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NUCLEIC ACID INSIGHTS

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PRECLINICAL AND TRANSLATIONAL TOOLS AND TECH

SPOTLIGHT

REVIEW

Reaching the CNS with therapeutic oligonucleotides via TfR1: navigating delivery and distribution challenges

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Oligonucleotides have emerged as a major therapeutic modality, with several approved drugs and a growing pipeline. Historically, broader adoption was limited by poor tissue uptake, but advances in delivery technologies have largely overcome these barriers for hepatic targeting. However, extrahepatic delivery, particularly to the central nervous system (CNS), remains a significant challenge. Oligonucleotides cannot cross the blood–brain barrier (BBB) and are typically administered intrathecally, an invasive procedure often resulting in restricted distribution and suboptimal outcomes. Increasing interest in oligonucleotide therapies for CNS disorders has spurred efforts to enable systemic delivery. Oligonucleotide conjugation to vehicles leveraging transferrin receptor 1 (TfR1) for BBB crossing is emerging as a leading CNS delivery strategy. Given the brain's extensive vascularization, this approach enables homogeneous distribution, reaching regions inaccessible via intrathecal injection. Here, we summarize the landscape of oligonucleotide therapies for CNS disorders and efforts to enable BBB crossing, with a focus on TfR1-based technologies.

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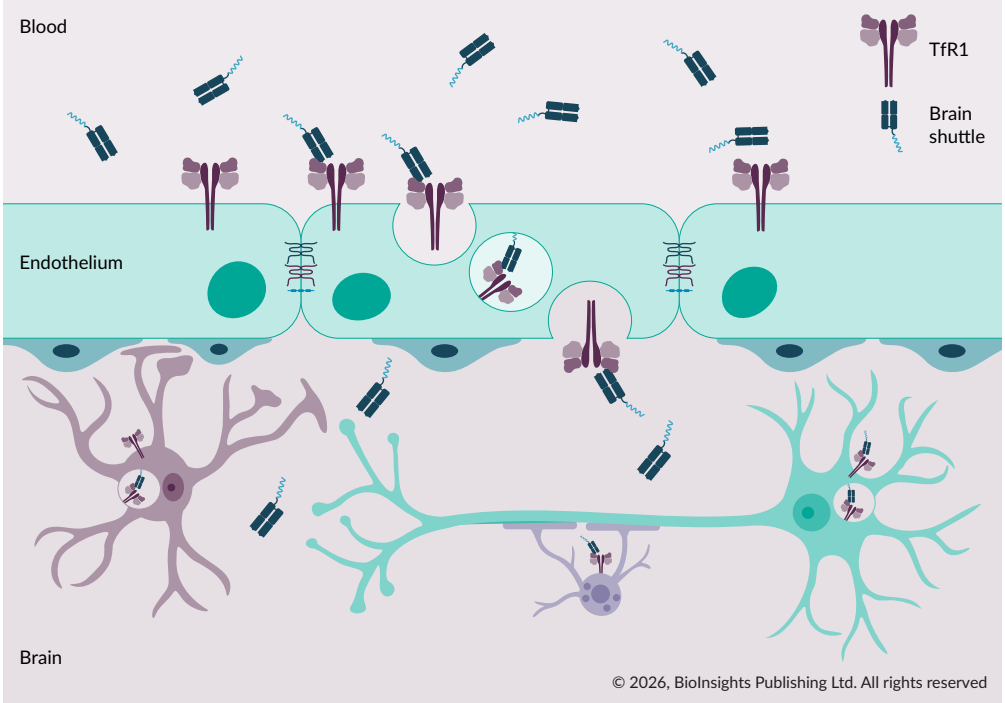
In the evolving landscape of modern medicine, oligonucleotides have emerged as transformative therapeutics, offering unique specificity in targeting the root causes of disease. Oligonucleotides operate through mechanisms such as RNA interference, antisense inhibition, splice modulation, and RNA editing, enabling them to reach previously 'undruggable' targets. Their versatility has opened doors to treating rare as well as common genetic disorders, cancers, and viral infections [1,2]. However, the clinical applicability of oligonucleotides is significantly constrained by poor tissue distribution and cellular uptake, particularly in extrahepatic

tissues such as the central nervous system (CNS) [3,4]. At present, oligonucleotide therapies for neurological disorders are administered via intrathecal (IT) injection, a burdensome approach that only achieves localized distribution and is applicable to a small subset of diseases. For these reasons, treatment of CNS diseases remains a vast unmet medical need.

Drug delivery to the CNS is challenging due to the blood-brain barrier (BBB), a formidable obstacle to the delivery of most large-molecule drugs and biologics [5]. The BBB is a selective, semipermeable structure lining the microvasculature of the CNS. BBB endothelial cells form tight junctions that limit the transport of hydrophilic molecules between the blood and the CNS [6]. A growing body of research has focused on exploiting receptors and carrier systems that undergo transcytosis across the BBB to enable efficient delivery of macromolecules from the systemic circulation to diverse cell types within the CNS. Among these approaches, platforms leveraging transferrin receptor 1 (TfR1) biology are currently the most advanced for enabling therapeutic access to the CNS

FIGURE 1

TfR1 targeting platforms leverage TfR1 transcytosis to cross the BBB and deliver to brain parenchymal cells.



 The 400–600 miles of vasculature in the brain are part of a specialized, conserved and complex structure, the BBB, that regulates the transfer of molecules from circulation into the brain parenchyma. This barrier allows the passage of small lipophilic molecules (<400 Da in size) but restricts the passage of larger and/or hydrophilic molecules [81]. The BBB is composed of multiple cell types: endothelial cells, vascular smooth muscle cells and astrocytes (or pericytes in capillaries). The endothelial cells at the BBB have a unique, specialized molecular architecture, expressing proteins that form tight and adherent junctions, which restrict the passive flow of molecules from the systemic blood into the brain parenchyma. TfR1 expressed by endothelial cells shuttles from the luminal to the parenchymal to transport cognate ligands across the BBB, whereas TfR1 expressed in brain parenchymal cells quickly cycles between the surface and the recycling endosome to enable ligand uptake. TfR1 biology can be leveraged by central nervous system drug delivery platforms to allow BBB crossing and parenchymal cell uptake of oligonucleotide conjugates. BBB: blood–brain barrier. TRf1: Transferrin receptor 1.

and unlocking treatments for neurological disorders **(Figure 1)**.

Neurological diseases, which affect an estimated 3.4 billion people worldwide – about 43% of the global population – remain a major unmet medical need, as current treatments largely provide symptomatic relief **[7–9]**. Oligonucleotide therapies that directly target the genetic defects underlying neurological disorders hold significant promise for treating neurodegenerative, neurodevelopmental, and neuromuscular conditions. Until recently, direct delivery through IT injection has been the only route of administration to enable delivery of antisense oligonucleotides (ASOs) to the brain **(Figure 2)** **[10]**. This approach has transformed treatment for spinal muscular atrophy (SMA) with Nusinersen, which is designed to boost expression of the protein called survival motor neuron (SMN) by promoting SMN2 exon 7 inclusion, improving patient survival and motor function **[11–13]**. Another example is Tofersen, an IT-delivered gapmer ASO that lowers toxic superoxide dismutase 1 (SOD1), developed for amyotrophic lateral sclerosis (ALS) caused by SOD1 **[14,15]**. Although IT injections achieve high drug concentrations in the spinal cord, accounting for successes in SMA and ALS, this delivery route provides negligible penetration into deep brain regions such as the caudate and putamen **(Figure 2)** **[10,16,17]**. For neurological diseases with widespread or deep brain pathology, the lack of distribution from IT administration can limit treatment efficacy. Indeed, several IT-delivered ASO programs for neurological diseases have been discontinued due to insufficient clinical benefit **[18–20]**.

Challenges with IT administration are even greater for standard siRNA scaffolds, which largely remain at the injection site and fail to distribute effectively **[21]**. To overcome this, molecular and chemical strategies are being developed to improve brain exposure and achieve uniform target engagement across the CNS **[22]**. Lipid conjugation is a promising approach to enable siRNA gene silencing in the CNS **[21,23]**. This approach underpins ALN–APP, a C16-lipid conjugated siRNA administered IT, now in clinical testing to reduce APP production in Alzheimer's disease (NCT06393712). Recent efforts aim to enable systemic delivery of oligonucleotides that penetrate the CNS. One approach is heteroduplex oligonucleotides (HDOs), where an ASO is paired with a cholesterol-conjugated sense strand **[24]**. This lipophilic design promotes BBB crossing and CNS exposure but requires high doses and has a narrow therapeutic index, highlighting the need for more selective strategies.

LEVERAGING TfR1 FOR CNS ACCESS

Receptor-mediated CNS delivery approaches

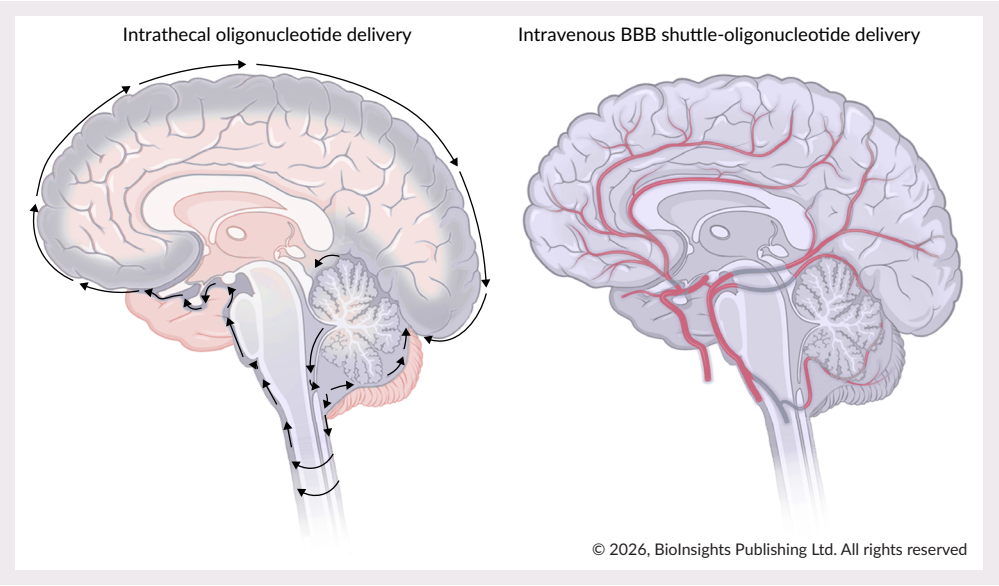
Efforts to deliver payloads such as recombinant proteins across the BBB began in the late 1980s with William Pardridge's pioneering work, which leveraged endogenous transport pathways to identify receptors capable of transcytosis, including TfR1 **(Figure 1)** **[25]**. Early clinical translation was achieved with lysosomal storage disorders using antibody–enzyme fusions, such as an anti-insulin receptor antibody linked to α-L-iduronidase for mucopolysaccharidosis type I, but the program was discontinued despite some benefit **[26]**. A successor approach using an anti-TfR1 antibody fused to iduronate-2-sulfatase was later approved for mucopolysaccharidosis type II, demonstrating the potential of receptor-mediated delivery for CNS pathologies **[27,28]**.

TfR1 biology and its role in iron transport

TfR1 is a type II transmembrane glycoprotein that forms a homodimer, each monomer comprising a short cytoplasmic

FIGURE 2

TfR1-mediated delivery of oligonucleotides after systemic administration shows broad CNS distribution compared to the limited distribution of oligonucleotides via intrathecal delivery.



 The diagram depicts a comparison of oligonucleotide distribution (purple shading) in the CNS after administration via lumbar intrathecal injection or after systemic intravenous administration as a TfR1-targeting conjugate. On the left, the oligonucleotide administered IT is carried by the flow of the cerebrospinal fluid within the CNS (arrows), thereby distributing to the spinal cord and the superficial layers of the cerebral and cerebellar cortex. However, this route of administration does not reach the subcortical layers or deep brain regions. On the right, the oligonucleotide administered systemically IV as a TfR1-targeting conjugate distributes homogenously to the spinal cord and throughout the brain, including deep brain structures.
BBB: blood–brain barrier. TRf1: Transferrin receptor 1.

tail, a single transmembrane helix, and a large extracellular ectodomain [29]. The ectodomain includes three regions: a protease-like domain near the membrane, a helical domain for dimerization, and an apical domain binding multiple ligands [30]. TfR1 binds diferric transferrin (holo-Tf) with high affinity to mediate iron uptake, but also interacts with heavy-chain ferritin and certain viral glycoproteins. Ligand binding triggers clathrin-mediated endocytosis, delivering iron to endosomes, where it is released under acidic conditions [31].

