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SPOTLIGHT

REVIEW

Unveiling the potential of RNA-targeting therapies

Rabia Khan and Loïc Roux

RNA-targeting therapies, particularly antisense oligonucleotides (ASOs), have emerged as a promising frontier in molecular medicine. This review explores the potential and challenges of these therapies, highlighting their unique advantages such as design flexibility, broad target range, and high specificity. We discuss the critical role of RNA structure in ASO design and efficacy, emphasizing the need for advanced computational tools in structure prediction and analysis. Recent advancements in artificial intelligence and machine learning are shown to significantly improve ASO design and toxicity prediction. While several ASO therapies have gained approval, we also examine the hurdles faced in clinical trials, including delivery challenges, toxicity concerns, and off-target effects. The paper concludes that the integration of cutting-edge computational methods, enhanced chemical modifications, and innovative delivery strategies is paving the way for more effective and safer ASO therapies, with an increasing number of RNA-targeting therapies expected to transition from laboratory to clinic as our understanding of RNA biology deepens and technologies evolve.

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In the year 2000, the sequencing of the human genome allowed scientists to read the book-of-life, the 3 billion base pairs that make up our genetic blueprint. This achievement challenged long-held central beliefs about what the genome contained, and our understanding of human health. Contrary to the central dogma of biology, where one gene encodes one protein and those proteins are the units-of-biology causing disease, it was evident that the majority of the human genome does not encode proteins, in the classical sense.

Quoting Dr Francis Collins: "Only a decade ago, most scientists thought humans had about 100,000 genes. When we analyzed

the working draft of the human genome sequence three years ago, we estimated there were about 30,000–35,000 genes, which surprised many."

This discovery highlighted the fact that a vast amount of our genome is transcribed and not translated [1]. Classical small-molecule drug discovery has targeted proteins, and the transcriptome has remained largely unexplored by drug discovery [2].

Oligonucleotide therapeutics unlock the potential of targeting RNA. Oligonucleotide therapeutics (OT) are chemically synthesized molecules that bind to complementary nucleic acids and have enabled the industry to unlock the potential of targeting RNA. A key advantage of oligonucleotide therapies is their remarkable specificity and ease of design. By leveraging the principles of Watson-Crick base pairing, researchers can often design highly specific lead compounds with only the knowledge of the target gene's sequence. Additionally, rapid screening methods can be employed to discover promising candidates, shortening the design-make-test cycles as compared to small molecules. The application of antisense oligonucleotides (ASOs) to modulate gene expression is quite broad, with the ability to selectively bind messenger RNA (mRNA), microRNA, or long non-coding RNA (lncRNA). This binding can trigger various responses, including RNA degradation, altered protein binding, or changes in splicing, ultimately affecting gene expression. Nonetheless, oligonucleotide-based medicines still face challenges in systemic delivery, tissue targeting, and cost of manufacturing [3,4].

Focusing exclusively on RNA primary sequences neglects the underappreciated contribution of structure to both RNA function and attempts to drug RNA molecules. RNA molecules are not simple, linear strings of information, but complex 3D biomolecules whose secondary and tertiary structures are critical for their function and druggability.

ADVANTAGES AND CHALLENGES OF ASO THERAPEUTICS

Compared to traditional small-molecule drugs, ASOs offer several advantages, including ease of design, breadth of addressable targets, and specificity. Yet, delivery of naked unmodified nucleic acids is not possible, due to the presence of nucleases in the blood, tissue, and extracellular fluids.

Immunogenicity was initially a significant concern, however, recent advancements in medicinal chemistry generating sophisticated modifications have helped mitigate some of these issues [5]. The oligonucleotide drugs brought to the market carry a small set of modifications pioneered by the first generation of companies, including backbone modifications such as phophorothiates, phosphoroamidates, 2′sugar modifications such as 2′fluoro, 2′*O*methyl and 2′-*O*-methoxyethyl, and modifications to the structure of the sugar, including constrained ethyl, locked nucleic acids, and morpholinos. The selective use of these chemistries has also been employed to define the mechanism of action from simple blocking to recruitment of nucleases for targeted degradation.

These modifications have been important in developing the drug's properties to optimize the affinity of the oligonucleotides to the target, metabolic stability, and favorable pharmacokinetic and pharmacodynamic properties [6]. However, a new set of concerns centered around off-target effects and safety (particularly hepatotoxicity) have emerged as more pressing issues [7]. Specifically, challenges that are emerging are hybridization-dependent (binding to other RNA targets in a sequence-dependent manner) and hybridization-independent (non-specific interactions between ASOs and proteins, facilitated by either nucleotide sequence or phosphorothioate backbone interactions), and pose clinical safety liabilities that need to be resolved [8].

In addition to toxicity issues, delivery to tissues outside the liver and the eye remains

a challenge, and delivery to the CNS via intrathecal injection can be challenging as a route of administration [5]. There are extensive efforts to conjugate ligands to oligonucleotide therapeutics to achieve more efficient liver targeting (e.g., *N*-acetylgalactosamine [GalNAc]) or to direct non-hepatic delivery.

Generally, targeted delivery approaches seek to direct the oligo to a specific tissue and cell type based on conjugating ligands to the OBM that selectively bind receptors on the surface of the desired cell type. Ligands range from small molecules such as GalNac, to peptides, to antibody-mediated ASO delivery. While these target-based delivery approaches broaden the application of oligo therapeutics, they remain limited by the cell/tissue selectivity of cell surface receptors and the effort required to identify ligands selective for such receptors.

Despite the modifications and innovation, these drugs need to be delivered at relatively high doses to ensure efficient cellular uptake and intracellular delivery. The delivered cargo needs to have a high capacity to undergo endosomal escape to ensure the drugs can reach their target sites in the cytosol, and eventually elicit biological effects at lower doses. This process of endosomal escape is not well understood and needs further development [9,10].

Despite these challenges, the ability to target previously 'undruggable' proteins and the vast non-coding RNA landscape has generated interest in oligonucleotide therapeutics.

RNA STRUCTURE-FUNCTION RELATIONSHIPS AND ASO DESIGN

Historically, ASO design has primarily centered on the sequence complementarity between the ASO and its target RNA [3], focused on Watson-Crick base pairing and overlooking the important of RNA structure. However, this simplistic approach does not account for the intricate nature of RNA molecules. The idea that RNA is a floppy, linear sequence is a legacy of RNA being considered simply a messenger between DNA and proteins. It is now well-established that RNA forms complex secondary and tertiary structures, with non-Watson Crick base pairing playing an important role in RNA structure [11]. Within a cellular context, RNA is decorated with RBPs or binding to DNA or other RNA. Understanding RNA architecture (the structure, function, and context of the RNA) within a cell, along with the RNA conformational diversity, can impact the hybridization and time to lead discovery [12].

Advancements in RNA structure prediction methods have combined computational and experimental approaches, with a recent review covering the methods in detail [13]. Advances in AI have also been applied to RNA structure predictions, and machine learning and deep learning technologies are now being utilized for more accurate RNA structure prediction [14,15]. However, limitations remain in current prediction models. While *in silico* ASO design has advanced, it is still limited by approximations in calculating affinity based solely on RNA-RNA/DNA sequences, and these tools do not consider long-range RNA interactions, non-Watson Crick Base Pair interactions, or tertiary structures. Most models consider only the most favorable secondary structure of the RNA, neglecting the dynamic nature of RNA structures and their tertiary interactions [16].

The importance of RNA structure in ASO design has been emphasized in recent studies. Using atomistic molecular dynamics simulations, researchers have demonstrated that ASO length and hairpin motifs significantly affect the stability of ASO-mRNA complexes. This suggests that considering RNA 2D structure and specific base pair interactions can lead to more effective ASO designs [12].

By incorporating computational tools and experimental techniques, scientists can predict and analyze RNA structures, identifying optimal binding sites and designing more effective ASOs. Moreover, non-canonical base pairing rules beyond Watson Crick A-U

and G-C create understudied structures like [17], kink-turns, and E-loops, all of which are themselves critical for non-coding RNA function. A structure-guided approach that incorporates RNA secondary and tertiary structure enhances the specificity, efficacy, and safety of ASOs, leading to more precise and potent therapeutic interventions. A recent review covers in detail the advancements in RNA structure prediction methods, many of which have combined computational and experimental approaches [13].

