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COMMENTARY

The chemistry and biology of oligonucleotide conjugation

Sritama Bose and Peter L Oliver

A major challenge associated with oligonucleotide nucleic acid therapeutics is the difficulty of these relatively large and often highly charged molecules to cross cellular membranes and reach their sites of action in the cytosol or nucleus. Consequently, unfavorable pharmacokinetic or pharmacodynamic properties and inefficient cellular uptake currently limit their full translational potential. In order to address these important issues, chemical conjugation of oligonucleotides with molecular transporters is used to improve nucleic acid therapeutic delivery and therefore enhance the clinical efficacy of these compounds.

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INTRODUCTION

Conjugates that improve the cellular uptake include lipophilic compounds such as cholesterol, tocopherol, fatty acids, and cell-penetrating peptides. In addition, more specialized tissue-specific delivery can be achieved by targeting cell-surface receptors using antibodies, peptides or small molecule targeting moieties such as the clinically approved carbohydrate conjugate N-acetyl galactosamine (GalNAc). Yet despite these recent advances, achieving efficient nucleic acid therapeutic delivery—especially to extrahepatic tissues—remains a major obstacle for translation. In this brief commentary, we provide an overview of specific current and future chemical conjugation approaches and how they influence the challenges of oligonucleotide delivery and the clinical approval path. The application of oligonucleotide delivery vehicles such as exosomes, lipid nanoparticles, or AAVs will not be covered here, but this subject is reviewed extensively elsewhere [1].

TYPES OF OLIGONUCLEOTIDE CONJUGATION

See **Figure 1.**

Lipid conjugation

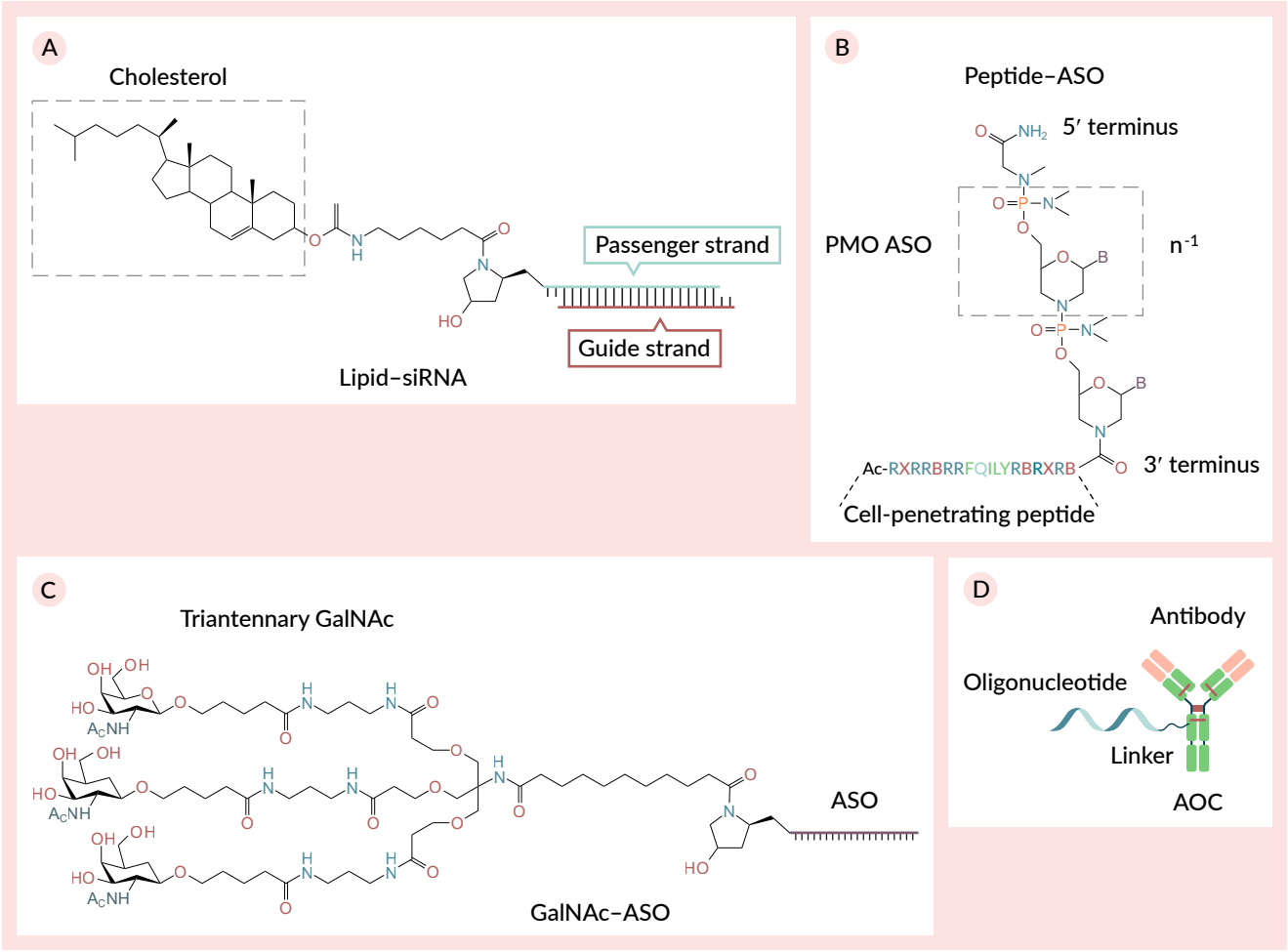
The addition of lipophilic moieties to antisense oligonucleotides (ASOs) and small

interfering RNAs (siRNAs) has been shown to significantly modulate their pharmacokinetic/pharmacodynamic (PK/PD) properties [2]. This class of conjugates enhance the interaction of the nucleic acid therapeutics (NAT) with albumin and serum lipoproteins, thus increasing blood-circulatory half-life and as a consequence, their biodistribution. Tocopherol (vitamin E) conjugates are used in a similar way; early studies demonstrated that siRNAs conjugated to α-tocopherol resulted in improved, potent silencing of apolipoprotein b (*Apob*) in the mouse liver [3]. In extra-hepatic tissues, there has been success with lipid conjugates such as palmitic acid for improving efficacy in muscle. For example, the potency of a splice modulating ASO was significantly enhanced using this conjugate in mouse skeletal and cardiac muscles, therefore permitting a lower therapeutic dose and improving the overall safety profile [4].

Two strategies can be used for the incorporation of lipids, fatty acids, or lipophilic small molecules into oligonucleotides, known as either pre-synthetic or post-synthetic. In

FIGURE 1

Oligonucleotide delivery strategies.