TfR1 rapid recycling (~15 min) supports continuous iron uptake and makes it an attractive target for drug delivery [32,33]. Highly expressed on brain endothelial cells, TfR1 enables iron transport into the CNS by binding holo-Tf on the luminal side, internalizing via endocytosis, and releasing iron to the brain [34].

Essential attributes of TfR1-targeting platforms for oligonucleotide transport

Key molecular attributes, such as binding affinity, epitope specificity, valency, and molecular size, play a critical role in the design of TfR1-targeting platforms for oligonucleotide delivery. Optimizing these parameters is fundamental for achieving efficient and safe oligonucleotide transport to target tissues, such as CNS **(Table 1)**.

The pioneering work of Sugo *et al.* described the use of a TfR1-binding antibody fragment to deliver siRNA to extrahepatic tissues such as muscle and heart,

TABLE 1

Key attributes of select platforms that leverage TfR1 for oligonucleotide delivery.

Platform	Format	Valence	TfR1 affinity	Oligonucleotide	Brain delivery
FORCE	Fab	Monovalent	2.6 nM [36]	PMO, ASO, siRNA	Yes
OTV	Fc + nonbinding Fab arms	Monovalent	110 nM [40]	ASO [40]	Yes
AOC	mAb	Bivalent	0.05 nM [39]	siRNA, PMO [39]	No
Bicycle	Cyclic peptide	Monovalent	2 nM [43]	ASO, siRNA [43]	No
TRiM	Fab	Monovalent	Undisclosed	siRNA [64]	Yes
Centyrin	Engineered protein	Monovalent	6 nM [73]	siRNA [73]	No

AOC: antibody oligonucleotide conjugates. ASO: antisense oligonucleotide. Fab: fragment antigen binding. Fc: fragment crystallizable. mAb: monoclonal antibody. OTV: oligonucleotide transport vehicle. PMO: phosphorodiamidate morpholino oligomer. siRNA: short interfering RNA. TRiM: Targeted RNAi Molecule.

achieving robust and durable target knockdown [35]. An anti-TfR1 fragment antigen binding (Fab) was selected as the TfR1-binding vehicle in the FORCE platform, which achieved robust delivery of oligonucleotides to both muscle and CNS [17,36–38]. Alternative platforms, such as antibody oligonucleotide conjugates (AOC), were designed using TfR1-targeting mAb for muscle, but not CNS delivery [39]. A different monoclonal antibody (mAb) format was used for the oligonucleotide transport vehicle (OTV) platform to facilitate delivery of ASOs to the CNS [40]. Specifically, the OTV platform employed a TfR1-binding site engineered into the fragment crystallizable (Fc) domain of the antibody, alongside nonbinding Fab arms of the native mAb structure [40]. While the majority of current platforms utilize antibody-derived binders for TfR1 targeting, it is expected that additional binding modalities may emerge as the field grows. For example, centyrins are a class of receptor-binding proteins based on the fibronectin scaffold that have been utilized to bind TfR1 to deliver an siRNA to muscle [41,42]. Another recent report demonstrated that a bicyclic peptide with high affinity to TfR1 delivers oligonucleotides to muscle in preclinical models [43]. Interestingly, at the time of writing, neither of these two formats appears to achieve productive CNS delivery (Table 1) [41,43].

Binding affinity to TfR1 influences both receptor engagement and systemic exposure. In the CNS space, both the high-affinity antibody in the J-Brain Cargo platform with K_D of 0.12 nM [27], the lower receptor affinity binder in the FORCE platform, with a K_D of 2.6 nM [36], as well as the OTV platform binder with the weakest K_D of 110 nM [40] all demonstrated delivery across the BBB. Early studies on anti-TfR1-mediated therapeutic delivery to the brain focused on high-affinity antibody binders, which cleared rapidly from circulation due to TfR1-mediated tissue uptake [27,44]. Subsequent studies suggested that weaker binding affinity to TfR1 may lead to greater uptake in the brain by enhancing the ability of the TfR1 binder to dissociate at the brain parenchyma side [45]. In addition, decreased binding to TfR1 might increase circulating exposure, enabling greater receptor-mediated

transcytosis [46]. On the other hand, effective CNS delivery achieved with Fabs and other low molecular weight binders with higher TfR1 affinity suggests that target binding can compete with faster clearance from circulation [36,47].

Epitope selection is essential to avoid interference with transferrin binding and maintain iron homeostasis. The structure of the TfR1 extracellular domain has been well-characterized and binding sites of native ligands are known, including Tf [31]. Given that disruption of Tf/TfR1 interactions can compromise iron homeostasis and thereby introduce significant safety liabilities, antibodies and other modalities directed against TfR1 are typically counter-screened to exclude this mechanism of interference [48]. However, anaemia is still a common feature observed in clinical trials with TfR1-targeted therapies [49–52]. The exact mechanism of anaemia associated with some platforms has not been described, but a number of parameters, including valency, size and binding epitope, either alone or in combination, may determine the degree of interference of conjugate binding with iron uptake.

Valency is an important parameter influencing TfR1-mediated CNS delivery. *In vitro* studies with anti-TfR1 antibodies suggested that bivalent engagement of TfR1 could lead to depletion of the receptor at the cell surface by inducing trafficking to the endolysosomal compartment and degradation of the receptor [53,54]. Barker *et al.* also reported degradation of TfR1 *in vivo* following administration of bivalent, but not monovalent binders, though these binders differed in affinity as well [40]. Many, though not all, TfR1 binding platforms utilize monovalent engagement to avoid both safety risks from receptor clustering and downregulation, as well as potential efficacy loss due to depletion of receptors on target cells [55]. Finally, molecular size affects circulation time and tissue penetration, with smaller binders such as Fabs offering enhanced tissue penetration and increased efficacy compared to the full-length antibodies [36,56].

ADVANCING TFR1-MEDIATED CNS DELIVERY TO THE CLINIC

Although the field of TfR1-mediated therapeutic delivery is expanding rapidly and supported by compelling preclinical evidence, only a limited number of TfR1-targeted platforms have advanced to the clinic. These programs encompass diverse modalities, including enzyme replacement therapies, antibodies, and oligonucleotide conjugates. A pioneering technology developed by JCR Pharmaceuticals, namely J-Brain Cargo, leverages a TfR1-targeting mAb to enable CNS delivery of a lysosomal enzyme iduronate-2-sulfatase (IDS) as payload and correct the clinical manifestations of Mucopolysaccharidosis type II (MPS II); this was approved in Japan in 2021 [28]. Another early technology, referred to by Denali Therapeutics Inc. as 'Enzyme Transport Vehicle™ (ETV), consists of an Fc-engineered TfR1-binding molecule fused to an enzymatic payload that is capable of crossing the BBB via TfR1-mediated transcytosis **(Figure 1)** [57]. DNL310 is an ETV with IDS as payload that, similar to J-Brain cargo, corrects the clinical manifestations of MPS II [58]. In a Phase 1/2 clinical trial (**NCT04251026**) in pediatric MPS II, weekly administration of DNL310 reduced accumulated CNS glycosaminoglycan storage, serum neurofilament light chain levels, and improvement in behavioral and cognitive clinical outcomes. This platform has shown anaemia in the clinic, which may be due to interference with endogenous TfR1 function [50].

A noteworthy example that supports the feasibility of TfR1-targeted delivery across the BBB is the bispecific monoclonal antibody, Trontinemab, which combines a TfR1-targeted Fab with the anti-amyloid-beta mAb gantenerumab [52]. In

a multiple-ascending-dose Phase 1b/2a study in Alzheimer's disease patients (NCT04639050), Trontinemab produced striking results in clearing of amyloid plaques such that ~90% patients became amyloid negative after 28 weeks of treatment. Although it remains to be determined whether Trontinemab can slow disease progression, these data are a direct demonstration that TfR1-mediated CNS delivery enhances the pharmacodynamic properties of the cognate payload.

Recent data generated with the FORCE platform utilizing a Fab binder demonstrated robust TfR1-mediated delivery of conjugated oligonucleotide therapeutics across the BBB for the treatment of CNS manifestations in neuromuscular diseases in preclinical studies [17]. Moreover, in a mouse model of Duchenne muscular dystrophy (DMD), FORCE resolved an anxiety-like phenotype, a hallmark of CNS disease in DMD [59, 60]. Of note, this platform demonstrated similar efficiency in both muscle and CNS delivery when compared to unconjugated oligonucleotides, with the platform inducing a 15–50 fold increase in delivery to both tissues [17]. Importantly, the approach is currently being tested in two clinical trials in Duchenne muscular dystrophy (DELIVER clinical trial NCT05524883) and myotonic dystrophy type 1 (DM1; ACHIEVE clinical trial NCT05481879), see Table 2. In the ACHIEVE trial, functional improvement was demonstrated in both muscle and CNS as measured via clinical data. Specifically, CNS-related benefit was observed via patient-reported outcome data,

TABLE 2

Transferrin receptor 1-mediated central nervous system-penetrant platforms with oligonucleotide payloads in clinical development for neuromuscular and neurological disorders.

Platform	Molecule	Target	Mechanism of action	Stage
FORCE	Zeleciment rostudirsen	DMD	Splice switching	Ph1/2 [38]
	Zeleciment basivarsen	DMPK	RNAseH-mediated degradation	Ph1/2 [37]
TRiM	ARO–MAPT	MAPT	RNAi	CTA [64]
OTV	DNL628	MAPT	RNAseH-mediated degradation	CTA [63]

TABLE 3

Receptor-mediated strategies for drug delivery to the central nervous system.

Receptor	Receptor density at the BBB (molecules/endothelial cells)*	Receptor internalization kinetics	Technology Stage
TfR1	~100,000–200,000 [74]	Fast [74]	Clinical
CD98hc	~100,000 [75]	Slow [76]	Preclinical
IGF1R	~10,000–30,000 [77]	Fast [78]	Preclinical
INSR	20,000–50,000 [77]	Fast [79]	Preclinical
LDLR	~30,000–80,000 [77]	Slow [80]	Preclinical

*Approximate receptor numbers from The Human Protein Atlas [82]. BBB: blood-brain barrier. CD98hc: cluster of differentiation 98 heavy chain. IGFR1: insulin-like growth factor 1 receptor. INSR: insulin receptor. LDLR: low density lipoprotein receptor. TfR1: transferrin receptor 1.

namely CNS subscales of the myotonic dystrophy health index, indicating functional improvement of CNS symptoms of DM1 such as cognition, communication, sleep, pain, emotional issues and fatigue [37]. This is the first clinical evidence of TfR1-mediated oligonucleotide delivery to the CNS (Table 2). Clinical data demonstrated a favorable safety profile, with no evidence of persistent related anaemia. This is in contrast to earlier CNS delivery technologies leveraging TfR1, which have shown detrimental effects on hematopoiesis, with anaemia observed in up to 30% of patients, and in some cases have led to discontinuation of clinical programs (NCT05450549) [61]. Importantly, the FORCE platform exhibits payload modularity, enabling the use of diverse chemistries, with clinical validation using both PMO and ASO modalities and preclinical demonstration of compatibility with additional payloads such as enzymes and siRNA [37,38,62]. Additional TfR1-mediated delivery platforms are progressing towards clinical development, both targeting *MAPT* for the treatment of Alzheimer's disease: one is using the OTV platform with an ASO payload [63] and another one using the Targeted RNAi Molecule (TRiM) platform with an siRNA payload [64]. Taken together, a growing number of therapeutic approaches focus on TfR1-mediated delivery of oligonucleotides, acknowledging its utility for reaching all regions of the brain for the best therapeutic impact for neurological diseases (Table 2).

TRANSLATION INSIGHT

Among the various strategies under investigation to improve CNS delivery of oligonucleotide therapeutics, receptor-mediated transport stands out as a particularly promising avenue for clinical translation. Within this category, TfR1-targeted conjugates have demonstrated superior potential for facilitating brain uptake, and a small number of molecules designed to deliver on the promise of TfR1-mediated distribution to tissue have advanced into the clinic (Table 2). These delivery platforms typically involve full-length monoclonal antibodies, antibody fragments, or engineered subdomains that are conjugated to oligonucleotides, forming CNS-penetrant complexes. Such antibody–oligonucleotide conjugates offer a compelling dual advantage: the high specificity of antibody-mediated targeting combined with the precise gene modulation capabilities of oligonucleotides. This synergy is especially valuable for neurological disorders, where targeted delivery across the BBB remains a major hurdle. As the field of TfR1-mediated delivery matures, researchers are actively exploring alternative receptor-mediated delivery pathways to achieve cell specificity and better compatibility with diverse therapeutic payloads. Interestingly, enzyme replacement therapy delivered via FORCE showed promise as a treatment capable of addressing both muscle and CNS manifestations in Pompe disease, setting this platform apart from other technologies in terms of modularity [62,63,65].