TOOLS AND TECHNIQUES TO UNDERSTAND RNA ARCHITECTURE

Secondary RNA structures significantly impact ASO binding. Ideally, ASOs target single-stranded, exposed regions or functional regions where the ASO can modify splicing as an example. However, complex structures within the RNA, decoration of the RNA with RBPs, or long-range RNA interactions and 3D conformations can hinder this process. A growing arsenal of tools exists to improve our understanding of RNA structure dynamics, for both long-range and short-range interactions.

EXPERIMENTAL TECHNIQUES FOR RNA STRUCTURE ELUCIDATION

While computational methods can predict RNA structures, their accuracy is often limited by factors like RNA length, with most predictive tools not capable of predicting more than 120 bps effectively. Moreover, most predictive tools cannot factor in non-Watson Crick base pairs effectively. Therefore, experimental data generation remains crucial for understanding RNA structures. RNA structure elucidation techniques include:

- Footprinting-based probing methods such as DMS-seq [18] , SHAPE-seq [19], and icSHAPE [20] modify RNA in a structure-specific manner, creating 'footprints' that reveal structural information. Other *in vivo* probing methods such as in-cell DMS-MaPseq [21] capture RNA structures within their native cellular environment, overcoming limitations of *in vitro* studies and capturing RNA's inherent cellular context. Building upon these foundations, chemical probing and high-throughput sequencing techniques like SHAPE-Seq [22] and STRUCTURE-Seq [23] enable simultaneous probing and mapping of thousands of RNA structures, providing comprehensive information.

- Proximity ligation-based probing methods: techniques like PARIS [24], SPLASH [25], and LIGR-seq [26] are more focused on long-range interactions and detection of base-pairing and interactions within RNA molecules.

- Given the vast RNA existing isoform heterogeneity, targeting of RNA can lead to isoform-specific targeting and new methods such as Nano DMS-MaP [27] can lead to isoform-specific structure determination [28].

RNA 3D PREDICTION METHODS

- NMR, Cryo-EM and SAXS [29] have been used to determine RNA 3D structure. These techniques offer structural information at low resolution, complementing data from probing experiments. The data produced by these techniques may not fully capture the structural heterogeneity of RNA in a cellular context and should be combined with other techniques to produce a fuller picture.

- Machine learning-based 3D predictions have seen increasing improvements with multiple methods being developed [30,15].

Combining RNA 2D, 3D, and long-range interaction can provide a comprehensive picture of the cellular RNA structural context, while functional data such as IRES elements [31] and RBP-binding data [32] can provide a whole picture of the functional RNA structures.

RECENT ADVANCEMENTS AND FUTURE PROSPECTS

In additional to RNA structure and optimal binding of the ASO to the RNA, other parameters such as off-targets, chemical modifications, toxicity, and delivery also need to be optimized for ASO design.

Researchers are developing new approaches to mitigate off-target effects and enhance safety profiles. For example, the BROTHERS (BRO) nanoarchitecture aims to reduce off-target interactions and enhance the safety profile of ASO drugs [7].

Recent advancements in computational methods have significantly improved our ability to predict and minimize toxicity in ASO development. One such tool is PFRED (Platform for RNAi and antisense oligonucleotide Evaluation and Design), a computational platform designed for the analysis and design of siRNA and antisense oligonucleotides (ASOs) [33].

PFRED incorporates several key features that address toxicity and off-target effects:

- Machine learning for efficacy prediction: PFRED uses a support vector machine (SVM) algorithm combined with diverse sets of oligonucleotide descriptors to predict siRNA functionality. For ASOs, the platform considers factors such as oligonucleotide length and chemical modifications in its activity models.
- Off-target effect prediction: the platform includes an off-target search module which, while not based on a structure-based off-targets, does serve as an effective annotation tool. It reports the number of cDNA off-target hits for 0, 1, and 2 mismatches, helping researchers prioritize and filter sequences.
- Toxicity considerations: PFRED incorporates a model derived from data on over 500 ASOs against 46 targets. It considers thermodynamic calculations, although these are currently limited to unmodified DNA ASOs.
- Sequence-based filtering: the platform filters out compounds that align with known human SNPs, sequence motifs associated with non-specific binding, and low complexity sequences with a high number of perfect off-target matches.

While PFRED represents a significant advancement in the field, it is important to note that some of its models, particularly those related to thermodynamic calculations, may have limitations when applied to heavily modified ASOs, such as those incorporating locked nucleic acids (LNA).

These computational methods allow for early elimination of potentially toxic ASOs from drug discovery pipelines, thereby improving safety assessments and increasing focus on promising candidates. As the field continues to evolve, we can expect further refinements in these *in silico* tools, potentially incorporating more advanced machine learning techniques and broader datasets to improve toxicity predictions for a wider range of ASO modifications.

CHALLENGES AND FUTURE DIRECTIONS

Despite these significant advancements, challenges remain. Accurate prediction, computational cost, and limitations in experimental validation methods continue to hamper the broader therapeutic application of ASOs. Future research is actively addressing these hurdles, focusing on refining computational tools for RNA structure prediction and

developing innovative experimental validation methods.

Several ASO therapies have faced challenges in clinical trials, providing valuable lessons for future development:

- BIIB078 for C9orf72 ALS: Biogen and Ionis ceased development due to a lack of efficacy compared to placebo, despite no significant safety issues **[34]**.
- Revusiran: clinical development for hereditary transthyretin-mediated amyloidosis was voluntarily stopped in 2016, leading to the development of AMVUTTRA with improved chemistry **[6]**.
- Mipomersen: while approved, this drug did not achieve successful marketing due to its risk:benefit profile, and availability of a superior small molecule (lopitamide) **[35]**.
- Eteplirsen: this drug showed weak physiological response due to reduced muscle absorption and rapid renal filtration **[36]**.

However, there have also been notable successes:

- Volanesorsen: the APPROACH study, a randomized, double-blind, placebo-controlled Phase 3 trial, demonstrated the efficacy of volanesorsen in treating familial chylomicronemia syndrome (FCS). The subsequent open-label extension study revealed sustained reductions in triglyceride levels and a 74% reduction in pancreatitis event rates compared to the preceding five-year period. However, safety concerns were noted, particularly thrombocytopenia, which occurred in about half the patients. Common adverse events also included injection site reactions. Despite its efficacy in significantly lowering triglyceride levels, these safety concerns led the US FDA to not grant marketing authorization in the USA in 2018 **[37]**.
- Golodirsen: approved by the FDA in December 2019 following positive results from a Phase 1/2 clinical trial, demonstrating increased dystrophin expression at the muscle level **[38]**.

Looking to the future, computational methods and technological developments are poised to play a crucial role in addressing these challenges and advancing ASO prediction and development:

- Advanced *in silico* tools: the development of more sophisticated computational models, such as those incorporating machine learning and artificial intelligence, will enhance our ability to predict ASO binding, efficacy, and toxicity. These tools will likely integrate diverse datasets, including RNA structure information, genomic data, and clinical outcomes, to provide more accurate predictions. The advent of large language models (LLMs) that can integrate heterogeneous datasets can further accelerate our ability to develop better, safer, and more effective oligonucleotide drugs.
- Improved RNA structure prediction: as computational power increases and algorithms become more refined, our ability to predict complex RNA structures will improve. This will lead to better design of ASOs that can effectively target specific RNA regions while minimizing off-target effects.
- AI-driven design optimization: machine learning algorithms will increasingly be used to optimize ASO sequences, chemical modifications, and delivery strategies. These AI systems could potentially design ASOs with improved tissue specificity, reduced toxicity, and enhanced efficacy.

- Integration of multiomics data: future computational approaches will likely integrate data from genomics, transcriptomics, and proteomics to provide a more comprehensive understanding of ASO interactions within cellular systems. This holistic approach could lead to more effective and safer ASO designs.

- Simulation of ASO-target interactions: advanced molecular dynamics simulations and quantum mechanical calculations may allow for more accurate modeling of ASO-target interactions at the atomic level. This could provide insights into the thermodynamics and kinetics of ASO binding, guiding the design of more effective therapies.

- Predictive toxicology models: the development of sophisticated *in silico* toxicology models will help identify potential safety issues earlier in the development process. These models could incorporate data from multiple sources, including historical clinical trial results, to improve their predictive power.

- Personalized ASO design platforms: computational platforms that can rapidly design and evaluate personalized ASOs based on an individual patient's genetic profile may become a reality. This could lead to more effective treatments for rare genetic disorders and cancer.