Schematics of four oligonucleotide conjugation platforms. (A) Lipid–siRNA conjugate where cholesterol is conjugated to the 3′ end of the passenger strand. (B) Peptide–ASO conjugate. An example of a PMO conjugated to a CPP. (C) GalNAc-ASO conjugate. $GalNAc_3$ moiety conjugated to an ASO. (D) A simplified schematic representation of an AOC. AOC: antibody–oilgonucleotide conjugate; ASO: antisense oligonucleotides; CPP: cell-penetrating peptide; $GalNAc_3$: triantennary N-acetylgalactosamine; PMO: phosphorodiamidate morpholino oligonucleotide; siRNA: small interfering RNA.

the pre-synthetic approach, the nucleotide monomers already carry the desired hydrophobic moiety prior to oligonucleotide synthesis, deprotection, and purification. That is, these modified nucleotides are incorporated into the oligonucleotide sequence during the usual phosphoramidite process, such as by direct addition to modified controlled pore glass resins used in solid-phase synthesis. This approach thus provides flexible options for points of attachment, as conjugation can be carried out either at 3′, 5′ or even between consecutive nucleotides. The most convenient technique is to attach the hydrophilic group at the 5′ end as a pre-synthesized phosphoramidite, while 3′-lipid attachment can be arduous since pre-tethered bioconjugation is required onto a solid support. In the post-synthetic approach, a small reactive functional group is introduced for coupling to the hydrophobic moiety after synthesis is complete. Although this method has the advantage of fewer steps involving a reactive side chain (e.g., azide, maleimide) and easier assembly using established solid-phase chemistry, this post-synthetic approach requires the handling of two macromolecules with solubility and stability issues and a limited number of compatible reactions available. As such, click chemistry is a commonly used technique to overcome this.

GalNAc conjugation

GalNAc is a carbohydrate moiety which binds to the asialoglycoprotein receptor (ASGR) and facilitates the uptake of oligonucleotides into hepatocytes by receptor-mediated endocytosis [5]. ASGR is highly expressed in the liver, and is recycled rapidly to the cell membrane, making it an ideal receptor for long-lasting, targeted delivery. The interaction between GalNAc and ASGR is also pH-dependent, hence the dissociation of the receptor and oligonucleotide conjugate occurs during acidification in the endosomal system, a key event in NAT intracellular processing that also facilitates recycling of ASGR. Of the five FDA-approved therapeutic siRNAs based on the GalNAc conjugation strategy, Inclisiran has currently the longest duration of therapeutic effect with twice-yearly administration [6]. Further optimization of this conjugate has included increasing the number of GalNAc moieties to tri- or tetravalent assemblies at the 3′ end of the siRNA to maximize potency. For further discussion of the preclinical and clinical advancement of GalNAc-conjugated oligonucleotide drugs, see Huang *et al.* [7].

Peptide conjugation

The conjugation of peptides, typically less than 20 amino acids in length, are an increasingly common choice for oligonucleotide NAT delivery. One major class are CPPs, with their specific ability to cross cellular membranes. Inspired by naturally occurring peptides such as the HIV Tat protein, and typically cationic, these molecules can vary in sequence, polarity, and structure [8]. A recent optimized example includes morpholino phosphorodiamidate oligomer (PMO)/peptide nucleic acid (PNA) internalization peptide 6a (Pip6a), a CPP formed of a hydrophobic core region flanked on each side by arginine-rich domains containing β-alanine and aminohexanoyl spacers. This peptide sequence has the ability to deliver associated cargoes across the plasma and endosomal membranes and is stable to serum proteolysis. In preclinical studies, it was shown that Pip6a conjugation to a PMO significantly enhanced ASO delivery into striated muscles of mice following systemic administration compared to unconjugated PMOs [9].

Peptide–oligonucleotide conjugates can be generated by post-synthetic coupling of the peptide and oligonucleotides after independent solid-phase syntheses steps. Alternatively, successive solid-phase assembly can be carried out on the same solid support. The latter holds advantages in terms of purification, yet the non-compatible syntheses methods of the two molecules has so far limited the

development of routine, practical methods [10]. Furthermore, often due to the structure and solubility of the peptide, post-synthetic peptide coupling generates a poor yield, while highly basic compounds interact with anionic oligonucleotides, impeding efficient conjugation. For further discussion of peptide-oligonucleotide conjugation strategies, see Klabenova *et al.* [11].

Antibody conjugation

Antibody–oligonucleotide conjugates (AOCs) are a novel and expanding class of synthetic chimeric biomolecules. Although antibodies are already established as delivery vehicles for other therapeutics, their application for NAT delivery is still in the early stages of development. Highly specific interactions between an antibody and a cell surface receptor have the potential to enable delivery to tissues and cells that are not accessible using other technologies. Various receptors have been targeted successfully for NAT delivery, such as the transferrin receptor (TfR1) that is highly expressed in cardiac and skeletal muscle. Initial pre-clinical focus has included conjugation of siRNAs to a full-length TfR1 monoclonal antibody in the context of myotonic dystrophy (DM1) and *DMPK* gene knockdown [12]. In parallel, smaller antibody fragment conjugates against TfR1 have been optimized, with the aim of reducing protein dose and toxicity [12]. More recently, the same receptor has proven to be a realistic target for central nervous system delivery from systemic administration. By engineering a lower-affinity Fc antibody domain conjugate, presence of TfR1 at the blood-brain barrier results in efficient on-target efficacy of ASO gene knockdown in the brain, with considerable promise for multiple central nervous system disorders [13].

For oligonucleotide-antibody conjugation, options include click chemistry or thiol–maleimide linkages. A standard method involves partially reducing the disulfide bonds of the antibody with tris(2-carboxyethyl)phosphine to generate a reactive cysteine and then conjugating with a maleimide linker-containing oligonucleotide. The reverse can also be performed by synthesizing the thiol-modified oligonucleotide on an automated synthesizer using standard phosphoramidite chemistry followed by conjugation with the antibody using heterobifunctional cross-linking reagents such as succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

METHODS OF CHEMICAL CONJUGATION AND LINKER CHEMISTRY

Of the various methods used for oligonucleotide conjugation, many focus on amide bonds, disulfides, and click chemistry. Amide conjugations can be performed using conventional amide coupling methods. Some commonly used reagents for amide coupling are 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU), and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The disulfide linkages can be synthesized by the reaction between two free thiol groups and are designed to be cleaved in the reductive environment of the endosome, facilitating efficacy. Disulfide bridges can be used specifically for peptide–oligonucleotide conjugates, where a cysteine containing amino acid can be used as a handle. Thiol linkers are commercially available for attachment to oligonucleotides at the 5′-end during solid-phase synthesis and can also be attached to the amino linkers. Thiol functionality is used for conjugation with maleimide-derived peptides via the thiol-ene click reaction that is widely applicable for labelling proteins; this reaction proceeds without a catalyst in aqueous buffers and results in covalent stabile linkages [14]. Due to its specificity and efficiency, click chemistry is also considered to be a useful

method for the generation of bioconjugates. Azide–alkyne click reactions with or without copper catalysts have the advantage of being performed in aqueous buffers at room temperature, thus making them biocompatible [15]. Copper-free click chemistry is the desired method, as copper-based catalyst can be difficult to remove post-reaction and can cause cytotoxicity in biological systems. Linkers for introducing an alkyne- or dibenzo-cyclooctyne group during oligonucleotide synthesis are available commercially and ligands can be easily derivatized for click reactions [16]. Azides and alkynes can be attached to terminal hydroxyl groups with phosphoramidite reagents or by coupling to amine linkers. Alkyne functionalities tethered to C5 of uridine are also employed for click reactions at the nucleobase.

Of note, the linker between the oligonucleotide and the conjugating partner is of great importance with regards to biophysical properties and can play an important role in the PK of the conjugate. Indeed, release from a bulky conjugate is often necessary for liberation of the NAT into the endosomal system (Figure 1). Click chemistry and the formation of amide bonds, for example, through the active ester method, yield covalent conjugates that will not be readily cleaved. These are used in cases when a more stable linkage is preferred where no dissociation from the carrier is necessary after successful intracellular uptake. The length of the linker is also crucial for successfully attaching larger molecules to oligonucleotides; longer linkers are often used in order to avoid a negative steric impact. Due to the high lipophilicity and poor solubility of the alkyl chains in aqueous buffers, this type of linker has a tendency for aggregation. Hence, PEG linkers are preferred for tethers exceeding about eight to ten atoms. In summary, the role of linkers is not limited to the attachment of two entities; composition, length, and other design features can greatly influence efficacy and PK/PD properties of the NAT cargo.