Receptors other than TfR1 may also be leveraged for delivery to the CNS (Table 3). Among these, insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) have garnered interest due to their roles in transporting endocrine peptides across the BBB. A recent publication described an IGF1R-targeting scFv-Fc fusion for delivery across the BBB to target α-synuclein preformed fibrils [66]. The challenge in leveraging these receptors for drug delivery is the potential for disrupting essential metabolic signaling [67–69]. Another promising candidate is low-density lipoprotein receptor-related protein 1 (LRP1), which is highly expressed in brain endothelial cells and capable of transporting a wide range of ligands. Early studies suggest it may be amenable to oligonucleotide conjugates as well [70]. Emerging targets such as cluster of differentiation 98 heavy chain (CD98hc)

are also under investigation – although less characterized, CD98hc holds promise for supporting transcytosis [71]. A dual targeting of CD98hc and TfR1 platform has also been explored for brain delivery [72]. This strategy leverages different properties of TfR1- and CD98hc-mediated brain uptake to achieve significantly higher brain concentrations than either vehicle alone. As delivery remains one of the central challenges in CNS therapeutics, expanding the repertoire of receptor targets beyond TfR1 could unlock new possibilities for treating neurological diseases. Future work will need to balance receptor accessibility, tissue specificity, frequency of dosing, and immunological safety to optimize these strategies for clinical translation.

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NUCLEIC ACID INSIGHTS

PRECLINICAL AND TRANSLATIONAL TOOLS AND TECH

SPOTLIGHT

Exploring multifunctional design-for-delivery approaches for nucleic acid therapeutics

Sasha Ebrahimi

INTERVIEW

"[...] there have been three key categories of strategies that have had a transformative impact on the field – chemical design, conjugation, and formulation-based approaches."

Jokūbas Leikauskas, Editor, BioInsights, speaks with **Sasha Ebrahimi**, Scientific Leader, Emerging Drug Delivery Platforms team at GSK, about advanced strategies to improve the developability and targeted delivery of nucleic acid therapeutics. In particular, they discuss emerging design-for-delivery approaches, such as ligand- and formulation-based multifunctional approaches for extrahepatic delivery. They also highlight key translational challenges such as immune activation, safety considerations under repeat dosing, and the growing need for scalable chemical and enzymatic synthesis methods to support increasingly complex oligonucleotide constructs.

Nucleic Acid Insights 2026; 3(1), 67–72 · DOI: 10.18609/nuc.2026.007

Q What are you currently working on?

SE I am currently a Scientific Leader within the Emerging Drug Delivery Platforms team at GSK. Our work focuses on identifying strategies to enhance the biochemical properties of emerging therapeutic modalities, such as antibody-drug conjugates and nucleic acids. By doing this, we aim to improve their developability and increase their likelihood of successful translation to the clinic.

Our research spans a variety of areas. For example, we are developing high-concentration formulations of protein-based therapeutics that can enable less frequent patient dosing. We also design strategies to control the molecular-level architecture of therapeutics in order to increase their potency. Additionally, we are developing drug delivery vehicles to ensure that therapeutics, such as nucleic acids, reach their intended targets in the body after injection.

Q What are the key challenges in the delivery of nucleic acid therapeutics?

SE There are several challenges, and they are present at almost every scale of biology encountered in the human body. At the organ level, the majority of systemically administered nucleic acid drugs accumulate in the liver, kidneys, or spleen. The question then becomes: how do you target other tissues or organs?

At the cellular level, I often like to draw comparisons with classic antibody- and small-molecule-based drugs. Therapeutic antibodies function outside cells, meaning they bind their targets in serum or on cell surfaces. Small molecules generally bind their targets inside cells, which works well because they are cell-permeable and can naturally enter cells. However, the situation is different with nucleic acids. The locations where they exert their effects are intracellular, for example, in the nucleus or cytoplasm, but nucleic acids are negatively charged, and the cell membrane is also negatively charged. The question then is: how do you get these structures into cells? This introduces an additional layer of complexity compared with other modalities.

At the subcellular level, even after nucleic acids enter a cell, they can become trapped in compartments such as endosomes, preventing them from engaging their therapeutic target. On top of that, there are nucleases inside cells that degrade nucleic acids. If a nucleic acid is degraded, it cannot bind its target and cannot have a therapeutic effect.

Q How can these hurdles be addressed?

SE In the early days of nucleic acid therapeutics, many of the aforementioned hurdles made development prohibitively challenging. Only through profound advances driven by fundamental research was the field able to identify strategies to transform sequences prone to degradation, inability to enter cells, or otherwise limited into sequences with enhanced properties. These advances significantly improved their prospects of success as clinically viable candidates.

Broadly speaking, there have been three key categories of strategies that have had a transformative impact on the field – chemical design, conjugation, and formulation-based approaches.

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Chronologically, chemical design was one of the earliest strategies. This involves modifying the sugars, backbone, or bases of nucleic acids to impart improved therapeutic properties. A classic example is the phosphorothioate backbone, which can reduce susceptibility to nuclease-mediated degradation and increase cellular uptake.

The second category involves attaching targeting ligands to nucleic acids. For instance, this could include conjugating a sugar or an antibody to a therapeutic that recognizes a receptor expressed in a tissue of interest. By recognizing and binding that receptor, the biodistribution of the nucleic acid can be altered to increase accumulation in the desired target tissue.

The third category consists of formulation-based approaches, with nanoparticles as a common example. Perhaps the most well-known example is the COVID-19 vaccine, where a lipid nanoparticle (LNP) facilitates delivery of the payload, in this case mRNA, into cells while also protecting it from degradation.

Q Accumulation of delivery vehicles at higher or repeat dosing is increasingly recognized as a limiting factor. From your experience, where does this issue most acutely manifest?

SE The best way to answer your question is by considering a comparison of vaccines versus therapeutics. When I give talks on delivery-based approaches for nucleic acids, I am sometimes asked a version of this question: if we were able to develop an LNP-based vaccine in such a short period of time, why is there not an LNP-based therapeutic for essentially any disease one could think of? Firstly, designing a therapeutic is different from creating a vaccine. Vaccines generally target immune cells, which are more broadly distributed throughout the body, whereas therapeutics are intended to target specific cells or tissues.

Secondly, vaccines typically require much less frequent dosing than therapeutics. Nucleic acid therapeutics, unlike vaccines, are often administered at higher doses and require chronic dosing. When frequent dosing is required, unwanted immune responses can arise, which makes translation into a chronic medicine more challenging. In fact, for clinically approved LNP-based therapeutics, patients are often given a steroid regimen before dosing to help control potential unwanted inflammation.

Taken together, the requirement for strict tissue targeting and immune responses are two factors that make therapeutic applications, particularly those involving chronic dosing, more difficult. These are challenges that the field is actively working on and beginning to solve.

Q What common themes have emerged in the 'design-for-delivery' of nucleic acid-based therapeutics?

SE Currently, there are three main themes in the delivery of nucleic acid therapeutics – extrahepatic delivery, multifunctional molecules, and complexity. Oligonucleotides are a good subclass of nucleic acids to focus on as an example to make the point.

The majority of approved oligonucleotide therapeutics are indicated for hepatic diseases because upon systemic administration, oligonucleotides are largely sequestered to the

"Importantly, as we move beyond niche indications and toward larger patient populations, increased molecular complexity poses additional challenges for commercial synthesis [...]"

liver. In other cases, local administration is an option if you want to target other organs, but it is not a particularly convenient route of delivery. As a result, oligonucleotide therapeutics today generally address niche diseases. A major area of interest in both academia and the pharmaceutical industry, as we look to expand the scope of targetable diseases, is extrahepatic delivery, for example, targeting the brain, the lung, and other organs.

Some of the earliest strategies I mentioned, particularly chemical design, are not particularly effective as levers for broad biodistribution outside the liver. That limitation is a major reason for the strong interest in targeting ligand- and formulation-based approaches. As a result, we are seeing a rise in what I would call multifunctional oligonucleotides – structures that combine molecules with distinct functions.

For example, in an oligonucleotide–antibody conjugate, the antibody directs the construct to the organ of interest, while the oligonucleotide provides the therapeutic effect. This kind of multifunctionality stands in contrast to the earliest structures used in the field, which were largely just oligonucleotides that relied on modifications to the sugar, backbone, or base to enhance their properties, rather than being combined with other molecular components.

With this rise in multifunctionality comes increased complexity, the third keyword. Adding a targeting ligand or a nanoparticle introduces its own set of challenges and intricacies, ultimately increasing practical complexity. If I am working with a nanoparticle, is it too polydisperse? Is there batch-to-batch variability in its structure? For many nanoparticles, the answer is yes, which can make translation to a commercial setting very challenging. Oligonucleotides themselves may not have significant stability concerns related to light exposure or elevated temperature, but if an antibody is attached as a delivery vehicle, an entirely new set of stability considerations is introduced.

Importantly, as we move beyond niche indications and toward larger patient populations, increased molecular complexity poses additional challenges for commercial synthesis while targeting more prevalent diseases means manufacturing much larger quantities of the drug. The question then becomes: are we equipped to synthesize these increasingly complex constructs at larger scales? In many cases, the answer is no. This is why the development of new chemical and enzymatic strategies for oligonucleotide synthesis is such an active area of research, and it is directly driven by these three themes, as discussed in our collaborative perspective and Op-ed articles published in *Nature Chemical Engineering* and STAT News **[1,2]**.

Q Lastly, what emerging technologies do you think could be transformative for nucleic acid delivery in the next 5–10 years?

SE There are many promising developments on the horizon. Firstly, advances in materials science and analytical chemistry are now enabling the synthesis and analysis of large libraries of materials in a high-throughput manner, and that is a real game-changer. For example, you can take large libraries of nanoparticles, systematically vary individual components within those nanoparticles, inject them into animals, and then track

their accumulation using approaches such as barcoding. This allows you to begin teasing out design rules and identifying which components of a nanoparticle promote accumulation in specific target organs. Most importantly, once you understand those design rules, you can begin to create so-called 'plug-and-play' platforms. In other words, you can rationally select a delivery vehicle based on where your nucleic acid needs to go in the body.

I am also very excited about the continued development of multifunctional nucleic acid structures. A good example of this is the CRISPR-Cas9 system, which contains both a protein and a nucleic acid component. We are already beginning to see the transformative potential of these systems as therapeutic modalities, particularly when paired with effective delivery vehicles. One especially remarkable recent example comes from personalized medicine – in an extraordinary case, a team of clinicians and scientists at Children's Hospital of Philadelphia and Penn Medicine developed a treatment for an infant, baby KJ Muldoon, who had a fatal genetic disorder [3]. The therapy was based on a CRISPR-Cas adenine base editor and was packaged for delivery in an LNP. Perhaps the most incredible aspect of this effort is that the entire process, from diagnosis to manufacturing of the therapeutic construct to treatment, was completed in just six months. That timeline may represent the fastest drug development effort in history.

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BIOGRAPHY

Sasha Ebrahimi earned his bachelors from the University of Illinois at Urbana-Champaign in 2016 with Highest Distinction in Chemical Engineering. He then went on to earn his PhD in 2021 at Northwestern University, Illinois, USA, as a Ryan Fellow working with Professor Chad Mirkin. Sasha joined GSK in 2021 where he is currently a Scientific Leader in the emerging drug delivery platforms team. Sasha's research at GSK has focused on enhancing the developability of emerging therapeutic modalities like antibody drug conjugates and oligonucleotides. This work has spanned areas such as the design of drug delivery vehicles for targeted delivery, the development of novel formulations for enhancing the *in vitro/in vivo* stability of therapeutics, and the engineering of the molecular architecture of these therapeutics to boost their potency. His contributions have been recognized with >20 awards of national and international scope, including the American Institute of Chemical Engineers (AIChE) 35 Under 35 award, the CASSS Next Generation Investigator award, and selection as a STAT News Wunderkind.

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NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES
ANALYTICS

REVIEW

Oligonucleotide impurity profiling by liquid chromatography–mass spectrometry

Huijun Tian and Hagen Cramer

Oligonucleotides (oligos) manufactured by solid-phase synthesis are an emerging class of drugs. Therapeutic oligonucleotides require stringent characterization of product-related impurities. Regulatory guidance demands identifying and quantifying both major and minor impurities. Oligonucleotide impurity profiling by liquid chromatography–mass spectrometry (LC–MS) is one of the specialized analytical techniques for oligonucleotide impurity characterization. A detailed overview is given covering the different types of impurities, the regulatory expectations regarding detection, identification and quantification of impurities, the analytical challenges associated with impurity profiling by LC–MS, and the practical considerations for developing LC–MS methods. A few examples of how to implement LC–MS workflows in alignment with regulatory expectations are presented. Overall, this mini-review intends to provide a background, and a jump-start for those who are new to oligonucleotide impurity profiling by LC–MS.