As these computational methods and technologies continue to evolve, they will synergize with advancements in experimental techniques, chemical modifications, and delivery systems. This integration of computational and experimental approaches promises to accelerate the development of safer and more effective ASO therapies, potentially expanding their application to a wider range

BOX 1

History of ASO approvals

- Mipomersen: RNase H1 MoA for homozygous familial hypercholesterolemia [40]
- Eteplirsen: exon skipping MoA for Duchenne's muscular dystrophy [8]
- Nusinersen: exon inclusion MoA for spinal muscular atrophy
- Patisiran: AGO2 MoA for hereditary amyloidosis [41]
- Inotersen: RNase H1 MoA for hereditary amyloidosis [42]
- Vutrisiran: RNase H1 MoA for hereditary amyloidosis [8]
- Milasen: splicing modulation MoA for ceroid lipofuscinosis 7 [43]
- Volanesorsen: RNase H1 MoA for familial chylomicronemia syndrome [44]
- Givosiran: AGO2 MoA for acute hepatic porphyria [45]
- Golodirsen: Exon skipping MoA for Duchenne's muscular dystrophy [46]
- Viltolanersen: Exon skipping for Duchnne's muscular dystrophy [8,47]
- Lumasiran: AGO2 MoA for hyperoxaluria [48]
- Inclisiran: AGO2 MoA for familial hypercholesterolemia [49]
- Casimersen: exon skipping MoA for Duchenne's muscular dystrophy [50]
- Tofersen: RNase H1 MoA for amyotrophic lateral sclerosis [8]

While fomiversen opened the door for antisense technologies, it also showed the potential for small molecules to outcompete. Fomiversen was only approved for a limited time until the advancement of highly active antiretroviral therapy [8]. Nusinersen is also showing similar challenges with the approval of risdiplam, which has taken a significant piece of the SMA market.

of diseases and offering new hope for patients with previously untreatable conditions.

TRANSLATION INSIGHT

A deep understanding of RNA structures is essential for designing effective ASOs, but rapid, large-scale data generation is improving our ability to engineer selectivity at an exponential pace. By incorporating structural insights and functional data into the design process, we can revolutionize the time to design of the ideal ASO. Continued advancements in computational techniques, experimental methods, and LLM technology, as evidenced by recent patents, promise an exciting future for precise and efficacious ASO-based therapies.

While challenges such as off-target effects and tissue-specific delivery remain, the field of ASO therapeutics continues to evolve rapidly. The integration of advanced computational methods, improved chemical modifications, and innovative delivery strategies is paving the way for more effective and safer ASO therapies. As our understanding of RNA biology deepens and technologies for ASO design and delivery improve, we can expect to see an increasing number of RNA-targeting therapies moving from the laboratory to the clinic.

In 1998, fomivirsen became the first FDA approved antisense on the market as a second line treatment for cytomegalovirus [39]. Since then another 14 antisense therapies were approved by regulatory bodies (Box 1).

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AFFILIATIONS

Rabia Khan
Founder/CEO,
Serna Bio,
London, UK

Loïc Roux
OligoTune Limited,
Cambridge,
UK

AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

INNOVATOR INSIGHT

Bridging the translational gap: humanized liver models as predictive tools for RNA therapeutic success

Matthew Baginski and Sara Donnelly

RNA therapeutics, particularly siRNAs, are advancing drug development, but traditional animal models often fail to predict human responses, leading to clinical trial failures. This article explores humanized liver chimeric mouse models, such as the PXB-mouse, as a solution to improve preclinical predictions of siRNA efficacy and safety. It highlights the limitations of conventional models and demonstrates, through case studies in viral hepatitis, metabolic disorders, and lipid regulation, how PXB-mice address challenges like off-target effects and therapeutic efficacy. These models offer a more human-relevant platform, potentially reducing late-stage failures and accelerating RNA therapeutic development. The PXB-mouse model features a liver that is highly engrafted with human hepatocytes, while other cell types remain of mouse origin—a factor to consider when assessing RNA therapeutics. Despite this limitation, humanized liver mice can provide greater confidence in the translational potential of investigational therapeutics than conventional animal models alone.

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The field of RNA therapeutics, with its potential for treating a wide range of diseases, continues to experience rapid growth and attracts significant investment. According to the American Society of Gene and Cell Therapy (ASGCT), as of Q2 2024, 30 RNA therapies have been approved globally and another 1,125 are currently in development (between preclinical and pre-registration stages) [1].

RNA therapeutics encapsulate several therapeutic modalities, including small interfering RNA (siRNA), messenger RNA (mRNA), and

antisense oligonucleotides (ASO) **(Table 1)**. IQVIA Pipeline Intelligence **[2]** noted that these three types dominate the development landscape, collectively representing 80% of the RNA therapeutics pipeline. Among these, siRNA is the most prevalent category from preclinical to pre-registration stages accounting for 37% of the pipeline. The range of therapeutic indications is very broad, with rare diseases topping the list of targets **(Figure 1)**.

Despite the promising number of therapies in development, the path from laboratory to clinic is fraught with challenges, particularly in translating preclinical findings to human outcomes. Of the RNA therapeutics that enter clinical trials, only a small percentage will successfully navigate all phases to reach market approval, underscoring the critical importance of accurate preclinical modeling in the successful advancement of these therapeutics.

Traditional animal models, while valuable, are often inadequate in predicting human responses due to species-specific differences in physiology, metabolism, and disease manifestation. This fundamental disconnect frequently leads to late-stage failures in clinical trials, leading to significant time and resource expenditures. Clinical failures can be attributed to a combination of reasons including a lack of clinical efficacy or toxicity (70–80%), poor drug properties (10–15%) or commercial reasons (10%) **[3]**. For RNA therapeutics in particular, delivery of the drug to target organs/tissues and toxicity due to off-target binding are some of the most significant challenges **[4]**.

In this article, we examine the use of humanized chimeric mouse models, specifically the PXB-mouse as a solution to improve preclinical predictions of the efficacy and safety of RNA therapeutics, with a particular focus on siRNAs due to their current prevalence. By addressing the limitations of traditional animal models, these advanced preclinical tools offer a more translatable platform for RNA therapeutic development, potentially reducing late-stage failures and accelerating the path to successful treatments.

CHALLENGES WITH TRADITIONAL ANIMAL MODELS

The development of RNA therapeutics, particularly siRNAs, faces several hurdles when relying on traditional animal models, such as mice, which can lead to misleading preclinical results.

Key limitations of traditional models include:

- Species-specific differences in physiology and metabolism
- Poor prediction of human pharmacokinetics and pharmacodynamics
- Inadequate modelling of human-specific infections (such as viral hepatitis)
- Inability to directly target human genes *in vivo* during preclinical studies

Together, these factors contribute to a significant translational gap, where promising results observed in animal studies fail to materialize in human trials.

PXB-MOUSE: A HUMANIZED LIVER CHIMERIC MODEL

Humanized liver chimeric models, such as the PXB-mouse (described below), have been developed to address the translational challenges. Such models provide a more human-relevant environment for the preclinical testing of RNA therapeutics.

The PXB-mouse is created using transgenic mice that allow for the ablation of endogenous mouse hepatocytes and accept the engraftment of xenotransplanted human hepatocytes **[5–7]**. This process results in a mouse with up to 95% of its liver repopulated with functional human hepatocytes. The outcome is a chimeric model that combines the benefits of a small animal model with the biological relevance of human liver tissue.

TABLE 1

Overview of gene-based therapeutics.

RNA therapeutics	RNA aptamer	
	ASO	
	RNAi	siRNA
		miRNA
	mRNA	mRNA Tx
		mRNA vaccines
		mRNA-based cell Tx
Gene editing	Nuclease	Meganuclease
		TALEN
		ZFN
	CRISPR/Cas	CRISPR/Cas
		Base editing
		Prime editing
	Epigenetic editing	

Features of the PXB-mouse include:

- Normal human liver histology and function **[8]**
- Human-specific metabolism and excretion pathways **[9–11]**
- Expression of human genes, mRNA, and proteins **[8,12]**
- Human-like lipoprotein profiles **[13]**
- Production of human albumin and human-like biliary excretion **[6,14,15]**
- Permissive to infection with HBV and HDV **[16,17]**

However, it should be noted that while human chimeric models, such as PXB-mouse, contain human hepatocytes in the liver, they lack the human liver immune and stromal cells that would also play a role in disease responses in humans.