SIGNIFICANCE OF OLIGONUCLEOTIDE CONJUGATION FOR CELLULAR DELIVERY, INTRACELLULAR TRAFFICKING, AND SAFETY

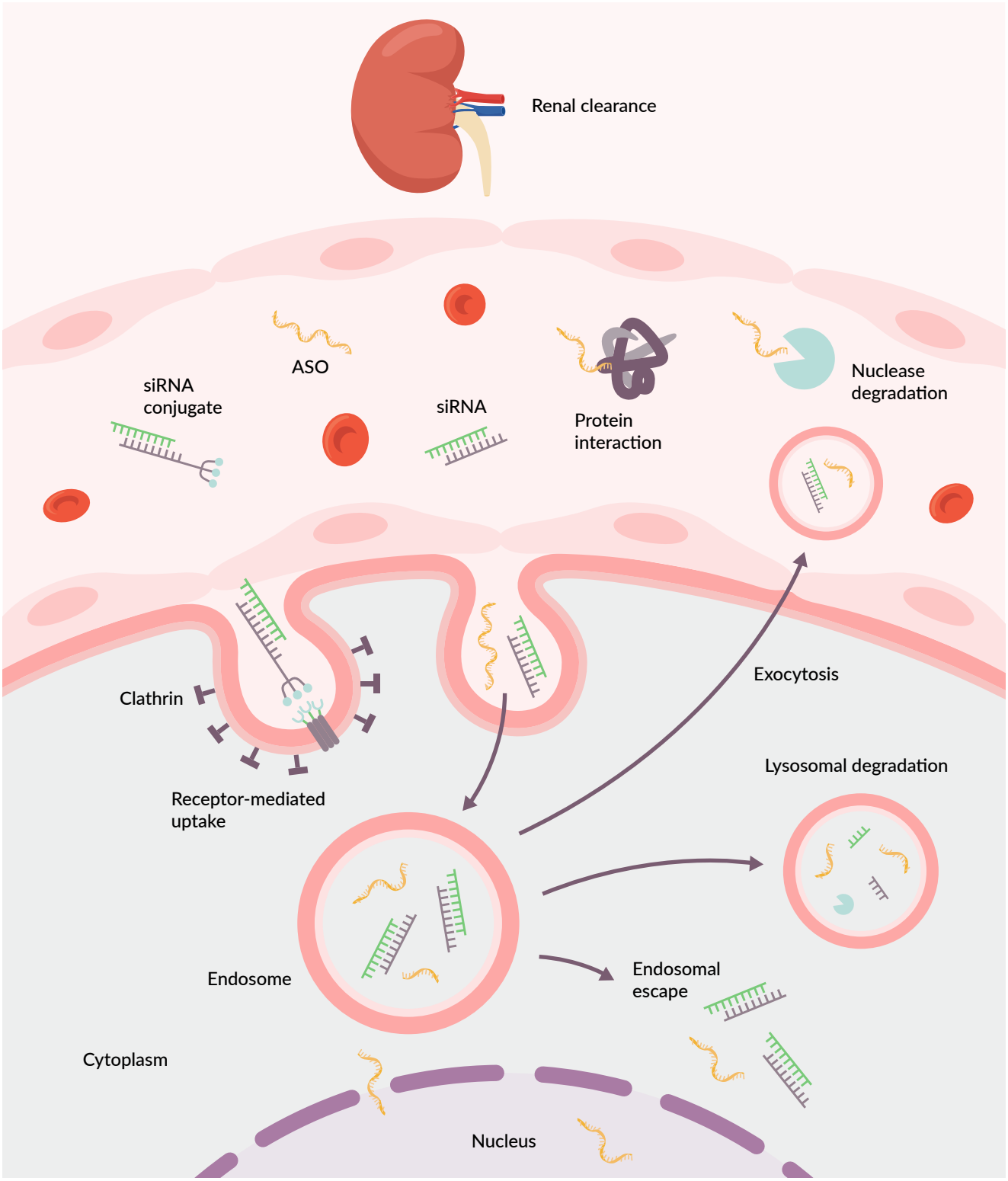
Delivery

It is acknowledged that some of the fundamental limitations of oligonucleotide therapeutics include the targeted delivery to tissues and efficient intracellular processing; the study of novel conjugation strategies is therefore focusing on these two critical areas [17]. An ideal conjugate will not only facilitate cellular uptake, but also provide selectivity to the organ of interest; this is particularly important if the target gene is widely expressed with an essential biochemical function. Other features include the complexities of systemic delivery to the ultimate fate of the NAT, where it needs to avoid nuclease degradation, unproductive sequestration by plasma proteins, and renal clearance (Figure 2). Of the small percentage of the NAT that evades these mechanisms, endocytic cellular uptake will be dependent on the type of conjugate employed.

Somewhat surprisingly given the advanced nature of the NAT industry, some of the molecular mechanisms underlying oligonucleotide cellular uptake are not fully understood. As introduced above, conjugates that target receptor-mediated uptake are an attractive option, whereby the endocytic mechanism and cellular specificity is known or can be tested empirically. For 'naked' ASOs with no conjugate, certain scavenger receptors play a role in facilitating uptake, although the size and charge of siRNAs render them highly inefficient for gymnotic uptake without assistance of a lipophilic molecule or ligand. For CPPs, detailed imaging and biochemical studies propose a number of internalization mechanisms, dependant on the charge properties of the conjugate. Typically containing arginine or lysine residues, CPPs can penetrate cellular membranes by interaction with

FIGURE 2

General cellular mechanisms of intracellular oligonucleotide delivery from the circulation.



ASOs or siRNAs need to avoid sequestration by proteins, nuclease digestion, and clearance by the kidneys for endocytic uptake; an example of receptor-mediated uptake of a triantennary $GalNAc_3$-conjugated siRNA is shown, in addition to the basic intracellular trafficking steps required for ASO/siRNA productivity. ASO: antisense oligonucleotide; siRNA: small interfering RNA.

negatively charged molecules on the surface, until a build-up begins to disrupt the lipid bilayer, forming pores for entry. Alternatively, endocytosis can occur via an energy-dependent mechanism, whereby accumulation of CPPs results in membrane invagination, with dynamin-mediated constriction of the clathrin or caveolin-coated vesicle that enters the endosomal pathway (Figure 2). Understanding these mechanisms is important; however, they do not address the issue of tissue-specific delivery, likely the most important topic for the NAT field to address in the short-term [18]. It is not the case for all applications, where direct delivery by injection—such as the intravitreal route to the eye—has made possible approved ASO therapies that lack any targeting conjugation [19].