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The accelerating clinical adoption of therapeutic oligonucleotides – including phosphorothioate antisense oligonucleotides (ASOs), double-stranded small interfering RNAs (siRNAs), splice-modulating oligos, immunostimulatory CpG motifs, DNA and RNA aptamers, and chemically complex conjugated architectures – has intensified demands for high-resolution analytical characterization throughout development and manufacturing [1–4]. Unlike traditional small molecules, synthetic oligonucleotides are generated through iterative solid-phase synthesis using phosphoramidite chemistry or enzymatic extension cycles that inherently produce a heterogeneous ensemble of closely related molecular species. These may include n−1/n+1 length variants, incomplete sulfurization or desulfurization products, depurination species, β-elimination fragments, 2′-modification loss, conjugation by-products, and a range of oxidative or nuclease-like degradation

products [2,4]. Many of these differ from the intended sequence by a single nucleotide or modification, yet they may alter potency, innate immune activation, or toxicology, motivating increasingly stringent impurity profiling requirements from regulatory agencies [5].

Liquid chromatography coupled to mass spectrometry (LC–MS) has consequently become the central tool for impurity surveillance in oligonucleotide therapeutics. Chromatographic approaches such as ion-pair reversed-phase (IP-RP), anion-exchange chromatography (AEX), hydrophilic interaction chromatography (HILIC), and mixed-mode phases provide distinct selectivities tuned to oligonucleotide charge state, hydrophobicity, and chemical modification patterns [6–8]. Advances in volatile ion-pairing reagents, column chemistry, and mobile-phase optimization have enabled MS-compatible, high-resolution separations capable of detecting impurities at sub-percent levels [6,9].

Parallel improvements in mass spectrometry – particularly high-resolution MS (HRMS) instruments capable of sub-ppm mass accuracy – have allowed the discrimination of subtle mass differences associated with single sulfur substitutions, terminal modifications, 2′-O substituents, or conjugate-specific moieties [8,10]. Fragmentation strategies including collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), electron-transfer dissociation (ETD), a hybrid ETD–HCD approach (EThcD), and ultraviolet photodissociation (UVPD) now support localized identification of modification sites, backbone integrity, and sequence scrambling, extending analytical depth far beyond what earlier approaches could achieve [11–13]. Methods leveraging ion mobility spectrometry (IMS), isotopic-pattern analysis, and charge-state deconvolution further enhance characterization of regioisomeric or conformationally heterogeneous impurities [14,15].

Despite these advances, oligonucleotide impurity profiling remains analytically challenging. Strong anionic charge density, extensive charge-state multiplicity, adduct formation with cations or ion-pairing reagents, and sequence-dependent ionization efficiency can compromise both chromatographic resolution and MS sensitivity [6]. Increasing structural complexity – including triantennary GalNAc-siRNA and ASO conjugates, antibody oligonucleotide conjugates (AOCs), phosphorothioates, lipid-modified constructs, and long ss/ds synthetic oligos – introduces additional chromatographic challenges or new impurity classes not fully addressed by standard workflows [3,4,6,7]. Concurrently, regulatory expectations toward quantitative, structure-specific impurity identification have become more rigorous, especially for late-stage development and commercial products [5].

This review provides a comprehensive assessment of LC–MS methodologies for oligonucleotide impurity profiling. We survey chromatographic strategies, ionization and fragmentation approaches, computational and deconvolution tools, and evolving regulatory considerations. Special attention is given to impurity classes associated with emerging therapeutic architectures and to analytical innovations – such as alternative stationary phases, multidimensional separations, and intact tandem mass (MS/MS) capabilities – that promise to enhance sensitivity, structural resolution, and throughput in future oligonucleotide workflows. A few examples of workflows are presented to demonstrate the oligonucleotide impurity analysis by LC–MS.

REGULATORY & QUALITY CONTROL CONSIDERATIONS

The development of therapeutic oligonucleotides requires rigorous characterization of product-related impurities, as even low-level variants may affect efficacy, safety,

or stability [16,17]. Although oligonucleotides fall outside the scope of ICH Q3A (Impurities in Drug Substance), ICH Q3B (Impurities in Drug Product), and ICH Q6A (Specifications for New Chemical Entities), the scientific principles underpinning these guidelines are still expected to be applied [18]. Table 1 lists the ICH guidelines applicable to oligonucleotides. A dedicated EMA Guideline on the Development and Manufacture of Oligonucleotides (EMA/CHMP/CVMP/QWP/262313/2024) is currently in draft form (July 2024) [19], with final publication anticipated in early 2026.

A foundational framework for oligonucleotide impurity classification was proposed by the Oligonucleotide Safety Working Group (OSWG) in a 2017 white paper [20], which organizes impurities into four classes and recommends that only Class IV impurities – those structurally distinct from both the parent and natural nucleic acids – require safety qualification.

Reporting, identification, and qualification thresholds

According to the draft EMA guideline (EMA/CHMP/CVMP/QWP/262313/202424), the reporting threshold for product-related impurities is determined by the analytical method's lower limit of quantification (LLOQ), which depends on the oligonucleotide's length and structural complexity.

The identification threshold defines the impurity level at which it is required to identify and characterize impurities of unknown structures. For most oligonucleotide drug substances, a threshold of 1.0%

TABLE 1

International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines.

Guideline	Topic	Application to oligonucleotides
Q1	Stability	Yes
Q2	Method validation	Yes
Q3a Q3b	Impurities in drug substance and drug product	Principles of the guidelines apply; limits for reporting, ID, and qualification are reviewed on a case-by-case basis
Q3c Q3d	Residual solvents and elemental impurities in drug substance and drug product	Yes
Q4	Pharmacopeia harmonization	Yes
Q6a (b)	API specification	Principles of the guideline apply but should be adapted for an oligonucleotide case-by-case
Q7	GMPs for API	Yes
Q8	Pharmaceutical development	Yes
Q9	GMP risk management	Yes
Q10	Pharmaceutical quality system	Yes
Q11	Development and manufacturing of drug substances	Yes
Q14	Analytical procedure development	Yes

API: application programming interface. Adapted from Table 14.2 in [18].

is considered acceptable and is typically aligned with limits for unspecified impurities in specifications.

The qualification threshold establishes the level at which a structurally defined impurity must undergo nonclinical qualification. A threshold of 1.5% is generally acceptable. Mutagenic impurities remain subject to ICH M7 and EMA/CVMP/SWP/377245/2016.

Specification limits for each defined impurity should be justified using batch analysis, with impurities above the qualification threshold requiring supporting toxicological data.

Impurity classification and qualification requirements

The OSWG white paper proposes organizing product-related impurities into four classes, summarized below **(Table 2)** [20,21].

Interpretation of the classification scheme

Class I impurities include those whose structure and sequence match major metabolites (e.g., terminally truncated forms). These require no qualification regardless of level.

Class II impurities contain only native nucleic acid features (e.g., phosphate diester forms) and likewise do not require qualification.

Class III impurities comprise sequence variants arising from internal substitutions, deletions, or insertions. These often co-elute or share mass spectra and are therefore quantified as a group initially.

- If the group total is <1.5%, no additional characterization or qualification is required
- If >1.5%, individual species must be identified and those exceeding 1.5% must be qualified

Class IV impurities include chemically modified or unknown structures and must be qualified if present above the 1.5% threshold. Minimizing these impurities through process optimization is preferred over conducting extensive toxicological studies. *In silico* or *in vitro* approaches may be considered when qualification is required. The CHMP SWP reflection paper on genotoxic potential (EMEA/CHMP/SWP/199726/2004) [22] may also apply.

Oligonucleotides themselves and their product-related impurities are not governed by ICH M7 but may require supplemental assessment under EMA genotoxicity guidance where relevant.

Impurity origins during solid-phase synthesis

Oligonucleotide synthesis proceeds through iterative cycles involving detritylation (deblocking), coupling, oxidation/sulfurization, and capping. Side reactions, incomplete steps, and degradation pathways may arise during any of these operations. As illustrated in **Figure 1** (adapted from Chris Oswald's workshop presentation, TIDES EU 2025), most of the Class I–III impurities originate directly from these cyclic synthetic transformations, whereas Class IV impurities typically arise from aberrant chemical reactions, backbone damage, or reactive raw materials.

COMPLEX IMPURITY LANDSCAPE

Synthesis of oligonucleotides (especially phosphorothioate-modified, conjugated, or chemically modified ones) can produce many types of impurities: truncated (shortmers), extended (longmers), internal insertion or internal deletion, adducts, depurinated species, deamination, base-loss, oxidated species, etc., as shown in **Table 3** [16,20,21].

Degradation during storage or under stress conditions can generate numerous

by-products, including those arising from oxidation, hydrolysis, N-1 formation, loss of nucleotide moieties from the 3′- and 5′-termini, depurination, formation of terminal phosphorothioates, and the production of ribose, ribophosphorothioates (Rp), and phosphoribophosphorothioates (pRp) [16,23]. Some impurities differ by only very small mass shifts (e.g., deamination), making them difficult to differentiate by single-quadruple mass spectrometry without chromatographically resolved for detection and quantification [24].

CHALLENGES IN OLIGONUCLEOTIDE IMPURITY PROFILING BY LC–MS

Oligonucleotide impurity analysis by LC–MS faces multiple technical hurdles.

TABLE 2

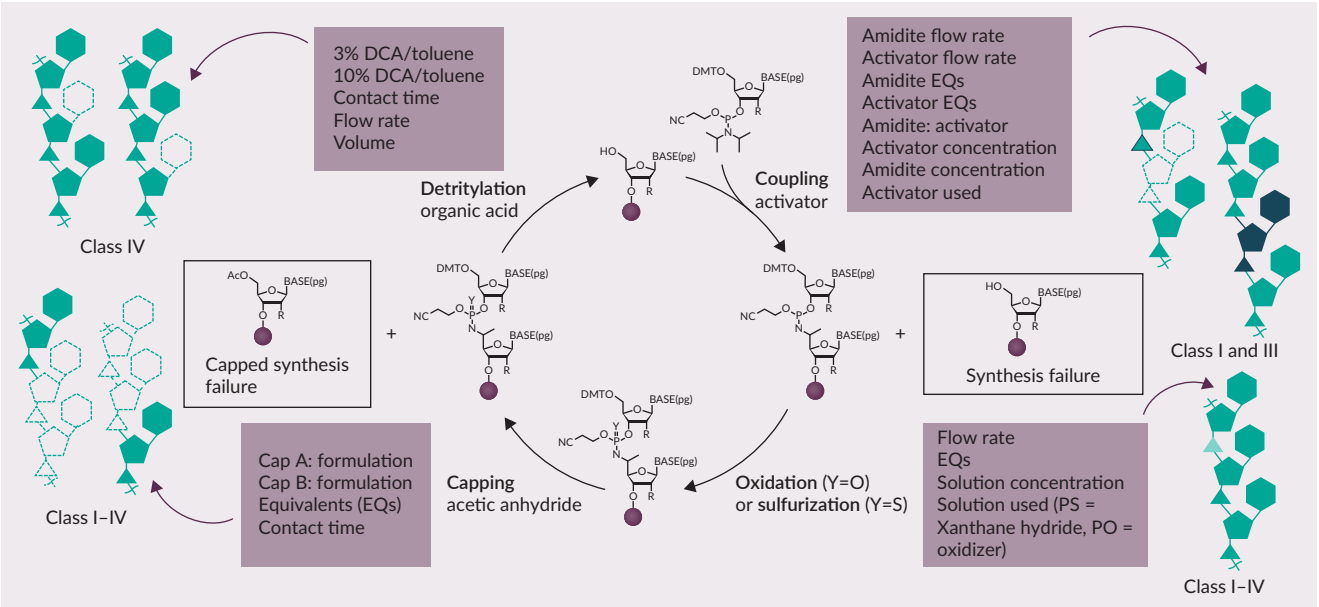
OSWG impurity classification.

Class	Description	Examples*	Safety assessment required?
Class I	Impurities identical to major metabolites; same sequence and structure as parent	3′/5′-truncated species; incomplete conjugates; single-stranded forms of duplex parent	No
Class II	Impurities containing only naturally occurring nucleic acid structural elements	Phosphate diester forms; oxidized bases; 2′–5′ linkages	No
Class III	Sequence variants of the parent oligonucleotide	n−1 or n+1 variants; internal deletions/additions; deaminated forms	No (unless individual species >1.5%)
Class IV	Impurities with structural elements not present in parent or natural nucleic acids	Base modifications; backbone alterations; depurinated species; unknown impurities	Yes

*Examples represent authors' interpretation [21]. Adapted from Capaldi *et al.* [20].

FIGURE 1

Generation of class I–IV impurities during solid-phase synthesis.