Given its unique characteristics, the PXB-mouse serves as an invaluable tool for studying responses to small molecule drugs, biologics, gene therapy delivery systems and RNA therapeutics. The presence of human hepatocytes allows for direct targeting of human genes *in vivo* at the preclinical stage, more relevant delivery to human hepatocytes, and simultaneous evaluation of toxicity and pharmacodynamics. Consequently, these models aid the early identification of potential efficacy and safety issues, leading to better-informed clinical trials.

HUMANIZED LIVER MODELS IN ACTION

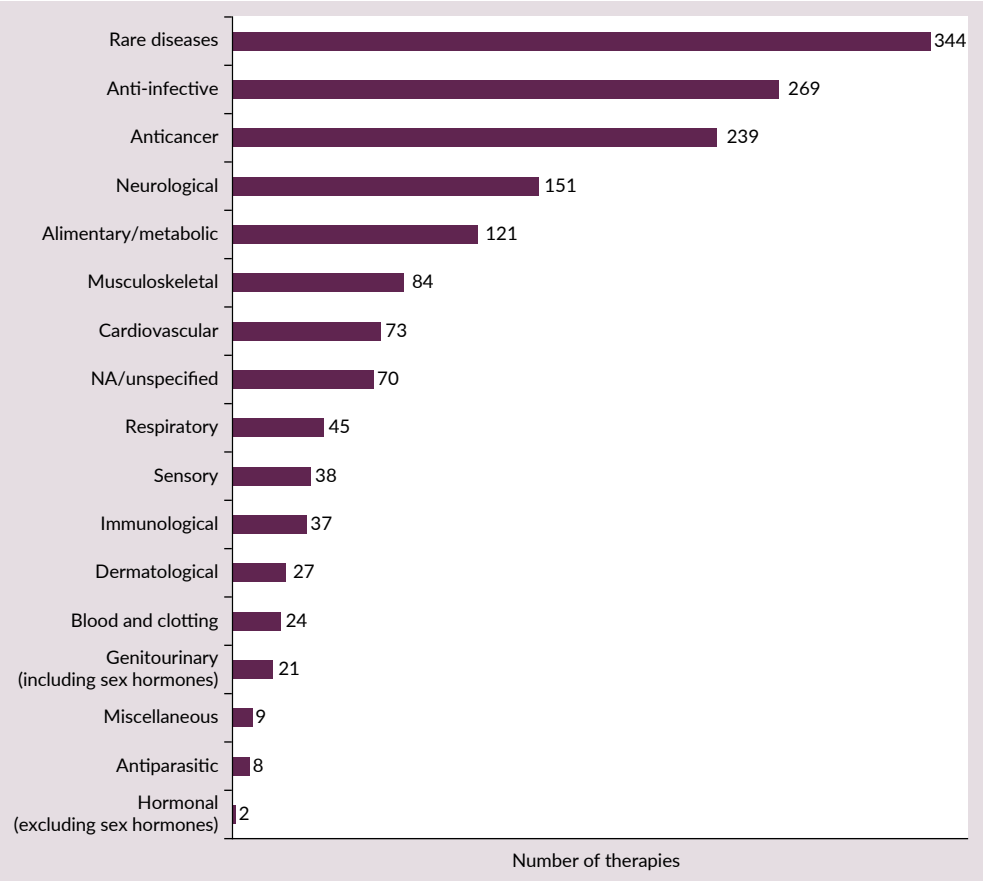
There are many reasons for the failure of most RNA therapeutics, including off-target effects, delivery challenges, target engagement challenges and low therapeutic efficacy. Despite these obstacles, humanized liver mouse models have consistently proven effective in recapitulating human outcomes in RNA therapeutic development. Here we examine four examples from a wide range of published literature that showcase the value of humanized liver mouse models in this field.

Case study 1 Overcoming off-target effects and safety challenges: RNAi therapeutics for chronic hepatitis B

Chronic hepatitis B virus (cHBV) infection remains a significant global health challenge;

FIGURE 1

RNA therapeutics innovation landscape: key therapeutic areas are shown by the number of RNA therapies currently in the pipeline (from preclinical to pre-registration) [1].



the WHO estimated 254 million people were living with the condition in 2022 plus 1.2 million new infections each year [18]. RNA interference (RNAi) therapeutics have shown promise in targeting cHBV, but safety concerns have hindered their development.

HBV is associated with the expression of various proteins including hepatitis B surface antigen (HBsAg). It is hypothesized that large quantities of HBsAg contribute to T- and B-cell dysfunction, impairing the host's ability to eradicate the HBV infection. A potential treatment involves reducing HBsAg using RNA interference via siRNA. Since there are overlapping templates within the X region of the HBV genome, a single siRNA could selectively and effectively target all HBV transcripts [19].

In a recent study, researchers used the PXB-mouse as a preclinical model to accurately predict the safety and tolerability of investigational RNAi therapeutics in healthy volunteers [19]. The study compared two siRNAs that target all major HBV mRNA transcripts: ALN-HBV and VIR-2218. These siRNAs have the same sequences, except that VIR-2218 has been chemically modified via enhanced stabilization chemistry plus (ESC+) resulting in a single substitution of a glycol nucleic acid modification within the seed region. This modification was an attempt to minimize off-target effects.

PXB-mice (12–18 weeks of age) received subcutaneous injections of ALN-HBV or VIR-2218 at doses of 12, 36, or 100 mg per kg of animal weight. Blood analysis over

seven weeks revealed markedly lower alanine aminotransferase (ALT) levels following administration of VIR-2218 compared to ALN-HBV, indicating reduced liver damage.

Importantly, these preclinical findings were supported by subsequent clinical studies in healthy volunteers. Here, the PXB-mouse model accurately predicted the improved safety profile of VIR-2218, demonstrating its value in assessing potential off-target effects and safety concerns early in the development process.

Case study 2
Overcoming delivery challenges: effective use of LNPs in PXB-mice

Effective delivery of RNAi therapeutics is as crucial as their sequence design **(Figure 2)**. Multiple options are available, but lipid nanoparticles (LNPs) have emerged as a popular choice for siRNA delivery due to their ability to target various tissues while protecting the siRNA from degradation. (Most recently, the spotlight was on LNPs as a key component of the COVID-19 vaccines [20,21]). Despite these advancements, issues in ensuring RNAi uptake by the correct tissue and achieving cross-species compatibility can complicate preclinical testing. This is where humanized liver mouse models offer a distinct advantage, helping to bridge these gaps by closely mimicking human responses to RNA therapeutics.

In a study by Okada *et al.*, an LNP encapsulated siRNA was effectively used to target Dock11, a host factor regulating covalently closed circular DNA (cccDNA) formation by HBV in PXB-mice [22]. cccDNA forms during HBV replication and acts as a viral reservoir in cells. The persistence of cccDNA and the inability to effectively target it with therapeutics is a key reason that a cure for HBV remains elusive.

In PXB-mice, the LNP-encapsulated siRNA targeting DOCK11 showed highly effective knockdown of human DOCK11 in PXB-mice and, importantly, a clear reduction in cccDNA levels. The study employed the same LNP formulation as that used in the FDA-approved drug Onpattro®, highlighting the cross-compatibility of human-tested LNPs in the PXB-mouse.

Another example of the effective use of LNPs in PXB-mice to predict human outcomes comes from a hepatitis delta virus (HDV) study [17]. HDV infects an estimated 10–20 million people globally and is associated with severe fulminant hepatitis, which often leads to cirrhosis and an increased risk of hepatocellular carcinoma. Despite the severity of the disease, there is an unmet clinical need for effective treatments.

HDV infection requires the presence of HBsAg; HDV can either establish itself as a superinfection in individuals already carrying HBV or through a simultaneous coinfection when a person is exposed to both HBV and HDV at the same time.

In this study, researchers used humanized mice dually infected with both HBV and HDV to evaluate the effectiveness of HBV-targeting siRNA therapy in controlling HDV infection, comparing it to a direct anti-HDV siRNA approach.

The results revealed that *in vivo* treatment with an anti-HBV RNAi agent successfully reduced both HBV and HDV viremia, showing the potential of this approach in managing HDV infection.

Specifically, treatment with ARB-1740, delivered via LNP technology, resulted in a 2.3 $\log_{10}$ reduction in HBV viremia and a 2.6 $\log_{10}$ decrease in serum HBsAg levels, which led to a subsequent 1.6 $\log_{10}$ reduction in HDV viremia. In contrast, HDV-targeting siRNA effectively inhibited HDV in both the blood and liver compartments without impacting HBV. Additionally, PEGylated interferon-alpha reduced HBV viremia by 2.0 $\log_{10}$ but did not affect HDV viremia under the conditions of this study. These findings demonstrate the inhibitory effect of ARB-1740 on HDV, supporting its potential as a therapeutic option.