Intracellular trafficking

Once internalized, NATs are transported to the early endosomal system, where a shift in luminal pH to the late endosomal state and subsequent membrane remodeling facilitates escape to the nucleus or cytoplasm (Figure 2). A majority, however, is likely degraded in lysosomes or maintained in vesicles and exocytosed. As such, conjugation or linker/conjugation combinations that can hijack this process to improve NAT productivity are being investigated. Non-cleavable linkers may provide the advantage of stability, with the NAT avoiding premature release in the circulation. Alternatively, cleavable linkers can provide some element of control over the disassembly of the conjugate from the oligonucleotide that might provide improved productivity. Well-studied examples include chemically cleavable linkers that are sensitive to the pH reduction in endosomal system, and recent advances are acid labile phosphoramide linkers that can be incorporated with an oligonucleotide during solid-phase synthesis [20]. CPP design can also be modified to directly enhance endosomal escape. Cationic sequences were thought to influence endosomal membrane destabilization or even effect luminal pH by acting as a proton 'sponge', however, recent quantitative studies do not support this [21]. More recent work has combined cyclic CPPs as an optimized endosomal escape vehicle (EEV) that show some improved exon-skipping efficacy *in vivo* [22]. In addition, a small molecule that shifts the accumulation of ASOs to a potentially more permeable extra-lysosomal compartment is reported to significantly enhance on-target potency [23]. Whether direct disruption of such fundamental aspects of intracellular trafficking biology will be detrimental to other cellular pathways in the longer-term remains to be investigated. For an excellent up-to-date summary of current approaches, see Dowdy [24].

Future approaches may focus on nuclear delivery and whether enhancing this important process is possible by novel ASO conjugation strategies. Recent work has tested short nuclear localization signals (NLSs) as peptide-ASO conjugates [25], while others have focused on more complex self-assembling amphiphilic peptides that form micellar nanostructures with a minimal NLS (KRKR) [26]. Whether such approaches can truly evade the inefficiency of endosomal entrapment of NATs certainly warrants further investigation, or if the intracellular endosomal 'slow-release' of specific NATs may in fact be highly beneficial for longer-term activity. It should also be noted that discussions regarding optimal cellular localization versus pharmacological activity are often limited by the resolution of imaging methods, in particular where oligonucleotides have been fluorescently labelled for detection and on-target efficacy data is derived from bulk cells or tissue. More sensitive, higher resolution detection methods will certainly assist more accurate quantification of such parameters [27,28].

Toxicity

With an ever-increasing number of ASO and siRNA products reaching clinical approval and in late-stage clinical trials, attention has

been drawn to improving the safety profiles of these compounds; in particular at the early pre-clinical phase, where the field is still building predictive models of toxicological properties while generating empirical data regarding the underlying mechanisms. It is noteworthy that the drug metabolism and pharmacokinetics (DMPK) of NATs certainly differs from small molecule drugs; indeed, the absorption, distribution, metabolism, and excretion (ADME) profiles of ASOs and siRNAs vary wildly depending on the backbone modification and conjugation type used (summarized in Takakusa *et al.* [29]). As such, the source of adverse toxicological events can be caused my multiple factors; hybridization-dependent off-target or over-active on-target gene regulation, as well as hybridization-independent mechanisms including sequestration of cellular RNA-binding proteins or immunostimulatory effects such as activation of the Toll-like receptor (TLR)9 by cytosine guanine (CpG) motifs [4]. NAT conjugations play a key role here, where careful consideration of ADME profiles in a compromized disease state may be critical to predict safety, although access to such models is not always straightforward or even practically possible.

Focusing on the role of NAT conjugates, no CPP-conjugated drugs have been approved by FDA to date; this is in part due to their rapid liver and renal clearance that limits target engagement. In addition, toxicity can be driven by the inherent positive charge of arginine-rich CPP content. This is improved by moving away from certain cationic properties, such as replacing a (RRRRRRRRRR) peptide with a (ACSSSPSKHCG) sequence [30], yet only now are the first molecular mechanisms being put forward to explain the cytotoxicity observed. In a recent study, it was demonstrated that cellular nucleic acids bind with high affinity to arginine-rich peptides, displacing multiple fundamental RNA- or DNA-binding factors that in turn influence metabolic pathways and contribute to immunogenicity [31]. With reference to GalNAc conjugations, their use in the clinic has facilitated retrospective analysis of safety and tolerability data from phase I trials. Given the increased potency of triantennary $GalNAc_3$ conjugated ASOs as compared to unconjugated ASOs of the same sequence, lower doses have been required for equivalent efficacy. Across several dose-ranging trials of both ASO types, the only significant increase in alanine transaminase (ALT) enzyme levels was observed at the higher doses of unconjugated ASO versus placebo. Furthermore, subcutaneous delivery of $GalNAc_3$-conjugated ASOs resulted in a 30-fold reduction in local cutaneous reactions compared to unconjugated ASOs; overall, these data support advances in both on-target activity and safety using oligonucleotide conjugation [32].

FUTURE OPPORTUNITIES

One strategy gaining considerable influence is the application of artificial intelligence and machine learning (ML) approaches for the high-throughput analysis of structural and experimental biology datasets to generate predictive models for conjugate design. For example, in the CPP field, there has been a need to expand the options beyond adapting natural protein fragments or focusing on cationic or amphipathic properties alone. Computational modelling has begun to predict new CPP sequences by integrating published data; however, the lack of standardization between independent, heterogeneous cellular uptake studies has limited this approach. As such, many *in silico* CPP databases provide valuable predictions, although they will require considerable empirical testing and experimental optimization [33]. Future work will thus benefit from focused, well-controlled benchmarking to train ML algorithms in the context of a specific lead oligonucleotide, with the parallel consideration of efficacy data and immunogenicity being essential for optimal pre-clinical predictive value.

One alternative approach is to identify novel protein sequences that would be predicted to internalize cells via interactions with known cell-specific surface receptors; these peptides may be related to the structure of known ligands, or to novel regions of the receptor that would permit 'bystander' internalization while avoiding aberrant receptor antagonism [34]. Again, this type of approach may generate many hundreds or thousands of potential conjugates for a given cell-type; this not only raises the issue of biological testing, but also how such peptide-oligonucleotide libraries can be synthesized at-scale. Examples include a 13-amino acid neurotensin peptide that binds with high affinity to the sortilin receptor, although efficacy was only enhanced by a small degree in the brain [35]. More recently, Altin *et al.* have developed an *in vitro* platform for performing complex proteomic assays against customizable targets by using DNA-barcoded peptides. Starting with a pool of DNA oligonucleotides encoding peptides of interest, this protocol outlines a fully *in vitro* and massively parallel procedure for synthesizing the encoded peptides and covalently linking each to a corresponding cDNA tag [36]. These types of high-throughput approaches are likely to be the future for large-scale screening pipelines.

Beyond targeted RNA degradation by ASOs or siRNAs, the fact that DNA and RNA molecules can interact highly specifically with transcription factors and RNA binding proteins opens up a completely new array of translational opportunities. For example, conjugating proteolysis-targeting chimera 'warheads' to a protein-binding oligonucleotide could be utilized for degradation of a specific transcription factor in the context of a disease. Such studies are in early development; however, there is much to learn regarding sequence optimization, linker chemistry options, and the practicalities of using such a compound in the clinic [37]. Yet, as this example demonstrates, the inherent properties of nucleic acids will often define quite readily the targeting aspect of a new drug concept; indeed, finding active molecules during the early preclinical phase may not be the rate-limiting step to translation. The challenge for the field is converting efficiently those leads into compounds with suitable PK/PD and safety profiles, combined with on-target efficacy in the specific cells or tissues of interest. Here is where diligent NAT conjugation and linker research and development—at both the chemistry and biology levels—is essential for the future success of oligonucleotide therapeutics.

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AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: LNPs

Regulatory strategies for developing and manufacturing RNA-LNPs

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"...it is particularly important to understand the technical and regulatory support that each equipment vendor is willing to provide when you select their manufacturing equipment to formulate your RNA-LNP."