DCA: dichloroacetic acid. EQ: equivalents. Refer to **Table 3** for details on the impurities formed during the process.

TABLE 3

Process and starting material-related impurities.

Impurity	Affected locations	Mass difference (ave.)	Source	Occurrence	Class*†
+ PS (phosphorothioate)	Backbone	+96.05*	Process	Occasionally	IV
+ PO (phosphate)	Backbone	+79.98*	Process	Occasionally	II
PS → PO conversion	Backbone	−16.07	Process (capping, deprotection, oxidation/sulfurization)	In all ONs containing PS bonds	II
Phosphorodithioate (S–P–S) or PO → PS conversion	Backbone	+16.07	Process (sulfurization)	Occasionally	IV
Cyclic phosphate (3′-truncation)	Backbone, ribose	+61.96	Process (deprotection)	Common in natural RNA	II
+ Chloral (trichloroacetaldehyde)	Backbone	+147.39	Raw material (DCA)	Rare	IV
+ 2′-O-tert-butyl dimethylsilyl (TBDMSi)	Ribose at rA, rG, rC, rU	+114.26	Process (deprotection)	Common in natural RNA	IV
+ DMT-C phosphonate	Internucleotide bond	+286.37	Process (detritylation)	Occasionally	IV
+ DMT-C phosphonate	5′ terminal phosphate	+366.10*	Process (detritylation)	Occasionally	IV
+ DMT	5′-O-position	+302.37	Process (detritylation)	Common	IV
– HF	2′-F pyrimidines	−19.99	Process (deprotection); lyophilization, storage	Common	I/II
– HF, + H_2O	2′-F pyrimidines	−2.00	Process (deprotection); storage	Very common	II
2′/3′ Isomer	Ribose; any RNA or 2′-mod RNA nucleotide	±0.00	Starting material	Common in very small amounts	II
Internucleotide linkage (3′–2′ migration)	Ribose; any RNA nucleoside	±0.00	Process (deprotection)	Common in natural RNA	II
Inverted base (5′–3′ inversion)	Ribose; any RNA or 2′-mod RNA nucleoside	±0.00	Starting material	Common in very small amounts	II
2′-O-methyl	Any MOE nucleoside	−44.05	Starting material	Common	I
2′-O-propyl	Any MOE nucleoside	−16.00	Starting material	Rare	II
2′-O-butyl	Any MOE nucleoside	−1.82	Starting material	Rare	IV
2′-O-(2-ethoxy-ethyl), EOE	Any MOE nucleoside	+14.03	Starting material	Common	IV
Branch-mer (amino group of base)	A, C, or G base	n + (n-x)	Loss of protecting group	Occasionally	IV
Branch-mer (O^6 of G-base)	G or I base	n + (n-x)	Unprotected O^6-position	Rare	IV
Depurination (abasic site)	A or G base	−117.12 or −133.11	Process (detritylation)	Common	IV
Depurination, Ethoxyacetal-formation	A or G base	−89.06 or −105.06	Process (deprotection)	Rare	IV
Deamination	5-Me-C, C, A, G base	+0.98	Process (deprotection)	Common in very small amounts	III

*Mainly observed on Shortmers. †Reflects authors personal opinion. ON: therapeutic oligonucleotides.

TABLE 3 (CONT.)

Process and starting material-related impurities (cont.).

Impurity	Affected locations	Mass difference (ave.)	Source	Occurrence	Class*†
2,6-Diaminopurine	G base	−0.98	Process (capping)	Rare	II
Transamination	C base; amino linker	+14.03	Process (deprotection)	Common if MeNH2 is used	IV
Acetyl remains on functional groups	C base; amino linker	+42.04	Process (capping; deprotection)	Common for ONs with amino linker	IV
N-β-methylamino acetamide (MAM)	Unknown	+85.10	Raw material (capping)	Rare	IV
N_4-acetyl-2,6-diaminopurine (ADP)	G base	+41.05	Process (capping)	Rare	IV
N^2-isobutyryl-2,6-diaminopurine (IDP)	G base	+69.11	Process (capping)	Rare	IV
3-(3-Acetyl-4-methylpyridine-2-one-6-yl)-2-aminoimidazole (AMPA)	A base	+98.04	Process (capping)	Rare	IV
+ Cyanethyl (CNET)	Heterocyclic bases, predominantly T base	+53.06	Process (deprotection)	Common	IV
Residual protection group; + isobutyryl	ibuG base	+70.08	Process (deprotection)	Common	IV
Residual protection group; + benzoyl	bzA base	+104.11	Process (deprotection)	Rare	IV
3-N4-Ethano-cytidine derivative	C base	+80.08	Process, thermal stress	Rare	IV
Depyrimidation (abasic side)	Any base	−52.03 or −94.07	Process (deprotection)	Common in RNA	I
8-oxo-formation	A, G base	+16.00	Process (oxidative stress)	Rare	II
5-Hydroxymethyl cytosine	5-Me-C base	+16.00	Process (oxidative stress)	Rare	IV
3′(n−1), 5′(n−1), 5′(n−2)	Terminally truncated	Varied	Process (coupling, oxidation/sulfurization, capping, deprotection)	In all ONs	I
n−1, n-X	Deletion sequences	Varied	Process (coupling, oxidation/sulfurization, capping, deprotection)	Common	III
n+1	Insertion	Varied	Process (coupling)	In all ONs	III

*Mainly observed on Shortmers. †Reflects authors personal opinion. ON: therapeutic oligonucleotides.

Instrument limitations are a major factor: while single-quadrupole systems can be used, their lower resolving power makes it difficult to distinguish closely related impurities (such as deamination species or exchange of U with C base resulting in a 1 Da mass difference), often requiring high-resolution MS, MS/MS, or ion-mobility–assisted methods. Data interpretation is another challenge due to complex spectra containing multiple charge states, adducts, and fragments, necessitating specialized software for accurate deconvolution. Chromatographic factors also complicate

analysis. Ion-pairing reagents such as TEA improve separation but can suppress ionization and contaminate MS systems. Nonvolatile buffer components require desalting to prevent ion suppression. Multiple charge states further increase spectral complexity, and detecting low-level impurities (<1%) requires sensitive, well-optimized methods. Adduct formation – particularly with sodium, potassium, ammonium, and various divalent metals – can distort or mask impurity signals. The ubiquitous existence of cations such as Na/K adduction cannot be prevented effectively due to the high charge density in nucleotidic species. Proper sample preparation, careful mobile-phase selection, and strategies such as 'standard' versus 'harsh' ionization conditions are needed to distinguish true impurities from adduct artifacts **[25,26]**. Ion-pairing reagents themselves can generate additional organic cation adducts, further complicating spectral interpretation.

- Instrument requirements: while single-quadrupole systems are usable (as shown by Agilent, Shimadzu and Waters) **[26–28]**, they are limited in resolving power compared to high-resolution instruments. This could make identification of very closely related impurities more challenging. For thorough characterization of species that fall below the resolution of single quadrupole MS, more advanced MS (high resolution or MS/MS system or additional techniques) may be needed (e.g., ion mobility – MS as the FDA work suggests) **[29,30]**;

- Data analysis and interpretation: because of the complexity of oligonucleotide spectra (many charge states, adducts, fragment ions), robust data-processing tools are needed. The Agilent note uses dedicated software ('Oligo Analysis Accelerator' for OpenLab CDS) to classify ions **[26]**;

- Ion-pairing reagents: selection of the ion-pairing reagent is critical for the resolution of impurities in oligonucleotides. Common LC mobile phases for oligonucleotides use ion-pairing (e.g., triethylamine [TEA]) to achieve adequate chromatographic resolution. But these reagents are often not very MS-friendly (ion suppression, contamination) and are therefore used at low concentrations **[31,32]**;

- Desalting: buffer components (especially nonvolatile ones) can suppress ionization or complicate MS; often need on-line or off-line desalting **[32]**;

- Charge states: oligonucleotides, when ionized by ESI, produce multiple charge states; deconvoluting the spectra can be nontrivial. High-resolution MS (HRMS) helps, for example, FTMS enables charge state determination from single m/z values of low-level impurities **[33]**;

- Low-level impurities: detecting very low-abundance impurities (e.g., <1%) demands high sensitivity and careful method design.

- Adducts: commonly sodium or other cation adducts complicate mass spectra. A list of adducts is included in **Table 4** **[34–39]**. In negative-mode electrospray ionization (ESI) LC–MS (mostly used for oligonucleotide analysis), oligonucleotides predominantly form multiply deprotonated molecular ions due to the acidic phosphate backbone, yielding a characteristic charge-state envelope of $[M-nH]^{n-}$ species that enables accurate intact-mass determination and deconvolution. In addition to these primary ions, metal-cation adducts – most commonly sodium and potassium – are frequently observed as mixed species

such as as $[M - (n + 1)H + Na]^{n-}$ or $[M - (n + 1)H + K]^{n-}$, which broaden isotopic distributions and complicate spectral interpretation. Ammonium adducts may also be present when volatile ammonium salts are used, often improving desolvation relative to alkali metals. Ion-pairing reagent–related species, including adducts or residual complexes with triethylamine (TEA), tributylamine (TBA), or dimethylbutylamine (DMBA), are common in IP-RP workflows and can suppress ionization or alter charge-state distributions if not carefully controlled. In positive-mode ESI LC–MS, oligonucleotides typically form a heterogeneous population of cationized species rather than clean protonated ions. The most common ions observed include weakly protonated molecules $[M+nH]^{n+}$, alkali-metal adducts (Na^+, K^+, Li^+), ammonium adducts, and complexes with ion-pairing reagents such as triethylamine (TEA), tributylamine (TBA), or dimethylbutylamine (DMBA). Sample preparation, system cleaning, LC methods and MS parametersmust be optimized to minimize them **[38]**. 'Standard' and 'harsh' ionization conditions could be used to differentiate adducts from 'authentic' impurities for identification and quantitative analysis **[25,26]**. Mitigation strategies include but are not limited to the following:

- Use organic bases (e.g., TEA, imidazole) to suppress alkali-metal adduction **[35]**;
- Use desalting procedures (precipitation, SPE, dialysis, on-line) to remove salt prior to MS **[39,40]**;
- Optimize mobile-phase purity, minimize non-volatile salts, use volatile buffers (e.g., alkylamine-acetate) and high-purity solvents;
- Periodically clean or recondition the LC system. The authors recommend using low concentration EDTA salt flush (such as 50 mM EDTA alkylamine salt) and low-pH flush (such as 10% acetic acid aqueous or organic solution) to remove metal salts (adsorbed or erosive) and prevent cumulative adduct build-up;
- Optimize the MS parameters (especially the cone voltage to shake off the salts and reduce the adduction).

COMMON LC–MS APPROACHES FOR OLIGONUCLEOTIDE IMPURITY PROFILING

The characterization of synthetic oligonucleotides and their impurities relies on a combination of chromatographic resolution, ionization behavior, and tandem mass spectrometric fragmentation pathways **[4,6]**. Electrospray ionization produces a distribution of charge states and a variety of adducts, including alkali-metal adducts (Na^+, K^+) and ion-pair–associated complexes formed through alkylamines such as triethylamine (TEA), tributylamine (TBA), or N,N-diisopropylethylamine (DIPEA). These adducts directly influence spectral quality, desolvation efficiency, and impurity detectability. Ion-pairing reagents alone can increase chromatographic selectivity in IP–RP LC but often elevate adduct formation and complicate deconvolution, whereas IP-RP LC in combination with fluorinated alcohols (HFIP/TEA, HFIP/DMBA) frequently improve the fidelity of MS-based impurity analysis by enhancing ionization efficiency, reducing metal adduct formation and improve chromatographical resolution. Anion-exchange chromatography (AEX) approaches have no ion-pairing reagent related adduct issues but are not compatible with mass spectrometry due to the high salt contents and resolution often lags IP-RP approaches. Hydrophilic

interaction liquid chromatography (HILIC) on the other hand is a more promising tool as it is adduct-minimizing and mass-compatible, but challenges remain [9].

Chromatographic strategies have evolved to address the complexity of therapeutic oligonucleotides. Ion-pairing reversed-phase (IP-RP) LC remains widely

TABLE 4

Common adducts for oligonucleotide analysis by LC–MS.