FIGURE 2

Overview of RNA therapeutic delivery methods.

Common delivery methods for RNA therapeutics

Chemical conjugates	Carriers
• Directly modify the therapeutic • Passive or active tissue targeting • Not applicable for mRNA delivery	• Encapsulate therapeutics for delivery • Useful for RNAi, ASO, mRNA, and more • Protect payload from degradation and renal clearance • Composition can be modulated for specific tissue targeting
GalNAc	Lipid nanoparticles (LNPs)
• Binds asialoglycoprotein receptor (ASGPR) • Liver targeting • Broad oligonucleotide compatibility • Multivalent GalNAc has improved receptor binding affinity	• ~100 nm in size • Usually contain cholesterol, a PEG-lipid, a helper lipid, and a cationic or ionizable lipid • Amine groups bind the RNA payload to generate stable particle
Antibodies	Polymers
• Tissue specificity can be achieved using different antibodies	• Polyphosphazenes • Dendrimers • Modulating charge, degradability, and molecular weight can affect RNA delivery

Note that, as anticipated by the investigators, the human chimeric mouse model showed no overt signs of liver damage (including cirrhosis or hepatocellular carcinoma) in either of the coinfection or superinfection studies described, mostly likely due to the lack of an adaptive immune response in this system.

Overall, these studies emphasize the value of the humanized liver mouse model in generating translationally relevant results, which can guide the selection of effective siRNA delivery methods.

Case study 3 Target engagement and therapeutic efficacy: hepatocyte-targeted siTAZ therapy in MASH

Metabolic dysfunction-associated steatohepatitis (MASH), formerly known as non-alcoholic steatohepatitis (NASH), is becoming the most common cause of liver disease. To date, therapies that have shown promise in mouse MASH models have not translated well to humans, underscoring the need for more predictive preclinical models [23,24].

It has been shown that MASH can be established in PXB-mice by feeding them a high fat diet [25]. PXB-mice fed these diets recapitulate the key features of human metabolic dysfunction-associated fatty liver disease (MAFLD)/MASH including hepatocyte ballooning, inflammation, and importantly, fibrosis.

Researchers used MASH diet-fed PXB-mice to test GalNAc-siTaz, an siRNA targeting the gene for TAZ, a transcriptional regulator [26]. The researchers chose this model reasoning that 'a mouse NASH model whose livers are populated with human hepatocytes would be particularly valuable in testing hepatocyte-targeted siRNA therapies'.

The PXB-mice were fed a high-fat, choline-deficient, L-amino acid-defined diet for 6 weeks to induce MASH. This was followed by 6-weekly injections of GalNAc-siTAZ or a control siRNA (GalNAc-control) while maintaining the MASH diet.

The results were promising. GalNAc-siTAZ lowered human hepatic TAZ and IHH (Indian hedgehog) a TAZ target that

TABLE 2

Alignment of humanized mouse models with CBER guidance.

CBER recommendation	Benefits of humanized mouse models
Select animal species that closely reflect the biological response expected in humans	Direct targeting of human genes is possible in the humanized liver of these mouse models
Consider physiological and anatomical comparability to humans	Liver expresses human metabolic pathways and transporters in physiologically relevant zonation patterns, and they also have humanized lipoprotein profiles [9,12,13]
Assess permissiveness/susceptibility to infection by, and replication of, viral vectors or microbial vectors for gene therapy	Humanized liver mice are permissive to AAV and adenovirus vectors, commonly used in gene therapies [29–31]
Evaluate immune tolerance to human cell therapy products or human transgenes expressed by gene therapy products	
Ensure feasibility of using the planned clinical delivery system/procedure	Amenable to therapeutically relevant delivery methods, including GalNAc-conjugated RNAs, LNPs and adeno-associated viruses (AAVs), and provide a system to directly test human-targeting RNAi therapeutics *in vivo*

promotes MASH fibrosis. In addition, treatment with GalNAc-siTAZ decreased liver inflammation, hepatocellular injury, hepatic fibrosis, and profibrogenic mediator expression compared to the control. These effects indicated that GalNAc-siTAZ decreased the progression of MASH in mice reconstituted with human hepatocytes.

This study demonstrates the value of humanized liver models in assessing both target engagement and therapeutic efficacy for complex metabolic liver diseases like MASH.

Case study 4 Target engagement and therapeutic efficacy: STP125G for hypertriglyceridemia

Hypertriglyceridemia, characterized by elevated triglyceride levels, is associated with an increased risk of cardiovascular diseases. In cases of severe hypertriglyceridemia (sHTG), where triglyceride levels exceed 1,000 mg/dL, the risk of developing acute pancreatitis is 5–10 times greater than in the general population.

STP125G, an siRNA therapeutic targeting apolipoprotein C3 (ApoC3), developed by Sirnanomics, was tested in PXB-mice to demonstrate its efficacy in reducing triglyceride levels [27]. ApoC3 is a key player in triglyceride metabolism and has recently been recognized as a factor influencing cardiovascular, metabolic, and neurological disease risk.

The study in PXB-mice showed a single dose of STP125G resulted in high-efficiency, durable knockdown of ApoC3, and significant reductions in both mRNA and protein levels of ApoC3 were observed up to 6 weeks post-treatment. Corresponding reductions in triglycerides and cholesterol were observed, returning to control levels by week 8.

The results in the humanized liver model provided strong support for ApoC3-targetting siRNA as a therapeutic approach in hypertriglyceridemia management, paving the way for clinical development.

FDA GUIDANCE ON CHOOSING ANIMAL MODELS FOR CELL AND GENE THERAPIES

The FDA's Center for Biological Evaluation and Research (CBER) has provided guidance on selecting relevant animal models for cell and gene therapy (CGT) assessments, including RNA therapeutics [28]. This guidance emphasizes key considerations that align with the advantages of humanized chimeric models (Table 2).

Humanized liver mice fit many of these criteria as their liver expresses human metabolic pathways and transporters in physiologically relevant zonation patterns, and they also have

humanized lipoprotein profiles [9,12,13]. Moreover, these models are amenable to therapeutically relevant delivery methods, including LNPs and adeno-associated viruses (AAVs), and provide a system to directly test human-targeting RNAi therapeutics *in vivo*.

Sponsors are encouraged to submit detailed species assessments as part of the preclinical section of the IND. To support these assessments, there are more than 300 publications featuring the PXB-mouse over the last 20 years, meaning it has been thoroughly characterized for a variety of applications including CGT, with a deep body of work to draw from when interpreting new data. Cumulatively, these studies show that the PXB-mouse is a valuable model for viral hepatitis and predicting human-specific response to therapeutics. Some limitations have also been identified, including dysregulation of some pathways where there is a mismatch in human and mouse signaling, for example, human growth factor (hGF) signaling [29]. In addition, the PK profiles of therapeutic compounds that show very high clearance in mice may not be accurate in humanized liver mouse models [10].

CBER also encourages using disease/injury animal models in preclinical studies for CGT products. Due to the unique features of CGT products—such as prolonged effects, persistence *in vivo*, complex mechanisms of action, and invasive routes of administration—using disease models rather than healthy animals is preferred for assessing activity and safety. As shown above, PXB-mouse can be used as a MASH model and is the gold standard for viral hepatitis. While the PXB-mouse is usually produced using healthy donor hepatocytes, it's also possible to generate disease models, such ornithine transcarbamylase deficiency (OTCD) mice [30].

It's important to note that no single model can perfectly predict the efficacy and safety of an investigational CGT product in humans. However, the PXB-mouse can be a key part of a comprehensive program of efficacy and safety testing, allowing for greater confidence when entering the clinic with a new therapeutic.

TRANSLATION INSIGHT

The development of RNA therapeutics, particularly siRNAs, represents a promising frontier in modern medicine. Yet, the path from preclinical studies to successful clinical outcomes is fraught with challenges, many of which stem from the limitations of traditional animal models.

Humanized liver chimeric models, such as the PXB-mouse, offer a powerful solution to these challenges. By providing a more human-relevant environment for preclinical testing, these models enable direct targeting of human genes *in vivo* and more accurate assessment of drug metabolism and pharmacokinetics. As a result, you obtain a better prediction of efficacy and safety in humans and can evaluate therapeutics against human-specific pathogens.