VIEWPOINT

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CONFLICTS IN REGULATORY PRECEDENCE

When the dust of filing the COVID-19 vaccines settled, it was obvious that there was a difference in RNA-LNP terminology, and it depended upon which Regulatory Authority you asked. As a prime example, the differences surrounding the regulatory framework for the lipids used to form the LNPs in Comirnaty™ and Spikevax™ were classified differently by the US FDA and EMA. The lack of consistency not only caused confusion in the industry, but it provided an opening for individuals to raise political opposition to the COVID-19 vaccines themselves. Since there wasn't a united Regulatory Authority front, not every member of the general population was able to trust such a novel technology, and an unnecessary distrust of RNA-LNP technology was allowed to take root and grow.

Obviously, the US FDA isn't the only Regulatory Authority out there. Europe has the EMA, China has the NMPA, and so on. I was discussing Regulatory Strategy, specifically involving the building blocks (i.e., lipids) of the LNPs, with my colleague, Laura Moreno of Curapath. Laura said, "It seems there's a bit of uncertainty surrounding the regulatory framework for novel functional excipients, like ionizable lipids, which are increasingly utilized in pharmaceutical formulations. From what I've observed, manufacturers often face challenges in determining which regulatory standards to adhere to, sometimes opting for guidelines intended for active pharmaceutical ingredients (APIs). It would be helpful to address this ambiguity and establish a unified approach to regulatory requirements for functional excipients. By doing so, it would provide manufacturers with clearer guidance, ultimately ensuring the safe and effective incorporation of these substances into pharmaceutical products."

This ties back to the differences in terminology/classification of the lipids used in Spikevax and Comirnaty. In the FDA approval that Spikevax received, the polyethylene glycol (PEG) lipid, and the ionizable lipid are not classified as excipients. Instead, they are listed as starting materials related to the API (i.e., the mRNA). In the EMA approval that Spikevax™ received, the polyethylene glycol (PEG) lipid, and the ionizable lipid are classified as excipients. Not only that, but they were functional lipids given the designation of novel excipients. In the FDA and EMA approvals that Comirnaty™ received, both regulatory authorities accepted the classification that the mRNA was the API, and the lipids were broken into two categories, excipients and novel excipients. This is important because there are increasing amounts of characterization work and safety data that the drug sponsor will have to provide associated with starting materials, functional/novel excipients, and APIs.

GAPS IN GUIDANCE

There is a certain degree of consternation that gaps in knowledge and a lack of precedence can create. Guidance from regulatory authorities has fallen behind the technology, and it fell behind a while ago. The technology development associated with non-viral drug delivery systems has outpaced the general regulatory guidance publications, and that uncertainty can also cause anxiety during drug development. For example, in April 2018, the FDA published their guidance document, *Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation*. By that point, there had been liposomal drug products on the market for over two decades. In 2012, I started working at a facility that manufactured commercially approved orphan drugs. That is when I learned of Abelcet®, a commercially approved drug product classified as a lipid complex. Abelcet received FDA approval in 1996, roughly 22 years before the FDA guidance appeared. What does this tell us? That it is possible to develop and

commercialize a lipid-based DDS without official general guidance documents [1].

However, it must also be noted that the 2018 guidance on liposome drug products does not include LNPs. Grant Henderson of Vernal Biosciences put it nicely when he said, "The majority of guidance documents and regulations were written before the recent growth in mRNA-LNP medicines. A review of guidance documents to clarify and ensure applicability to mRNA products (and in some cases, biologics in general) would certainly benefit the industry. For example, many of the methods that are being applied to mRNA-LNP medicines don't fit nicely into an ICH Q2 or Q14 framework, adding uncertainty and potentially complicating development and qualification."

I was speaking with my colleagues Christian Cobaugh and Grant Henderson of Vernal Biosciences about mRNA-LNP formulation characterization. Christian said, "It's important to note that it is incumbent on sponsors, not the FDA, to develop and defend a control strategy *vis-à-vis* quality control (QC), instead of the other way around. The regulator may have basic expectations that a sponsor knows how to develop and apply phase-appropriate control strategies, but most authorities that approve clinical trials and authorize market approvals have not changed their general guidelines specifically for mRNA medicines." Grant said, "I believe that the FDA response and participation in the development of COVID vaccines should not be viewed as a permanent change, and unfortunately, it was that experience that is still providing guidance for the RNA industry as it exists today. A return to pre-pandemic norms would be beneficial, both in terms of CMC as well as in terms of expectations around program deliverables and timelines, which are still being held in comparison to the pandemic era."

They both bring up good points. I think there is a wide misconception that the regulatory authorities are the experts in biopharmaceutical technology, drug development, and commercialization. They are not; they are the experts of the regulations. This is not to say that the FDA shouldn't be consulted with as a resource. Quite the opposite, the FDA should be contacted early and frequently during the application process, because they are there to provide guidance. However, it is up to the drug sponsor and CRDMO to develop and establish an agreed upon Quality Target Product Profile (QTPP). It is up to the drug sponsor to provide justification of safety and efficacy. The level of FDA involvement with the COVID-19 vaccines is the exception to the rule, and it has caused a skewing of expectations related to product development and speed to the clinic that can be difficult to reconcile with drug sponsor and investor expectations.

QTPP development and characterization of these different types of ATMP should be thought of on an individual product basis. The method/rationale of establishing specification limits for an individual platform technology will not carry across all platform technologies. It will vary from product to product. The most frequent answer I give is, "That is formulation-specific." That is why it is up to you to make the most of your time when meeting with the regulatory authorities. I also say that when Regulatory Affairs and Technical Support Teams have their scheduled meetings with the regulatory authority, invite your corresponding team member from the CRDMO that you are using (if you are using one). I think it is important to have those associated with cGMP manufacturing activities be able to directly hear it from the horse's mouth, so to speak. Everyone needs to be aligned when moving forward. You also need to be transparent with the regulatory authority. "We are not enemies, but friends." Believe it or not, the regulatory authorities want you to be successful. So, don't be afraid to consult with them and ask them about the approach to classification of Critical Quality Attributes (CQA).

When working through establishing CQAs, Christian advises that you ask the

regulatory authority, "When it comes to a quality attribute with a poorly understood relationship between dosing (route of administration, dose level) and safety, and that is challenging to quantify, help us understand the strategy around setting a specification." That is a sound approach because you are being upfront about what you do and do not know. The regulatory authority can then inform you of the phase-appropriate expectations for your drug substance/drug product because there are still things that we don't have answers for in an FDA general guidance for industry. Some answers you won't know until you step into the arena. Christian went on to give this example. "Many of the product-related impurities are poorly understood, including associated safety-related effects. Additionally, the specific compositions of those impurities is often unknown, making it difficult to create accurate reference standards. For example, cell-based studies, animal studies, and our understanding of RNA viruses suggests that double-stranded (ds) RNA, a process-related impurity common to most *in vitro* transcription processes, is a safety risk. However, regardless of a target product profile, it's nearly impossible to empirically state how much dsRNA is unsafe. Further clouding the dsRNA issues, several different types of dsRNA have been described in scientific literature, but the test methods common to a QC lab setting are unable to accurately describe which types are present in any given sample. So, we start this chicken-and-egg problem that prevents us from developing a reference standard that accurately 'describes' the impurities."