Adduct	Description/formula	Mass change (Da)	Notes
Na^+	Sodium adduct	+22	Common contaminant from glassware or buffers; shifts m/z by +22 Da per sodium ion
K^+	Potassium adduct	+38	Often observed with biological samples; +38 Da per potassium ion
Li^+	Lithium adduct	+7	Less common; +7 Da per lithium; can be used intentionally to improve desolvation in ESI
NH_4^+	Ammonium adduct	+18	Common from ammonium acetate or bicarbonate buffers; +18 Da per NH_4^+; volatile and generally improves desolvation
Mg^{2+}	Magnesium adduct	+24	+24 Da per Mg^{2+}; can stabilize higher-order structures (quadruplexes); less common in standard LC–MS unless intentionally added; usually minimized by EDTA or low-pH flushing
Ca^{2+}	Calcium adduct	+40	+40 Da per Ca^{2+}; similar effects as Mg^{2+}, usually avoided; usually minimized by EDTA or low pH flushing
Cu^{2+}	Copper adduct	+62.5	+62.5 Da per ($-2H+Cu^{2+}$); similar effects as Mg^{2+}, usually minimized by EDTA or low pH flushing
Na^+/K^+ clusters	Multiple sodium/potassium ions per oligo	Varied	Can complicate deconvolution for large oligos
NH_4^+ clusters	Multiple ammonium ions	+18 × n	Common when ammonium salts are used as mobile-phase additives
EDTA	EDTA adduct	+292.1	+292.1 per EDTA, common when EDTA is used as sample or mobile-phase additive
Sulfate/phosphate	Sulfate/phosphate adduct	+97.0	+97.0 from sulfate or phosphate buffer or contamination, usually avoided by flushing water extensively
Organic cation adducts	e.g., TEA^+, TBA^+	Varied	Can suppress or broaden MS peaks if excess reagent remains; must optimize buffer composition.
Ion-pairing reagent related			
Triethylamine (TEA^+)	+101 Da per TEA		Common in TEA/HFIP buffers; bulky, can reduce ionization efficiency if excessive
Tributylamine (TBA^+)	+185 Da per TBA	+185	Often used for longer oligonucleotides; more hydrophobic, stronger retention; can suppress ESI if not optimized
Dimethylbutylamine ($DMBA^+$)	+115 Da per DMBA	+115	Occasionally used as milder ion-pairing agent
N,N-diisopropylethylamine ($DIPEA^+$)	+259 Da per DIPEA	+259	Common ion-pairing reagent; can form weak adducts
Hexylamine (HA^+)	+101 Da per HA	+101	Used in short oligo separations; can form weak adducts
Amine oxide (e.g., oxidized TBA^+)	+201.3 Da per TBA oxide	+201.3	Observed if amine is oxidized during the storage or ionization; can be reduced if storing the amine solution at refrigerated conditions once opened and using freshly prepared mobile phases

Data from [34–39].

used due to its robustness and compatibility with mass spectrometry, enabling resolution of length variants (n−1 truncations), diastereomers [41,42], and phosphodiester impurities. Hydrophilic interaction liquid chromatography (HILIC) offers high retention for charged oligonucleotides while minimizing metal adduction and has recently been applied to conjugated or highly modified oligos. Anion-exchange chromatography (AEX) provides strong selectivity for charge-based variants, while modern 2D-LC formats – such as AEX → IP-RP or SCX (strong cation exchange chromatography) → HILIC – enhance peak capacity and facilitate low-level impurity isolation. Such orthogonal 2D separations now support both impurity discovery workflows and high-throughput screening in early development.

Here are common strategies and chromatographic modes from the literature:

- Ion-pairing reversed-phase (IP-RP) + MS
  - Selection of the ion-pairing reagent: effect of multiple ion-pairing reagents was investigated for oligonucleotide impurity analysis. For example, six ion-pairing reagents (triethylamine, tripropylamine, hexylamine, N,N-dimethylbutylamine, dibutylamine, N,N-diisopropylethylamine) were compared by Gong L [31] and eight ion-pairing reagents (diethylamine, triethylamine, diisopropylamine, dipropylamine, hexylamine, dibutylamine, octylamine, dihexylamine) in two concentrations (10 mM, 100 mM) buffered with acetic acid were investigated for oligonucleotide impurity analysis by Kadlecová *et al.* [43] while a method was proposed to classify the hydrophobicity of 13 alkylamines by their retention in RP-LC [44];
  - Very common one: use ion-pairing reversed-phase LC (e.g., C18) with TEA/HFIP mobile phases [43,44]. Adequate separation of full-length and truncated oligos are generally achieved even for ASOs which contain mostly PS bonds as HFIP suppresses peak broadening resulting from the diastereoisomers. More lipophilic sugar modification such as 2′-O-MOE or 2′-O-Me are resulting in retention time shifts and elute later than their DNA analogue and can lead to loss in resolution if the oligonucleotide contains different types of sugar modifications. The same trend can be observed for more lipophilic backbone modifications. 5′-terminal lipophilic conjugates tend to result in a large retention time shift making the chromatographic separation easier since the conjugate is coupled to the growing chain at the last step. Then MS (often ESI in negative mode) is used to detect and deconvolute charge states.
- 2D LC (2D-LC) + MS
  - To improve separation (especially of closely related impurities), 2D-LC has been used: for instance, heart-cutting between anion-exchange (AEX) and reversed-phase [45]. Example: AEX (first dimension) separates based on charge; then transfer selected fractions ('heart-cut') into a second dimension (e.g., HILIC) that is MS-compatible for desalting and further separation [32];
  - Another 2D method: quinine-based weak anion exchange is used in the first dimension, then IP-RP is used in the second dimension, enabling sensitive MS detection of siRNA or oligonucleotide impurities [46].
- Ion-pairing hydrophilic interaction LC (IP-HILIC)
  - A more MS-friendly mode. For example, IP-HILIC has been used to

separate very subtle impurities (like deaminated oligonucleotides) that differ by <1Da [24]. Adjustment of ion-pairing reagent, pH, and temperature are key parameters to optimize separation [24];

- Two reviews regarding using IP-HILIC chromatography for oligonucleotide impurity analysis were published in 2024 [9,47].

- High-resolution MS (HRMS)

  - Use of TOF (time of flight), Orbitrap and FT-ICR (Fourier transform ion cyclotron resonance) MS allows precise mass measurement, charge state determination, and modeling of isotopic distributions, which is useful for low-level impurities [33];

  - High mass accuracy helps confirm the chemical composition of impurities (truncated, internal deletion, adducts, etc.) [33,48].

- Routine/QC-MS (nominal mass)

  - For routine impurity profiling, a single-quadrupole MS (nominal mass) system can work, as shown in the Ionis platform method by Rentel *et al.* [25]. However, such a method relies on quantification of many impurities by mass as chromatographic resolution is not possible, increasing complexity. The authors believe that it is advisable to accomplish at least partial resolution of all major impurities chromatographically, which is often possible for ASOs or siRNA after scouting for different method conditions including different aliphatic amines, organic solvents, different columns (chemistry/dimensions) and column temperatures. Once a sufficiently adequate resolution has been achieved through scouting, Design of Experiments (DOE) can be performed to further optimize resolution. In case the resolution is only partial, quantification of impurity by mass should be compared to quantification by UV to show that the quantification by UV gives similar results. Only in cases where the resolution is insufficient to allow for a quantification by UV, a quantification by mass would need to be established [1,43,44]. Vendor application notes [26–28] have shown how to detect n−1 (truncated) impurities, adducts, etc., using LC-UV-MS. Software is readily available to categorize and label impurities automatically by just providing the sequence information [28];

  - Agilent application note: their InfinityLab Pro iQ Plus LC/MS system (unit mass detector with deconvolution software) is used to identify full-length, n−1, n+1, depurinated, alkylated species, etc. [26].

- MS/MS (fragmentation/sequencing)

Once a candidate impurity is detected at the intact-mass level, MS/MS fragmentation is essential for confirming its identity and localizing structural variations [11–13,49,50]. Following chromatographic separation, tandem MS enables precise annotation of modifications, truncations, and other impurity features. Enzymatic digestion approaches, such as Nuclease P1–based bottom-up RNA sequencing, may also be employed for structural confirmation [51].

Collision-induced dissociation (CID) generates primarily a/B/Y ions through slow, ergodic energy deposition. Although broadly applicable, CID often produces substantial base loss and yields limited sequence information for long or heavily modified oligonucleotides. Higher-energy collisional dissociation (HCD) improves

fragmentation richness, producing mixed a/B/C and x/y/z ions, and is particularly effective for phosphorothioate (PS) oligonucleotides. Nonergodic activation methods such as electron-transfer dissociation (ETD) yield predominantly C/Z ions while preserving labile modifications, supporting localization of PS linkages, ribose alterations, and conjugation sites. EThcD, which couples ETD with supplemental collisional activation, produces complementary C/Z and a/B/Y ions and often provides the most complete sequence coverage for structurally complex antisense oligonucleotides. Ultraviolet photodissociation (UVPD) delivers extensive high-energy fragmentation across nearly all ion types (a, b, c, d, w, x, y, z), enabling unmatched resolution of sequence isomers, n−1 variants, and other low-abundance impurities.

Oligonucleotide fragmentation follows McLuckey–Hirokawa nomenclature **(Figure 2)**, wherein a, b, and c ions retain charge on the 5′ fragment, x, y, and z ions retain charge on the 3′ fragment, and w and d ions reflect internal-cleavage pathways [52–55]. Base-loss ions such as [M–B]are characteristic of CID/HCD. Because each activation method biases toward a specific ion-type distribution, method selection directly influences the confidence and depth of impurity identification.

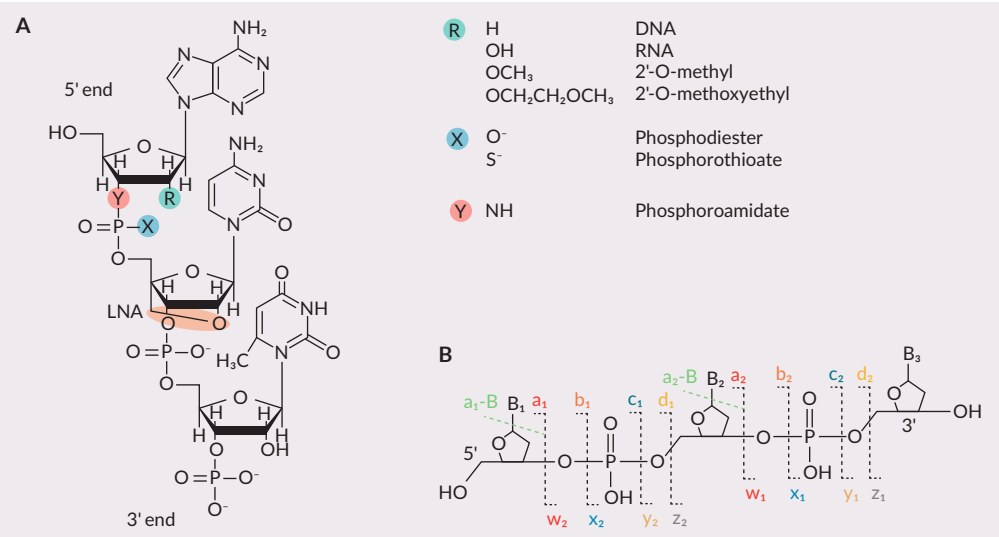
Together, the integration of chromatographic selectivity, adduct management, and tailored MS/MS activation strategies enables comprehensive structural elucidation of oligonucleotide impurities. Coupling orthogonal LC workflows with advanced fragmentation techniques such as ETD, EThcD, and UVPD provides unprecedented analytical clarity, supporting both impurity characterization and deeper mechanistic insight into synthetic failure pathways.

EXAMPLE OF A RECOMMENDED WORKFLOW FOR OLIGONUCLEOTIDE IMPURITY PROFILING BY LC–MS

The workflow for oligonucleotide impurity profiling by LC–MS includes optimized steps from sample preparation through

FIGURE 2

Oligonucleotide structure with typical modifications and McLuckey/Hirokawa nomenclature for fragmentation by MS.



(A) RNA with modifications. (B) McLuckey/Hirokawa nomenclature for fragmentation. LNA: locked nucleic acid. Reprinted from **[56]**).

reporting. Samples are typically diluted to 5–50 µg/mL in low-salt conditions, with desalting, or SPE recommended for PMOs (phosphorodiamidate morpholino oligomers as PMOs lack the negatively charged phosphate backbone typical of DNA and RNA and have poor ionization efficiency in conventional ESI negative mode, often requiring specialized conditions such as positive-mode ESI, alternative ion-pairing reagents, or HILIC-based separations). Chromatographic method choice depends on oligonucleotide type: IP-RP is preferred for PS ASOs and siRNAs, HILIC for PMOs and lipid conjugates, and 2D-LC for challenging low-abundance or matrix-suppressed impurities. Mass spectrometry is generally run in high-resolution negative mode, using ion-pairing systems that minimize adduct formation. Deconvolution should validate charge-state symmetry, minimize adduct contributions, and confirm peak purity. Fragmentation is selected based on analytical goals – EThcD or ETD for sequence confirmation, CID/HCD for truncation impurities, and UVPD for oxidation mapping, PS stereochemistry or modified RNA. For reporting, impurities ≥1.0% must be identified, with structural assignments required for truncations, oxidized/desulfurized species, and conjugation-site impurities. Any newly observed impurity above this threshold (≥1.0%) should be supported with MS evidence.