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AFFILIATIONS

Matthew Baginski
Executive Vice President,
Strategy and Business Development,
PhoenixBio USA,
New York, NY, USA

Sara Donnelly PhD
Director, Research Planning & Business Development,
PhoenixBio USA,
New York, NY, USA

AUTHORSHIP & CONFLICT OF INTEREST

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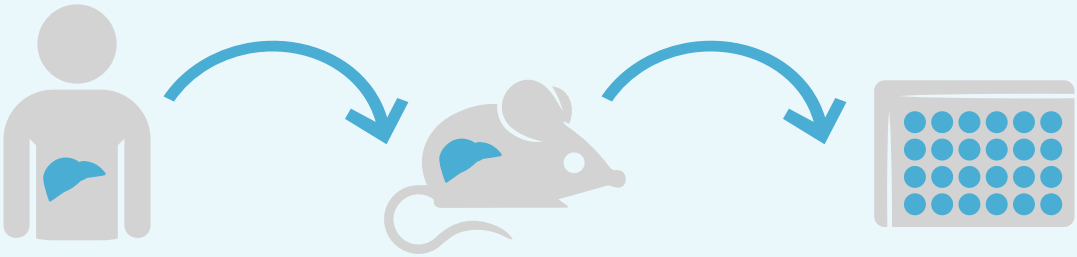

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SPOTLIGHT

INTERVIEW

Tackling the key challenges for therapeutic oligonucleotide manufacture: cost, scale, and sustainability

In this wide-ranging interview **David McCall**, Senior Editor, BioInsights, and **Ben Andrews**, Senior Manager in Strategic External Development, GlaxoSmithKline, discuss the key challenges and opportunities for successful oligonucleotide manufacturing at scale, the merits and drawbacks of various synthesis options, and current efforts to mitigate the industry's environmental impact.

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Q What are you working on right now?

BA: I'm an organic chemist by training and these days I'm working mainly on process development for therapeutic oligonucleotides. Currently I'm in the middle of a number of QbD work packages including DoE studies and robustness/confirmation work on

"...a lot of work is going on in the industry at the moment to optimize solid-phase synthesis in order to bring the cost down and improve sustainability."

oligonucleotide synthesis and purification steps. These are my two focus areas when it comes to the process.

I'm also supporting manufacturing campaigns to make material for clinical trials. Supporting these trials and working on marketing applications are among my key deliverables at the moment. I'm writing various chemistry sections for regulatory filings, and I'm also doing some new technology work, including assessing novel methods of synthesis.

Q Can you frame for us the current key trends and challenges in oligonucleotide synthesis as you see them?

BA: The three main challenges facing oligonucleotide manufacturing are cost, sustainability, and scalability.

Oligonucleotides are very expensive to make; somewhere in the ballpark of US$300 per gram. They cost much more than small molecules, which isn't very surprising as they are larger and more complicated. They're more like peptides or antibodies in that respect. Regarding sustainability, they require lots of raw materials to make and they generate a lot of waste—around 4,300 kg of materials used for every 1 kg of drug substance produced. That is a lot of materials, and a lot of waste! And finally, scalability: with the current equipment that we use for synthesis, the maximum batch size is approximately 1,600 mmol/ batch, which translates to about 7 kg/batch. Obviously, if you have a high-volume oligonucleotide product to manufacture, it is going to involve a lot of batches.

There is progress being made in addressing these issues. For example, a lot of work is going on in the industry at the moment to optimize solid-phase synthesis in order to bring the cost down and improve sustainability. This involves approaches like reducing the equivalents of the amidite monomers (which are very expensive), reducing solvent wash volumes, and eliminating unnecessary steps.

There are efforts to improve sustainability via approaches like solvent recycling, which involves recovering, repurifying, and re-using the solvents, especially acetonitrile. There's also enzymatic synthesis, which is trying to address sustainability through moving to fully aqueous systems and avoiding the use of hazardous organic solvents.

Finally, alternative synthesis methods like liquid-phase synthesis are being explored to help to address scalability, because you are not limited to the current hardware and can scale up to larger batches in solution.

Q Can you expand on the relative pros and cons of solid-phase, liquid-phase, and enzyme-based oligonucleotide synthesis?

BA: Solid-phase synthesis is the current state-of-the-art. It has been around for decades and is a very mature technology that performs really well. You can typically achieve a 50–60% overall yield from start to finish of the process, and it also produces high-quality material: >90% purity after downstream processing, which is an amazing achievement when you consider how large and complicated these molecules are. In addition, it is very versatile. You can make whatever type of oligonucleotide you want by using the appropriate monomers and linkages. So overall, solid-phase synthesis is very robust and reliable, and performs really well.

The issues with solid-phase synthesis are the ones I touched on earlier—cost, sustainability, and scalability. The expensive monomers and the excesses of reagents that you have to use to drive the reactions to completion in order to achieve that high yield and high purity do contribute to the cost and sustainability issues.

Liquid phase oligonucleotide synthesis is a relatively new technology. It works in a similar way to solid phase, but is in solution instead of on a solid support. With solid-phase chemistry, the oligonucleotide is covalently attached to the polystyrene beads in the column. To wash the reagents between steps and stop them interfering with one another, you simply need to wash a solvent through the column. Liquid phase has to do this in different ways, however. It uses either precipitation or membrane filtration to remove these reagents between the steps. But otherwise, the chemistry is similar.

Liquid phase approaches are being developed primarily to address the scalability issue. If you are synthesizing in liquid phase, then you can use larger vessels to scale-up—you are not limited by the current hardware that solid phase uses. There is also the potential to monitor reactions, which you cannot do in solid phase. For example, you can take samples and analyze them to see if the coupling reaction has progressed to completion.

In terms of potential issues for liquid phase, I think it now provides similar purity to solid phase, but everything just takes longer. Flushing materials through a column takes a matter of minutes. With liquid phase, though, you have to charge your reagents, stir for a while, quench or precipitate, and then filter. Lengthier cycle times mean more time in plant and potentially, more cost. Your oligonucleotide is also exposed to the reagents for longer, so there may be greater potential for impurity formation. It takes 45 minutes for one solid-phase cycle to complete versus several hours for a liquid-phase cycle. That means that instead of processing a batch in a day, it might take you the best part of a week. There are more calculations to be done to compare these technologies—factoring in things like cycle time, time in plant, cost, sustainability, and so on.

Enzymatic approaches are also relatively new. They use simpler monomers with fewer, smaller protecting groups, which improves the atom economy. You have cheaper and simpler starting materials, which is potentially more environmentally friendly. Furthermore, moving away from organic solvents and into aqueous systems is better for sustainability. And as with

liquid phase, you are not limited by equipment size—you can essentially do it in whatever size vessels you have.

There are different types of enzymatic synthesis. There is single base extension, where you add one monomer at a time, or there is templated ligation, where you have a template and you bring in short fragments which bind to the template, and then join them together with an enzyme. Templated ligation can actually produce better quality material because impurities in your fragments are rejected from the template and don't join together. So you can actually skip the chromatography step and still get high quality material out at the end, and that's another benefit.

In terms of potential issues with enzymatic synthesis, it's not really an off-the-shelf technology as such. It takes time to develop and optimize an enzymatic ligation and you have to do enzyme evolution work for the ligase enzymes in order to tolerate the linkages you are making and the modifications at the 2′ positions. There is more work to do upfront, which means it is not quite as versatile as solid-phase synthesis.

Q How do you predict these approaches will evolve in terms of their relative popularity and future application in the space?

BA: I'm a big fan of solid-phase synthesis. I expect that to be retained as the backbone of oligonucleotide manufacturing going forward, as it performs so well. And improvements will continue to be made in order to bring down cost and improve sustainability.

The other technologies will find their place, though. For liquid phase, this may be for the large-scale synthesis of oligonucleotide fragments that can feed into enzymatic templated ligation. Enzymatic synthesis will likely come into its own for large volume oligonucleotides, which would justify the upfront development work—perhaps also for longer oligonucleotides, as you do see a drop-off in yield for longer linear synthesis. If you can join the fragments together in a convergent synthesis, that will potentially improve yield and purity.

Q Can you tell us about the current state-of-the-art in impurity synthesis?

BA: It is crucial to have impurity samples for analytical method development. We need samples of the various impurities in order to show that our analytical methods can detect them. It's a key part of the work that we do. Amidite impurities are required to develop starting material methods, and oligonucleotide impurities are required to develop drug substance methods.

We also use them in spiking and purging work. Some of the amidite monomer impurities in the starting materials are critical because they can react to form oligonucleotide drug

substance impurities. We spike them in, see how they track through the process, and see if they are purged. If they are not purged, we set specifications in the starting materials to control them.