ADVANCING TECHNOLOGY AND CPPS

Ultimately, your RNA-LNP regulatory strategy will need to meet or exceed the requirements as established by the regulatory authority of your target market. If you are going into multiple markets, you will need to relay that information to all members of your supply chain and document it in the CMC section of your submissions. I was speaking with my colleague, Lin Jin of CATUG Biotechnology, about the dynamic between meeting both client and regulatory authority expectations. Lin said, "When we start getting ready to manufacture GMP batches, we are consistently having biopharma companies coming and wanting to discuss batch-to-batch variations, potency assay variations, and LNP stability. So, you address that. The NMPA calls for more extensive biophysical characterization than other regions—for example, performing cryo-EM for LNP development. Apart from physiochemical properties, potency studies are also required for stability evaluation. We had some clients' programs back in 2021 that required *in vivo* release data, which still holds true for those programs. So, you have to be able to customize your CMC strategy and pay attention to process controls."

There are a lot of new ways to manufacture nanoparticles at the development and cGMP scales that did not exist prior to the COVID-19 pandemic. For example, **Table 1** shows the results of a survey of the commercial nanoparticle manufacturing equipment market from April 2024. There are Critical Process Parameters (CPPs) associated with the control of each of these systems. Understanding their impact on the quality and efficacy of the drug substance/drug product will need to be fully defined when progressing through the development and scale-up cycles, especially when switching from one 'technology type' to another. Thus, it is particularly important to understand the technical and regulatory support that each equipment vendor is willing to provide when you select their manufacturing equipment to formulate your RNA-LNP. This information will need to be documented in the CMC section of your submission.

TABLE 1

Commercially available lipid nanoparticle (LNP) manufacturing equipment.

Company	Model	Technology	R&D/cGMP	Max flow rate	Min/max volume	EE
Avanti	Mini	Extrusion	R&D	*	0.025 mL/1 mL	*
CDMO	In-house[1]	Solvent injection	R&D/GMP	As needed	As needed	*
CDMO	In-house[1]	Extrusion	R&D/GMP	As needed	As needed	*
DIANT	LARU	Solvent injection	R&D	400 mL/min	20 mL/*[2]	*
DIANT	LiFT	Solvent injection	cGMP	2 L/min	*/*[2]	*
DIANT	LiFT HP	Solvent injection	cGMP	20 L/min	*/*[2]	*
Evonik	Lipex	Extrusion	R&D/cGMP	*	1 mL/>100 L	>90%
FDX Fluid Dynamix	EUREKA	FDmiX solvent injection	R&D	100 mL/min	2 mL/100 mL	>95%
FDX Fluid Dynamix	Curiosity	FDmiX solvent injection	R&D	16 mL/min	0.5 mL/10 mL	>95
FDX Fluid Dynamix	Curiosity Multi-Use	FDmiX solvent injection	R&D	16 mL/min	0.5 mL/10 mL	>95
FDX Fluid Dynamix	FDmiX Platform	FDmiX solvent injection	cGMP	>1 L/min	0.5 mL/*[2]	>95
Fluigent	Nanoparticle Production Station	Microfluidic	R&D	*	*/*	*
GEA	Xstream Lab Homogenizer 1000	Homogenization	R&D/cGMP	9 L/h	*/*	*
GEA	Xstream Lab Homogenizer 2000	Homogenization	R&D/cGMP	20 L/h	*/*	*
GEA	Ariete Series	Homogenization	R&D/cGMP	>1,500 L/h	*/*	*
GEA	One Series	Homogenization	cGMP	10,000 L/h	*/*	*
GEA	Plug & Play Pharma Skid	Homogenization	cGMP	1,100 L/h	*/*	*
G&G Technologies	Custom	Solvent injection	cGMP	*	*/*	*
Helix Biotech	Nova BT	Solvent injection	R&D	200 mL/min	0.1 mL/60 mL	*
Helix Biotech	TWIST	Extrusion	R&D	*	0.025 mL/1 mL	*
Inside Therapeutics	Custom	Microfluidic	R&D	30 mL/min	0.025 mL/0.25 mL	>90%
Inside Therapeutics	TAMARA	Microfluidic	R&D	*	0.2 mL/10 mL	>95%
Knauer	IJM NanoScaler	Solvent injection	R&D	50 mL/min	1 mL/10 L	>90%
Knauer	IJM SingleCore NanoProducer	Solvent injection	cGMP	60 L/hr	10 L/100 L	>90%
Knauer	IJM DuoCore NanoProducer	Solvent injection	cGMP	120 L/h	10 L/>1,000 L	>90%
Knauer	Custom	Solvent injection	cGMP	*	*/*	*
Leon Nanodrugs	NANOlab	Solvent injection	R&D	500 mL/min	1 mL/*[2]	*

*Unknown due to mixed reports or a lack of information/data published.
[1]Nonproprietary equipment created by the manufacturing site to facilitate technology transfers into their facilities.
[2]Marketed as a continuous manufacturing process, not on a per batch basis.
[3]The flow rate is preset and not adjustable.
[4]Upgrade to the GMP unit available that allows for max flow rate=1.6 L/min.
[5]Maximum amount per mixing module.

TABLE 1 (CONT.)

Commercially available lipid nanoparticle (LNP) manufacturing equipment.

Company	Model	Technology	R&D/cGMP	Max flow rate	Min/max volume	EE
Leon Nanodrugs	NANOme	Solvent injection	cGMP	120 mL/min	200 mL/1.2 L	*
Leon Nanodrugs	NANOus	Solvent injection	cGMP	1.2 L/min	*/*[2]	*
Microfluidics	LM10	Homogenization	R&D	600 mL/min	30 mL/*	*
Microfluidics	LM20	Homogenization	R&D	90 mL/min	14 mL/*	*
Microfluidics	M110P	Homogenization	cGMP	120 mL/min	50 mL/*	*
Microfluidics	M110EH	Homogenization	cGMP	450 mL/min	120 mL/100 L	*
Microfluidics	M815	Homogenization	cGMP	1.2 L/min	1.5 L/*	*
Microfluidics	M7125	Homogenization	cGMP	7.56 L/min	5.0 L/*	*
Microfluidics	M7250	Homogenization	cGMP	15.12 L/min	5.0 L/*	*
Micropore	AXFmini	Micromixing	R&D	150 mL/min	1 mL/10 L	>90%
Micropore	AXFone	Micromixing	cGMP	200 L/h	5 mL/>100 L	>90%
Micropore	AXFn	Micromixing	cGMP	1,500 L/h	30 mL/>1,000 L	*
Micropore	AXF Pathfinder Series	Micromixing	R&D	200 mL/min	0.2 mL/5L	*
PNI	Spark	NxGen™ mixing	R&D	N/A[3]	0.025 mL/0.25 mL	>90%
PNI	Ignite	NxGen mixing	R&D	200 mL/min	1.0/60 mL	>90%
PNI	Blaze	NxGen mixing	R&D	115 mL/min	10 mL/1 L	>90%
PNI	Blaze+	NxGen mixing	R&D	115 mL/min	10 mL/10 L	>90%
PNI	GMP System	NxGen mixing	cGMP	200 mL/min[4]	10 mL/*	>90%
PNI	Commercial Formulation System	NxGen mixing	cGMP	48 L/h	0.5 L/400 L	>90%
PNI	Modular Commercial Formulation Skid	NxGen mixing	cGMP	48 L/h	*/400 L[5]	>90%
PreciGenome	Flex-S	Microfluidic	R&D	4 mL/min	0.1 mL/2 mL	>85%
PreciGenome	Flex-M	Microfluidic	R&D	5 mL/min	1 mL/12 mL	>85%
PreciGenome	PRO	Microfluidic	R&D	20 mL/min	2 mL/200 mL	>85%
PreciGenome	Max RUO	Microfluidic	R&D	4.8 L/h	50 mL/1 L	>85%
PreciGenome	Max GMP	Microfluidic	cGMP	40 L/h	50 mL/1 L	>85%
PreciGenome	Max+	Microfluidic	cGMP	>10 L/h	*/>10 L	>85%
Quantoom Biosciences	Ncapsulate	*	*	*	*/*[2]	*
Unchained Labs	Sunscreen	Microfluidic	R&D	30 mL/min	0.105 mL/2 mL	>90%
Unchained Labs	Sunshine	Microfluidic	R&D	30 mL/min	0.32/20 mL	>90%

*Unknown due to mixed reports or a lack of information/data published.
[1]Nonproprietary equipment created by the manufacturing site to facilitate technology transfers into their facilities.
[2]Marketed as a continuous manufacturing process, not on a per batch basis.
[3]The flow rate is preset and not adjustable.
[4]Upgrade to the GMP unit available that allows for max flow rate=1.6 L/min.
[5]Maximum amount per mixing module.

