboration targets.

Lundbeck and Contera Pharma collaborate to develop RNA-targeting therapies for neurological diseases [11]

Lundbeck and Contera Pharma have entered a strategic research collaboration to develop RNA-targeting oligonucleotide-based medicines for serious neurological conditions with significant unmet medical needs. Leveraging Lundbeck's extensive neuroscience experience and Contera Pharma's proprietary RNA discovery platforms (AttackPoint discovery®, OligoDisc®, and SpliceMatrix®), the partnership aims to identify and optimize novel therapies targeting molecular factors involved in disease progression.

Secarna Pharmaceuticals and Scenic Biotech collaborate to develop a disease-modifying oligonucleotide therapy targeting novel drug target [12]

Secarna Pharmaceuticals and Scenic Biotech have entered a discovery and co-development agreement to develop a first-in-class disease-modifying oligonucleotide therapy targeting a novel drug target identified by Scenic Biotech's proprietary Cell-Seq platform. Secarna will lead the oligonucleotide discovery efforts using its AI-powered OligoCreator® platform, while Scenic Biotech will contribute its expertise in target and disease biology. The collaboration aims to address rare genetic disorders and broader disease conditions by leveraging the synergies of both companies' platforms to bring new treatments to patients.

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Jokūbas Leikauskas is Commissioning Editor of *Nucleic Acid Insights*