In terms of the synthesis of these impurities, there are several CROs out there who can make amidite impurities. It is custom synthesis in a similar way to small molecule impurities. There are also CROs who can make oligonucleotide impurities, and many of them are pretty straightforward to make. Others, such as abasic impurities, are a bit more challenging to synthesize, and require custom amidites. They are also less stable, so you need to take precautions.

Q Can you talk us through your approach to oligonucleotide process development and optimization?

BA: As I mentioned earlier, we follow a QbD workflow at GSK, and do DoE studies on individual synthesis steps such as deprotection, coupling, sulfurization and capping. We study each step individually, and then bring it together at the end. This gives us the process understanding on each step that we need. We do a similar thing for purification. For the simpler downstream steps, like lyophilization, we use a combination of DoE and modeling from first principles.

Next, we move into robustness and confirmation work in order to establish the design space. This is a set of parameter ranges for your process, and if you stay within those ranges your process should operate successfully and give you material of appropriate quality that passes specification. This design space is not just a series of set points where you have to operate, but is an area in which you can work. It gives you some flexibility.

This flexibility means you can make some small changes within that design space without having to re-file with the regulators. For example, when you move to a different manufacturing site, or if you change pieces of equipment and you need to make some minor changes, you have the flexibility to do that. That is the big benefit of this approach.

Q You discussed scalability as a key challenge for the field. What are your thoughts on how this can best be addressed?

BA: I am not certain what is limiting scalability at the moment, to be honest. I mentioned that the maximum scale is approximately 1,600 mmol/batch, and 7 kg/synthesis batch. I used to think that it was an engineering issue, because as you scale up, the columns get wider and the challenge becomes how to distribute the reagents effectively across that increased column diameter. But is that actually true or just a myth? Could it be that the equipment for larger-scale synthesis simply doesn't exist yet?

One way to address the issue with current technology is to combine multiple syntheses batches. For example, perform three syntheses, then three purifications, then combine them into one batch for downstream processing. So, you go from three to one—from three 7 kg batches to one 21 kg batch. That approach also reduces your analytical burden when releasing the drug substance.

Approaches like liquid-phase synthesis are also trying to address the scalability issue, as I mentioned earlier. However, another option is doing solid phase on a larger scale. Some people with peptide expertise are using stirred-tank reactors with a filter at the bottom. You have your solid support, you add your reagents, you stir for a while, and then you filter. People are having reasonable success with that and achieving material of equivalent quality, following some optimization.

One question I do have, though, is what the maximum batch size is that we would be happy running? It is very expensive; one batch currently costs around US$2 million. So, while it might be technically feasible to scale up to hundreds or even thousands of liters, if that costs US$10 or $20 million a batch, how happy are we take on that much risk? How many eggs do we want to put in one basket, so to speak? That is a decision that needs to be considered from a business perspective.

Q To what extent is continuous manufacturing an option in this space?

BA: There are possibilities for continuous manufacturing. Perhaps not for the actual synthesis yet, but for the downstream steps there are certainly options. Multicolumn continuous solvent gradient purification (MCSGP) is an approach where you are constantly adding crude material to a pair of columns and you are re-purifying mixed fractions, so you are not losing product. This can be operated in a continuous manner.

There are also options for continuous tangential flow filtration (TFF) by utilizing single-pass TFF. Thin film evaporation (TFE) already operates in a continuous mode and instead of isolating by lyophilization, you can run either continuous spray drying if you want a solid, or just go straight into solution API. In the future, I can imagine a process that has multiple synthesis batches that all feed into continuous purification and downstream operations.

Q You are involved in two consortia that are focusing on the key topic of sustainability. Can you discuss these efforts, and share your thoughts on the next steps in this particular arena?

BA: I am part of the **American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable Oligonucleotide Subteam**, which is a highly

"We are looking into the technical feasibility of solvent recovery in oligo synthesis, and the regulatory and quality implications too."

sustainability-focused consortium. Several pharmaceutical companies with an interest in oligonucleotide manufacturing and sustainability are collaborating on that. We published a white paper a few years ago and gathered sustainability metrics on oligonucleotide manufacturing, which is where this figure of 4,300 kg of materials used per 1 kg of drug substance came from—it is an average taken from eight compounds that we had in development in order to create a baseline of the current manufacturing process [1]. We also provide ignition grants for oligonucleotide sustainability research.

We are currently working on a similar project to gather sustainability metrics on amidite material consumption to help complete the picture, because our previous effort only focused on oligonucleotide manufacturing. It didn't include the monomer synthesis, but of course, the monomers themselves are very large and complicated. They are about the same as a small molecule drug in terms of their size and complexity. I have a feeling that the materials number will actually double when you include the amidite and all the reagents and solvents that you use to make the monomers!

The other consortium I am involved with is the **European Pharma Oligonucleotide Consortium**. Again, this is a group of pharmaceutical companies with an interest in therapeutic oligonucleotide manufacturing. Since this is a relatively new modality, there is not much regulatory guidance out there yet. The idea was that a group of us would get together and collaborate in a pre-competitive sense to harmonize our ways of working and thinking, propose best practice in different areas, and maybe try to influence the regulatory environment somewhat.

We have published various white papers on different topics. Some projects have completed and some are still ongoing. I was involved in the impurity purge factor team, and I am now involved in the solvent recovery team. We are looking into the technical feasibility of solvent recovery in oligo synthesis, and the regulatory and quality implications too. I recommend that people take a look at the **publications** we have produced on various topics, because they are very interesting and useful.

Q What will your key priorities be for the foreseeable future?

BA: A key goal for me at the moment is to support GSK's oligonucleotide projects. I would love to get one over the line—onto the market and into the hands of patients. Much of my current work is focusing on late-stage activities such as file writing.

Another goal would then be to apply my knowledge to new projects. I have had lots of experience working on antisense oligonucleotides. I'd like to bring that to bear on new compounds and other platforms and modalities, perhaps siRNAs. That would a fun challenge!

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BIOGRAPHY

BEN ANDREWS is a Senior Manager in Strategic External Development at GlaxoSmithKline, Stevenage, UK. He has a background in organic chemistry with a PhD and postdoc in the field of Asymmetric Synthesis. In his 21 years at GlaxoSmithKline, he has contributed to the development of several mid- to late-phase compounds, from small molecules to oligonucleotides. He has 14 years of experience of oligonucleotide manufacturing with expertise in synthesis, purification and isolation. He is a longstanding member of the ACS GCIPR oligonucleotide team and the European Pharma Oligonucleotide Consortium (EPOC).

AFFILIATION

Ben Andrews
Senior Manager,
Strategic External Development,
GlaxoSmithKline,
Stevenage, UK

AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: RNA/DNA DELIVERY OF GENOME EDITING PLATFORMS

SPOTLIGHT

INTERVIEW

Improving lipid nanoparticle formulations for targeted delivery of genome editors

Although there have been significant innovations in the lipid nanoparticle (LNP) field in recent times, researchers face complex challenges including stability and target specificity. **David McCall**, Senior Editor, BioInsights, speaks with **Rohit Sharma**, Assistant Project Scientist, Innovative Genomics Institute, University of California, Berkeley, about methods to overcome these lingering issues and realize the potential of LNPs to revolutionize therapeutic strategies, particularly in the burgeoning area of *in vivo* genome editor delivery.

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Q What are you working on right now?

RS: I work at the Innovative Genomics Institute in the Department of Bioengineering at the University of California, Berkeley with Professor Niren Murthy, a leader in bioengineering and drug delivery. Our lab is interdisciplinary, bringing together team members from different backgrounds. I feel fortunate to be at UC Berkeley, where we have amazing opportunities to collaborate with scientists and clinicians from various fields. This creates an outstanding, multidisciplinary environment for conducting high-level research.

I'm currently working on several projects, with a primary focus on obesity. Obesity is a global issue, and we are taking a new approach by using LNPs. We use LNPs to deliver mRNA or small interfering RNA (siRNA) to fat cells (white fat) to transform them into beige fat, which is healthier. Early experiments in obese mice show promising results, including weight loss.