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NUCLEIC ACID INSIGHTS

FORMULATION AND DELIVERY: LNPS

INTERVIEW

Key challenges and future opportunities for LNP-based delivery

Lipid nanoparticles (LNPs) face a number of hurdles, including diverse challenges and considerations around safety, efficiency, and freedom to operate. **David McCall**, Senior Editor, BioInsights, talks to **Julien Couture-Senécal**, Co-Founder and Director of Azane Therapeutics, about the key remaining questions surrounding LNPs as they continue to gain traction in the nucleic acid delivery space and beyond.

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Q What are you working on right now?

JCS: I co-founded Azane Therapeutics while pursuing my PhD research at the University of Toronto in the Institute of Biomedical Engineering. My doctoral thesis focuses on studying the effect of ionizable lipid chemistry on the reactogenicity and immunogenicity of mRNA vaccines.

I am working in the laboratory of Dr Omar F Khan, an Assistant Professor at the University of Toronto Institute of Biomedical Engineering, the Canada Research Chair in Nucleic Acid

"...some of the most promising ongoing clinical programs are the ones leveraging the adjuvant properties of LNPs..."

Therapeutics, and my Co-Founder at Azane Therapeutics. We started working on new lipid nanoparticles (LNPs) for mRNA delivery while witnessing the impact of mRNA vaccines during the COVID-19 pandemic. Through our research, we discovered potent and chemically diverse ionizable lipids. This motivated us to pursue clinical applications of these delivery molecules through Azane Therapeutics.

Q What are you excited about in the nucleic acid world currently, with particular relevance to utilizing LNPs?

JCS: RNA medicine has the potential to bring new life-altering treatments to patients for various indications, from enzyme deficiencies to autoimmune diseases. LNPs are the leading vehicle for the cytosolic delivery of mRNA due to its labile nature. In my opinion, some of the most promising ongoing clinical programs are the ones leveraging the adjuvant properties of LNPs such as mRNA vaccines against various infectious diseases beyond COVID-19, as well as for cancer.

Another area that I am excited about is *in vivo* gene editing, as many companies are moving away from viral vectors in favor of LNPs. I am looking forward to seeing innovation in this space focused on enabling LNPs to deliver to specific cells and tissues.

Q Turning to some of the challenges that the LNP field faces, firstly, what would you pick out as the key current concerns related to targeted delivery?

JCS: Devising safe and scalable approaches to LNP delivery beyond the liver is a huge challenge for the field. Current strategies for extrahepatic delivery involve conjugating ligands onto the surface of LNPs or incorporating additional lipid components to modulate surface properties. In mice, we know how to skew functional delivery of the mRNA payload between the liver, spleen, and lungs after an intravenous injection. However, we have a poor understanding of how the properties of the LNPs affect biodistribution and toxicity, and how the results that we are seeing in mice translate to humans.

The second big challenge is that when LNPs do reach their target cell type in the right organ, they still need to escape rapidly from endosomes to deliver their payload—the mRNA needs to get into the cytosol where it can be translated into the encoded protein. In other words, it

doesn't matter if you are getting into the right cell if you are not functionally delivering your payload to the cytosol. As a result, in addition to targeting the right cell, more work needs to be done to increase the efficiency of endosomal escape.

Q What about challenges relating to safety?

JCS: A key focus of my research at Azane Therapeutics is the biodegradability of ionizable lipids. There is an unmet need to develop safer and more rapidly biodegradable ionizable lipids. Ionizable lipids are foreign to the body and can cause toxicity as they start accumulating inside cells and activating inflammatory pathways. The rapid clearance of foreign lipid components from the body is a key safety consideration for repeated dosing in chronic disease indications, which may involve multiple injections per week. Another safety challenge of LNPs, specifically for intravenous administration, is to minimize inflammatory responses and eliminate infusion-related reactions without the need to co-administer antihistamines and corticosteroids.

Q And regarding considerations around freedom to operate?

JCS: Key intellectual property litigations on ionizable lipid structures and LNP compositions haven't been resolved yet, so the picture is a little murky in terms of what it means to have 'freedom to operate'. The outcomes of these patent disputes will tell us where we as a field really stand on these issues. I anticipate that future drug products will have unique and differentiated ionizable lipids, as is already the case for Onpattro, Comirnaty, and Spikevax, not only to maximize therapeutic efficacy but also to avoid infringement and minimize licensing costs.

Q Tell us more about Azane Therapeutics and your approach to LNP R&D—what differentiates it?

JCS: The foundational technology of Azane Therapeutics stems from a strong intellectual property portfolio from our research on ionizable lipids at the University of Toronto. Our expertise focuses on the chemistry of ionizable lipids and their structure-activity relationships. Our name, Azane, references our focus on amine-based ionizable lipids.

The optimization of LNPs is a multivariate problem. The ionizable lipid component drastically affects the properties of a given LNP, so I believe it should be optimized for each payload and indication to maximize a drug's therapeutic potential. At Azane, we use a chemistry-based approach to optimized LNPs for each unique application.

"At the moment, it is unknown whether we can truly separate delivery efficacy from the reactogenicity associated with LNPs."

Q What is your vision for the future evolution and application of LNPs?

JCS: The first area of improvement that comes to mind is the efficiency of endosomal escape. In my experience, a small fraction of LNPs that enter a cell deliver mRNA to the cytosol. The challenge is to improve endosomal escape while reducing the body's inflammatory response. At the moment, it is unknown whether we can truly separate delivery efficacy from the reactogenicity associated with LNPs. These are important questions that we are tackling at Azane through the rational design of new, biodegradable ionizable lipids.

Secondly, as the field moves towards more complex approaches like gene editing that require multiple RNA payloads, we need to develop new lipids that efficiently co-encapsulate all necessary RNA components into one LNP. Current studies suggest that a single LNP can only encapsulate single digit copies of mRNA. Increasing the packing efficiency of an LNP will maximize the simultaneous delivery of multiple synergistic components to the same cell.

Lastly, it is critical to improve our understanding of how the surface properties of a nanoparticle impact its protein corona and cellular biodistribution. We have a reasonable understanding of factors influencing ApoE-dependent delivery to the liver, but little is known beyond that with respect to other organs and routes of administration. I think the solution is to use 'first principles thinking' to improve our understanding of how LNP chemistry drives activity.

Q Finally, can you sum up one or two key goals or priorities that you have for your own work at Azane over the foreseeable future?