Sample preparation

- Dilute to 5–50 µg/mL in low-salt buffer or EDTA or water
- For PMOs: desalting or SPE is recommended prior to LC–MS

Chromatographic method selection

- Ion-pairing reversed-phase (IP-RP)
  - Best for: PS ASOs, siRNAs, duplexes
  - Ion-pairing reagents: TEA, DIPEA, DMBA, tributylamine
  - Strengths: excellent retention, robust impurity separation
  - Weaknesses: metal interactions; system conditioning is required

HILIC

- Best for: lipid conjugates, charged-neutral hybrids, PMOs
- Strengths: compatible with MS, excellent for isobaric impurities
- Weaknesses: require careful control of water gradient

2D-LC (such as AEX → IP-RP, AEX → HILIC, SCX → HILIC, WAX → HILIC, etc.)

- Best for: low-level impurities, matrix suppression, oxidized species
- Strengths: heart-cut selectivity, sharp peaks, improved sensitivity

Mass spectrometry acquisition

- Resolution ≥60,000 (Orbitrap) or TOF equivalent
- Negative mode for most oligonucleotides; positive mode optional for conjugates
- Use adduct-minimizing conditions (HFIP/TEA or DMBA/AA)

Deconvolution

- Use sliding-window or Bayesian deconvolution
- Check:

- Charge envelope symmetry
- Adduct suppression
- Peak purity

Decide on fragmentation strategy

The MS fragmentation strategy is selected based on the type of task to be performed, as shown in **Table 5**.

Reporting and release criteria

- Identify impurities ≥0.5% (or lower if possible)
- Structural assignment required for:
  - n−1 and n−2 truncations
  - Oxidized + desulfurized species
  - Conjugation-site impurities

Provide MS evidence for any new impurity ≥0.5% (or lower if possible).

PRACTICAL CONSIDERATIONS IN METHOD DEVELOPMENT

Effective method development for oligonucleotide LC–MS requires careful control of sample quality, chromatographic conditions, MS settings, data analysis, and validation. Cleanliness is crucial – desalting minimizes ion suppression, and sample concentration must balance sensitivity with avoiding ion-source saturation. System cleanliness is crucial as well to avoid ion adducts/interferences. Chromatographic choices should match the oligonucleotide type: C18 columns for ion-pairing reversed phase and HILIC columns for IP-HILIC. Mobile phases must be MS-compatible and volatile, and gradients optimized to resolve subtle impurities. Elevated temperatures (e.g., ~80 °C) can improve separation, particularly in HILIC while high temperature may also lead to degradation depending on the chemistry. In MS acquisition, negative mode is standard, with scan ranges and resolution tuned for expected charge states and high-resolution deconvolution. Adduct formation must be minimized through low-salt conditions and proper source optimization. Data analysis relies on accurate deconvolution, impurity quantitation via extracted ion chromatograms, and MS/MS confirmation when structural ambiguity remains. For QC use, methods must be qualified or validated per ICH Q2(R2), including assessments of sensitivity, specificity, linearity, accuracy, precision, and solution/mobile-phase stability.

Sample preparation

- Depending on the sample matrix, desalting could be critical. Oligonucleotide solutions must be sufficiently 'clean' to minimize inorganic salt adducts;

TABLE 5

Decide on fragmentation strategy.

Purpose	Recommended activation
Sequence confirmation	EThcD > ETD > HCD
Impurity assignment (n−1, n−x)	CID/HCD
Oxidation mapping	HCD or UVPD
PS stereochemistry assessment	ETD/UVPD
Modified RNA/conjugates	UVPD

- Concentration strength: enough material required to see low-abundance species, but ion source overloading needs to be avoided.

LC conditions

- Choose a compatible column: C18 for IP-RP, or HILIC for IP-HILIC. While C18 is the work horse, other column types for RP might be used as well. Conjugates might require a different type of column. For cholesterol or lipid conjugates, C4 columns often work best. Sometime, phenyl columns work better than C18 **[1]**
- Mobile phase: volatile buffers (e.g., alkylamine acetate) are preferred for MS compatibility; balance the resolution and minimize nonvolatile salts;
- Gradient optimization: to resolve not just full-length from truncated species but also from more closely related impurities;
- Temperature: high temperatures (e.g., 80 °C) have been used to improve resolution in HILIC **[24]** while most IP-RP methods use 50–60 °C **[1]**.

MS parameters

- Ionization mode: choose a suitable (the negative ionization mode is commonly used for most oligonucleotides while the positive ionization mode is recommended for PMOs);
- Scan range: depends on expected charge states;
- Resolution: for HRMS methods, higher resolving power is needed to deconvolute isotopic envelopes;
- Adduct control: optimize source conditions, maybe include source cleaning, use low salt, etc.

Data analysis

- Deconvolution: because of multiple charge states, a deconvolution software is needed to deconvolute to a zero-charge mass;
- Quantitation: using extracted ion chromatograms (EIC) for each impurity;
- Sensitivity threshold: define what 'impurity' means (e.g., lower limit of detection, percent relative to main species);
- Confirmation: for structurally ambiguous impurities, use MS/MS to get sequence-level confirmation.

Validation

- If used for QC, method needs to be qualified for early phase clinical development or validated per ICH Q2(R2) (LOD/LOQ, specificity, linearity, precision, accuracy, reproducibility, mobile phase/solution stability, etc.).

EXAMPLES FROM LITERATURE

Heart-cutting 2D-LC–MS/MS for impurity identification [45]

A recent study by Zhou *et al.* **[45]** applied heart-cutting 2D liquid chromatography (2D-LC) coupled to tandem mass spectrometry (MS/MS) to characterize impurities in therapeutic oligonucleotides. Traditional LC–MS methods often fail to resolve subtle but critical impurities – such as oxidation and hydrolysis products – because of limited chromatographic selectivity or MS-incompatible mobile phases. To improve separation, several orthogonal LC combinations have been investigated, including HILIC × WAX **[7]**, IP-RP × IP-RP, IP-RP × HILIC, RP × RIPC, IP-RP × SAX and

AEX × IP-RP ([45] and references within). However, many of these pairings suffer from poor MS compatibility or extended run times.

Heart-cutting 2D-LC as shown in **Figure 3** resolves these challenges by transferring a precisely defined region of interest (the heart cut) from the first dimension into an orthogonal second separation. This approach improves resolution, reduces matrix interferences, and supports MS-compatible conditions.

In the workflow by Zhou *et al.* [45]:

- First dimension: anion-exchange chromatography (AEX) separates oligonucleotides by charge (column 1: DNA-PAC 100);
- Second dimension: ion-pairing reversed-phase chromatography (IP-RP-LC, column 2: C18) provides high-efficiency, MS-compatible separation prior to MS/MS detection.

Applied to two approved RNAi therapeutics – givosiran and patisiran – the method identified 3 and 20 impurities, respectively. Data-dependent MS/MS provided detailed fragmentation information, enabling molecular weight confirmation and structural elucidation.

This heart-cutting 2D-LC–MS/MS strategy represents a major advancement in oligonucleotide impurity profiling, offering high sensitivity, orthogonality, and reliable structural identification.

Ion-pairing hydrophilic interaction chromatography (IP-HILIC) for resolving deaminated impurities [24]

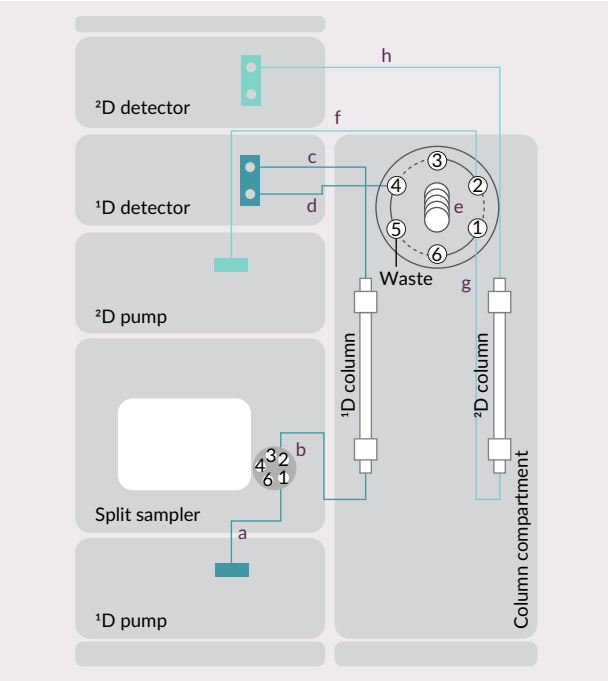
Tutiš *et al.* [24] introduced ion-pairing hydrophilic interaction chromatography (IP-HILIC) as an alternative MS-compatible separation mode for detecting deaminated impurities in therapeutic phosphorothioate oligonucleotides. Deamination (mass shift: +0.98 Da) can occur at multiple nucleobase positions when exposed to extended periods of time to high pH buffers (20 mM NaOH), used for the purification of pH stable oligonucleotides such as ASOs and is notoriously difficult to detect in MS due to minimal mass differences relative to the full-length product (FLP).

The authors demonstrated, as shown in **Figures 4 and 5**, that IP-HILIC, using triethylamine acetate and a BEH amide column, effectively resolves deaminated variants from:

- The FLP of a GalNAc-conjugated 16-mer phosphorothioate oligonucleotide, and
- The corresponding non-conjugated FLP (NC-FLP).

FIGURE 3

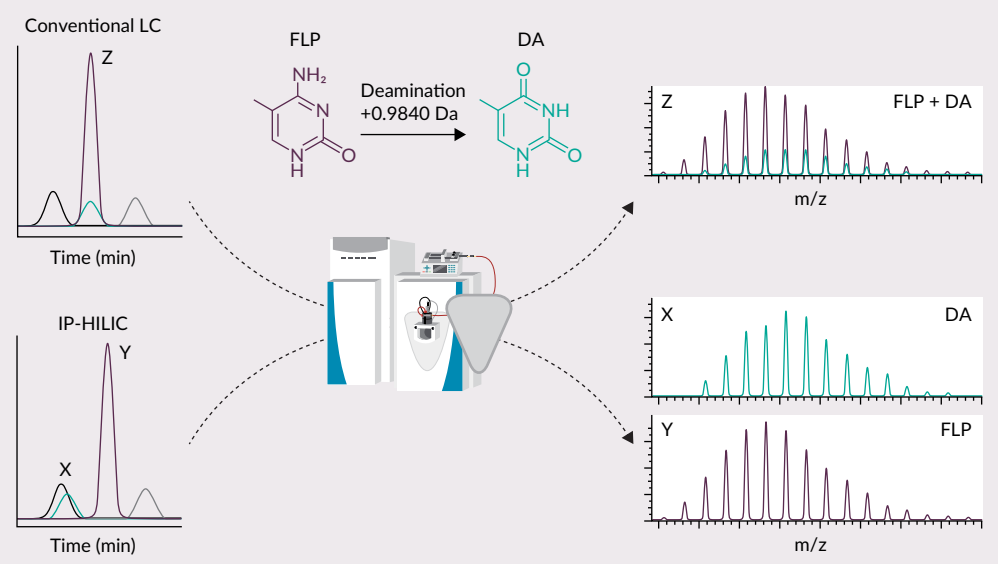
Diagram for heart-cutting 2D-LC.



(A) Viper capillary. (B) active pre-heater. (C) Post-column cooler. (D) Viper capillary. (E) Loops. (F) Viper capillary. (G) Active pre-heater. (H) Post-column cooler. (I) Viper capillary. (J) Viper capillary. Detector 1: UV. Detector 2: MS or MS/MS. Reprinted from Thermo Scientific Technical Note 73298 [57].

FIGURE 4

IP-HILIC-UV-MS separation and isotope patterns for deaminated impurities.



Republished from [24].

Although deaminated species and n−1 impurities coeluted, MS clearly differentiated them due to their distinct mass differences. The study further illustrates (in **Figure 6**) how ion-pairing reagent hydrophobicity alters retention behavior in HILIC versus RPLC (reverse phase liquid chromatography), significantly improving separation selectivity.

Single-quadrupole LC–MS impurity profiling (Agilent application note + ionis golden method) [25,26]

An Agilent application note demonstrated that single-quadrupole LC–MS instruments can effectively profile impurities in a 20-mer phosphorothioate oligonucleotide, detecting ~25 ions above 0.2% relative abundance using the established IP-RP-LC–MS method.