We are also collaborating with clinicians at UCSF on glioblastoma and ovarian cancer. Glioblastoma is an aggressive brain cancer, and we are using LNPs to deliver CRISPR machinery to combat it—specifically, CRISPRoff, an epigenome editor that can induce stable, long-lasting gene silencing. Early experiments are showing good results, and we are aiming to publish our initial findings soon. For ovarian cancer, we are investigating the delivery of therapeutic mRNA to activate T cells and B cells. We are also using siRNA to silence specific genes that cause the disease. In another project, we are testing different LNP formulations on iPSC-derived cortical and motor neurons to understand the effects of CRISPR editing and toxicity, since neurons are more sensitive to toxicity.

We are also focusing on CNS diseases, particularly targeting neurons in mouse experiments. The brain is one of the most challenging areas to deliver LNPs, and our goal is to find the best formulation to efficiently deliver CRISPR machinery to neurons with minimal toxicity. This could lead to potential treatments for Alzheimer's disease, Huntington's disease, and other neurological disorders.

Q Can you frame the challenges in employing LNPs specifically for the *in vivo* delivery of genome editors?

RS: One of the major challenges is off-target effects. Most LNPs, when used *in vivo*, primarily end up in the liver and spleen, which is not ideal. We want LNPs to reach specific tissues and perform their intended functions with minimal side effects. Developing LNPs that are more targeted to specific tissues or diseases will be beneficial for their future use in a wide range of applications.

In addition to off-target effects, there are several significant general challenges in LNP research. One of these is endosomal entrapment. The goal is to create LNP formulations that can quickly escape from the endosome, ensuring a high volume of the payload reaches the target cells or tissues.

Another important challenge is finding lipids that minimize toxicity. High toxicity and immune response are bottlenecks in LNP research; even empty LNPs can trigger significant cytokine responses. Developing lipids with minimal toxicity is essential for the success of LNPs in clinical settings. Stability and integrity are also crucial for the long-term use of LNPs. As seen with COVID-19 vaccines like those from Moderna and Pfizer, creating LNPs that remain stable over extended periods is difficult, especially in regions of the world without adequate storage facilities. Many countries still lack the infrastructure to store LNPs at −80 °C, making stability a significant concern. Since LNPs encapsulate specific mRNA or genome editor payloads, maintaining their stability is vital for their effectiveness.

"By optimizing lipid composition, we can enhance stability and cellular uptake, which in turn increases overall biodistribution."

Immunogenicity, alongside toxicity, is a significant issue. The strong immune response triggered by a single dose of LNP-based therapy is a major drawback. Additionally, improving biodistribution and pharmacokinetics is crucial. LNPs often do not reach the entire target tissue, instead accumulating in non-specific regions, which limits their effectiveness in treating various diseases.

Q How are you addressing the current limitations in stability?

RS: An LNP consists of four key components: an ionizable lipid, a helper lipid, cholesterol, and polyethylene glycol (PEG). The stability of LNPs depends on these components. While most lipid-based LNP formulations remain stable at −80 °C for extended periods, their stability decreases significantly at room temperature or 4 °C, typically within a week. To enhance LNP stability, we need to explore alternative lipids or develop new formulations using lipid classes that remain stable under these conditions, where traditional lipids fall short. For example, incorporating zwitterionic head groups can provide electrostatic stability, reducing the likelihood of protein corona formation and aggregation *in vivo*. Additionally, varying the PEG chain length (from short to long) and introducing new polymers as a replacement of PEG can optimize stability. Lyophilizing LNPs with suitable cryoprotectants can further improve long-term storage and transport stability.

Q And the limitations in biodistribution and target specificity?

RS: Our lab is continually developing new lipids, polymers, peptides, targeting ligands, and antibodies linked with LNPs to improve biodistribution. By optimizing lipid composition, we can enhance stability and cellular uptake, which in turn increases overall biodistribution.

Different lipids influence pharmacokinetics and tissue targeting, and adjusting the size and charge of LNPs affects circulation time and distribution. Smaller, neutral particles typically show improved biodistribution. PEG plays a crucial role in LNP biodistribution, reducing immunogenicity and aggregation while increasing circulation time. However, increasing the PEG content beyond 10% results in diminished LNP performance, as it reduces endosomal escape efficiency. So, while higher PEG levels can improve immunogenicity and distribution, they also lower the transfection rate, making it essential to balance these factors. To address this, our lab is developing a new class of lipids designed to reduce toxicity and improve LNP

"...one of the major challenges with LNPs is their low transfection efficiency, particularly when targeting complex cancer tissues."

distribution. Recently, we published a paper in *Nature Nanotechnology* introducing 'acid-degradable LNPs'. Compared to traditional LNPs, these new formulations demonstrated better biodistribution, covering a larger tissue area in mice, and exhibited lower toxicity. In summary, designing new lipids that reduce toxicity, improve biodistribution, and stabilize LNP structure is key to advancing this technology.

Q What are your expectations in terms of the continued evolution of both genome editor platforms and LNP technology—and where might we see convergence between the two that will create opportunities for novel therapeutic development?

RS: This is certainly a crucial question for the future of LNPs. Initially, LNPs were used to deliver simple nucleic acids like DNA or mRNA. However, as the field has evolved, increasingly complex technologies such as base editing and prime editing have been developed.

Furthermore, one of the major challenges with LNPs is their low transfection efficiency, particularly when targeting complex cancer tissues. For instance, in glioblastoma, the tightly packed cancer cells make it difficult for LNPs to penetrate beyond the surface, preventing them from reaching cancer stem cells. This can lead to recurrence after initial treatment.

To address this, we need to develop LNP formulations that can effectively reach cancer cells or other hard-to-transfect cells that current formulations struggle to access. Additionally, improving the transfection efficiency of LNPs for prime and base editing is critical, especially for addressing genetic disorders. Traditional CRISPR/Cas9 systems have limitations due to off-target effects, which base and prime editing aim to improve. However, no LNP formulation currently exists that can deliver a high percentage of prime editing machinery to target cells or tissues, with editing rates typically around 1–5%. This is insufficient for sustainable therapeutic outcomes, and we need to find the right combination of LNPs and genome editors to improve this efficiency.

Moreover, when targeting brain disorders, LNPs face the challenge of crossing the blood-brain barrier after systemic delivery. We need targeted formulations that can deliver their payload effectively with a single IV injection.

As mentioned above, another persistent challenge with LNPs is toxicity, primarily caused by the ionizable lipid component. While modifying the ionizable component can reduce toxicity, it also reduces transfection efficiency. The future of LNP development should focus on identifying a new class of lipid formulations that are less toxic, more biodegradable, capable of efficient endosomal escape, and able to encapsulate a high percentage of editing molecules, all while targeting tissues that are currently inaccessible with existing formulations.

Q What are some key goals for your work in the foreseeable future?

RS: I'm lucky to work with great teams at IGI, UC Berkeley, Gladstone Institute, and UCSF, each focusing on different research areas. This teamwork helps us learn and improve. One challenge I have faced is delivering prime editing tools using LNP systems. This is tough because prime editing is complicated, and the success rate is usually only around 1–2%, which is disappointing. We are working to improve this efficiency so we can achieve better results in treating diseases. I'm also interested in creating LNP formulations for cancer treatment. Although there is promising research here, it is still early because cancer is complex, and it's difficult to deliver LNPs to the right locations in the body. Our goal is to develop LNP formulations that enhance genome editing efficiency, target hard-to-reach cells, and minimize side effects.

BIOGRAPHY

ROHIT SHARMA is an Assistant Project Scientist in the Department of Bioengineering at the University of California, Berkeley, Berkeley, CA, USA working in Professor Niren Murthy's research group at the Innovative Genomics Institute. His current research focuses on developing treatments for obesity using LNP/mRNA/siRNA complexes. He is also actively involved in collaborative projects targeting glioblastoma, gynecological cancers, and other neurological disorders. Through the LNP platform, Rohit aims to deliver gene-editing complexes as potential gene therapy treatments and to deepen the understanding of gene-editing mechanisms. Before joining Berkeley, Rohit completed his postdoctoral training at the Comprehensive Cancer Center in Puerto Rico, where he gained expertise in tissue culture and mouse model studies related to ovarian cancer, under the mentorship of Professor Pablo Vivas. During his PhD, he worked on enzyme nanoparticles for biofuel production and participated in projects related to protein drug delivery. His PhD in Applied Biochemistry and Biotechnology was completed under the guidance of Professor Kai Griebenow. Rohit earned his master's in Biotechnology and bachelor's degree in India.

AFFILIATION

Rohit Sharma
Assistant Project Scientist,
Department of Bioengineering,
University of California, Berkeley,
Berkeley, CA, USA

AUTHORSHIP & CONFLICT OF INTEREST

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