JCS: I'll be able to share more information later this year, so stay tuned! Right now, our key goal is to solidify a preclinical internal pipeline that leverages the strengths of our LNPs. We are also looking to find the right partners to help accelerate the translation of nanomedicines to the clinic.

AFFILIATION

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NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES: TARGETING & DELIVERY

INTERVIEW

Pushing the boundaries of siRNA targeting and delivery

Although siRNA therapeutics have made significant progress in terms of their stability and safety, key challenges relating to the specificity and efficiency of delivery remain. **David McCall**, Senior Editor, BioInsights, talks to **Merle Fuchs**, Co-Founder and CEO of PRAMOMOLECULAR GmbH, about promising approaches to address this issue and continue expanding the range of applications available to oligonucleotide therapeutics.

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Q Tell us about your background in the nucleic acids space.

MF: I am a molecular biologist by training. I did my PhD at the lab of Nobel Prize winner Manfred Eigen, at the Max Planck Institute for Multidisciplinary Sciences in Göttingen. It was a great place to learn interdisciplinary work, which is something I really love. After my PhD, I worked for nearly 25 years as a consultant for high-tech startups as well as mature companies. I had my own company, and I am co-founder of an additional seven high-tech startups. One of those is BianoGMP, which was recently acquired by EUROAPI. BianoGMP produces

"...we see different organ preferences in the combination of siRNA and lipid-based delivery molecules, with higher organ preference for the lung, heart, and pancreas..."

GMP-compliant therapeutic oligonucleotides. It was through this work that we became aware of the problem of delivery of therapeutic oligonucleotides to locations other than the liver. Around the same time, we also became aware of the research of a particular academic group whose leader spent nearly his whole academic life developing covalent, lipid-based delivery molecules for mononucleotides. Our idea was that if these molecules work for mononucleotides, they may also work for oligonucleotides. We took our own money and started our first animal experiment. We only looked at three organs: the liver, lung, and kidneys. With the first delivery molecule we observed a high silencing efficiency in the lung, but not in the kidneys and not in the liver, which was quite interesting.

With these results, we managed to acquire funding from the German Ministry of Economics. The grant is called EXIST-Forschungstransfer and it's the most important and prestigious funding for pre-seed high-tech projects in Germany. With this money, we were able to generate additional data and start PRAMOMOLECULAR in 2021.

Q Can you frame for us the key challenges in targeting and delivery of siRNA therapeutics, both historically and currently?

MF: Historically, the challenges were stability and safety, but these issues have now been resolved. Today, the important topics are endosomal escape, cell entry, transfection rate outside of the liver, and specificity/organ preference.

Q Can you tell us more about PRAMOMOLECULAR and its focus?

MF: 'Pramo' means 'ferry' in Esperanto, and our name was chosen because we utilize covalent lipid-based 'ferry' molecules. We couple defined lipids via defined chemical bonds directly to the siRNA, which helps to stabilize the siRNA and to ferry it through the cell membrane. Interestingly, we see different organ preferences in the combination of siRNA and lipid-based delivery molecules, with higher organ preference for the lung, heart, and pancreas, but a comparatively much lower preference for the liver, kidneys and skeletal muscle.

Currently, we are focusing on KRAS mutations in non-small cell lung cancer and in colon cancer for local applications. We have also started to search for siRNAs against heart proteins for use in heart failure, but this work is at a very early phase. We have concentrated initially on

the organs where we see the highest transfection rates. PRAMOMOLECULAR doesn't know precisely why we have this organ preference, but we see it and we use it. Biology still involves an element of alchemy!

Q What differentiates PRAMOMOLECULAR's approach?

MF: I believe we have the most straightforward covalent lipid-based delivery system out there for our target organs, particularly if you compare our approach to our most important technological competitor, DTx Pharma. There is not a lot of data about them available, but what we have seen is that they use several delivery molecules per each siRNA, and we only need one. They are able to target neuromuscular disorders via neuroproteins, and we address these proteins in our three target organs. DTx was bought by Novartis last year for US$500 million fixed with a potential further US$500 million in milestone-dependent payments. We think we also have a very interesting approach and could build a similar story.

Q Looking across the wider oligonucleotide field today, where do you currently see exciting innovations that can expand the application and the success of these technologies in the therapeutic setting?

MF: I think covalent delivery is the cutting edge at the moment. There had been lots of siRNA approaches using nanoparticulate systems, but if we look at the technologies that are in the clinical phases, most of them are either naked siRNA, or a combination of covalent delivery molecule and siRNA. I believe we will also see more and more innovation in the local delivery applications because we see that even here, delivery is a problem. It is not enough to directly introduce your drug into the organ. For example, at the beginning of this year, the US FDA didn't accept the data from Sylentis' Phase 3 clinical trial of a naked siRNA against dry eye disease due to inefficiency. We think that even for these local applications, you should use a delivery molecule. Our technology's particular advantage is that it makes it easy to do this. Our delivery molecules can be coupled as an additional phosphoamidite during the last step of oligonucleotide synthesis, and it is very easy to test whether you can increase the transfection rate, even for local applications.

There are lots of efforts underway utilizing lipid-based delivery molecules. For example, at the end of 2022, Alnylam Pharmaceuticals published a *Nature Biotech* paper where they showed data from their own lipid-based delivery molecule. Most of the data addressed the central nervous system, but they also had a dataset where they addressed the lung. We compared their data with our own. For the biodistribution data, we used non-optimized siRNAs, and Alnylam used highly optimized siRNAs. They were able to show, after systemic application, a silencing rate of 40% whereas we, with our non-optimized *in vivo* systemic application,

"...our proprietary linker sterically aligns the siRNA and lipids in a particularly favorable way for membrane transition."

showed 50%, which would suggest that our molecule may be the more efficient of the two. We believe that our proprietary linker sterically aligns the siRNA and lipids in a particularly favorable way for membrane transition.

Q And looking to the future, what for you will be some key directions for innovation in the oligos field, both in terms of technology platform/modality development and specific indications?

MF: In the beginning, nearly everybody in the field concentrated on orphan diseases but today, we see lots of other diseases being approached. Cancer is a hot topic, but poses a lot of problems. We have seen clinical data from cancer trials—for example, from Silexion Therapeutics (formerly Silenseed). They had a local approach and were not able to show high efficiency, but I believe they are working to improve their delivery technology. Cancer will be an increasingly key focus moving forward.

Very recently, the University of Nashville, TN, published another delivery approach based on lipid-based, non-particulate delivery systems in *Nature Communications*. Like us, they covalently couple long-chain lipids to siRNA via a chemical linker. But they also use several hydrophilic 'spacers'. In three xenograft mouse models of human triple-negative breast cancer, they achieved an impressive reduction in tumor growth by silencing the MCL-1 mRNA. I also wonder how many innovations in the field of *N*-acetylgalactosamine (GalNAc) are possible. Lots of molecules have entered clinical trials using the GalNAc system and this will remain a hot topic. And there are a number of associated approaches to optimize endosomal escape, but I don't think any of those have entered first-in-human trials as yet.

Q Finally, can you sum up one or two key goals that you have for your own work/role over the foreseeable future?

MF: We are still in a quite early preclinical phase, but we are now concentrating on strategic preclinical development, with an aim of entering the clinic in 2026.

My most important job at this stage is to communicate. We are very interested in partnerships with both small and medium-sized enterprises but also with big pharma. My core responsibility is to find enough money to finance our approach.

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AUTHORSHIP & CONFLICT OF INTEREST

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