Accurate impurity profiling requires full-scan acquisition across the main chromatographic peak and may demand mass-range coverage beyond m/z 2000, a challenge for many unit-mass detectors. The Agilent InfinityLab Pro iQ Plus provides enhanced ion transmission at high mass ranges, enabling robust impurity detection suitable for QC environments.

A key step in the workflow involves discriminating true impurities from adducts by comparing spectra acquired under:

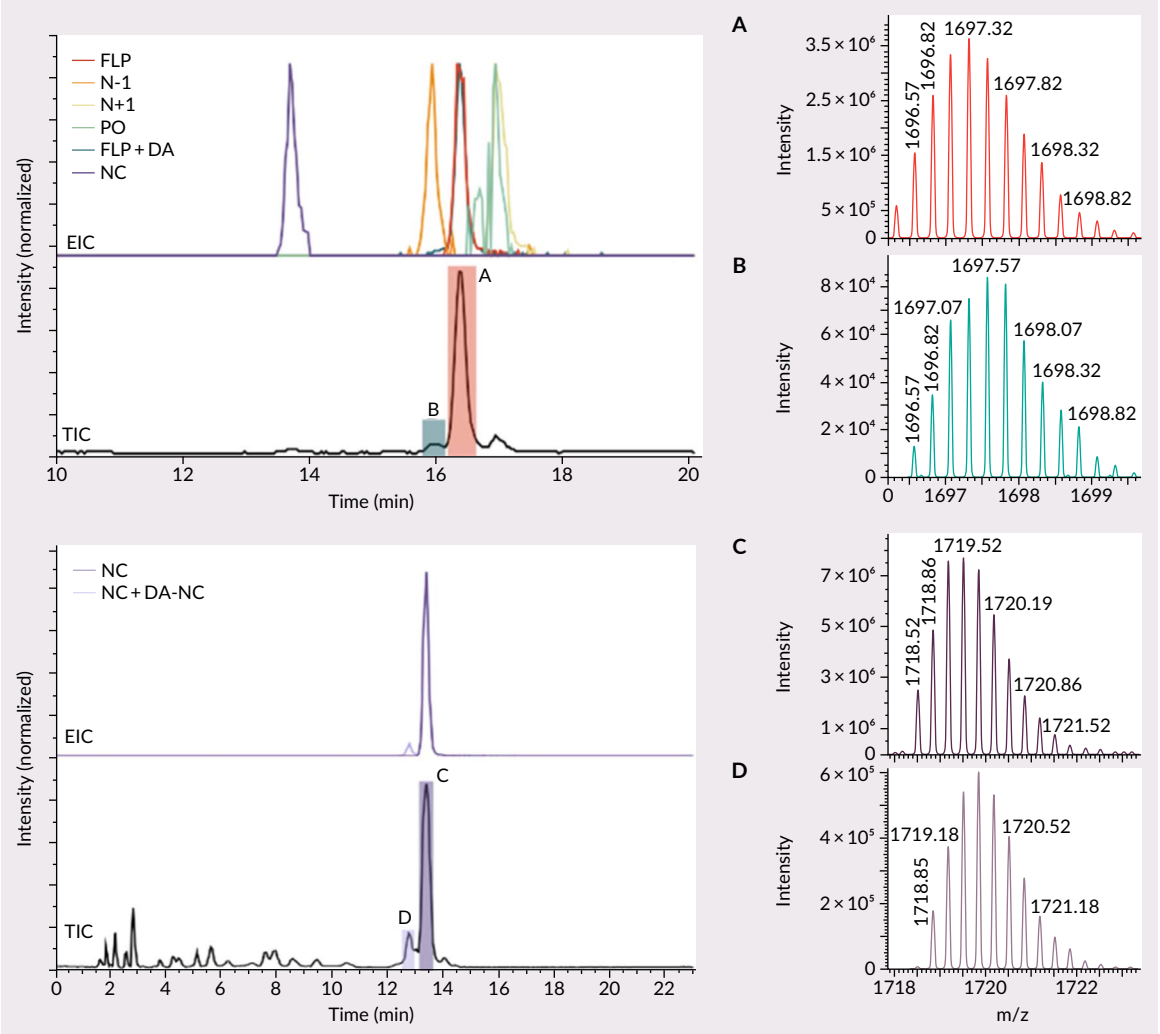
- Standard ESI conditions, and
- Harsh (higher-temperature) conditions, which suppress adduct formation.

As shown in **Figure 7**, ions that disappear under harsh conditions are classified as adducts. This approach enabled precise impurity assignments within the 4− charge state region.

Rental *et al.* [25] published a similar workflow, which was developed and used as the platform IP-HPLC-UV-MS method for different oligonucleotide drug substances and products. **Figure 8** shows the mass spectrum of the main peak with assigned impurities and adducts. This IP-HPLC-UV-MS method for assay, purity (combined UV and MS purities) and impurity

FIGURE 5

Deaminated species resolved from FLP by IP-HILIC-UV-MS.



Left: IP-HILIC-UV-MS of (top) mixture of FLP and impurities at 2% and (bottom) mixture of NC with DA-NC at 2% total-ion chromatograms (TIC) and extracted-ion chromatograms (EICs; ±5 ppm m/z) for 1696.32 (FLP), 1611.07 (N – 1), 1783.58 (N + 1), 1694.33 (PO), 1697.32 (FLP and DA), 1719.52 (NC), and 1718.52 (NC and DA-NC) for the mixtures are plotted. Right: mass spectra showing the isotope patterns of the **(A and B)** [M–4H]4– ions and **(C and D)** [M–3H]3– ions observed in the peaks highlighted in the corresponding TICs. NC: non-conjugated FLP. DA: deaminated FLP. DA-NC: deaminated non-conjugated FLP. Republished from [24].

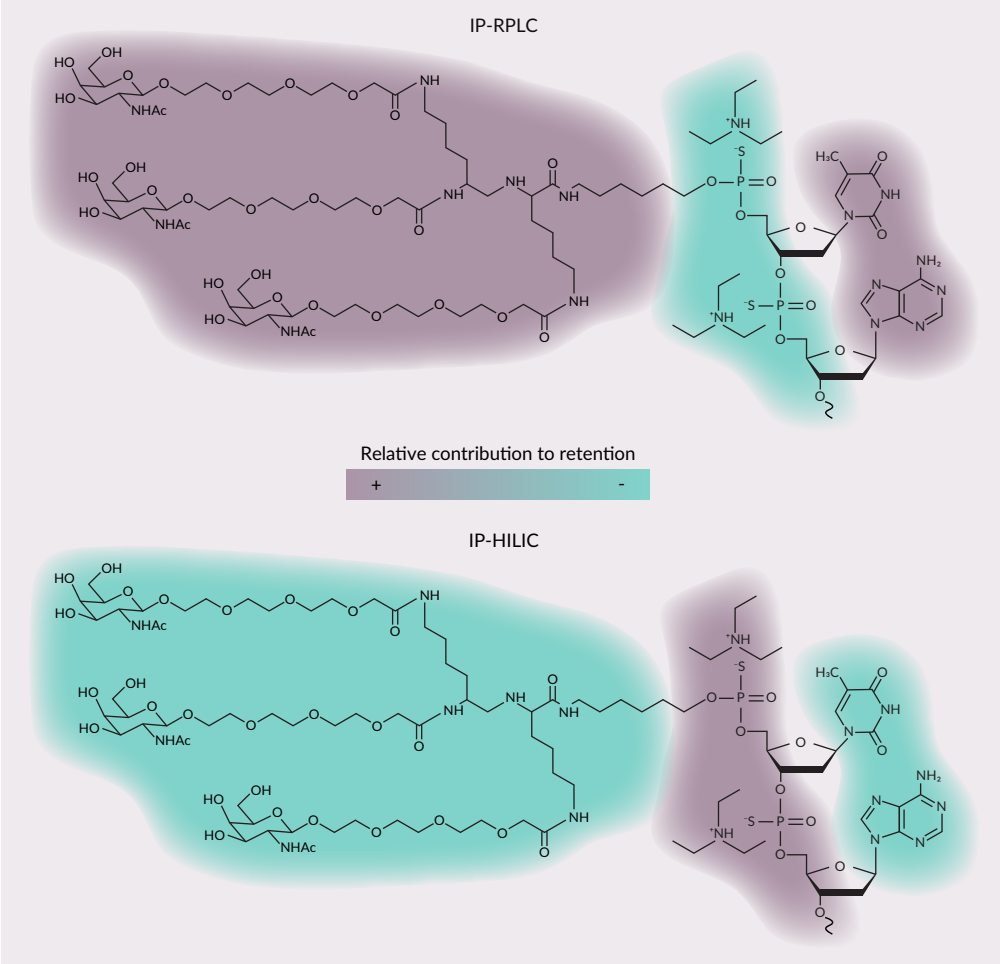
profile analysis has been validated for >40 different oligonucleotide sequences, showing consistent results across the platform. The method has been accepted by regulatory agencies in >50 countries for release and stability testing of mipomersen (KYNAMRO®), nusinersen (SPINRAZA®), volanesorsen (WAYLIVRA™), tofersen (Qalsody®) and an additional 60+ drugs in various stages of clinical development (in Ionis and Biogen).

Integrated Waters Xevo G3 QTof workflow for intact mass and MS/MS sequencing [30]

The Waters Xevo™ G3 QTof workflow [30] integrates high-resolution QTof-MS with dedicated informatics to support intact mass analysis and MS/MS sequencing of synthetic oligonucleotides and their impurities. Operated within the compliant-ready waters_connect™ environment,

FIGURE 6

Mechanistic schematic of IPR effects in IP-HILIC vs IP-RPLC.



Republished from [24].

the system enables seamless acquisition, deconvolution, and fragment-ion annotation.

Two software applications streamline impurity characterization:

- INTACT™ Mass App: automated deconvolution and mass assignment for FLP and impurity candidates
- CONFIRM™ Sequence App: automated fragment-ion annotation from DIA (MSE) and targeted MS/MS data, enabling detection of sequence deletions, insertions, scrambling, and chemical modifications

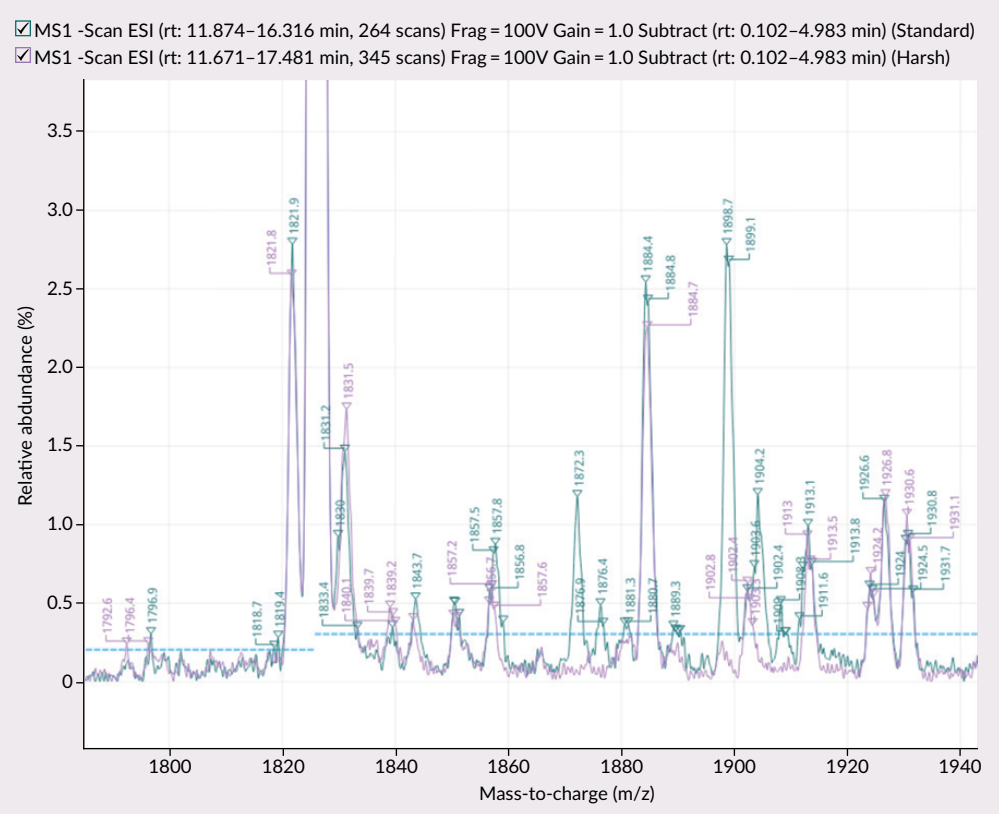
Coupled with the ACQUITY™ Premier UPLC, the system provides a sensitive and robust platform suitable for both characterization studies and QC workflows. In a representative experiment, 16 low-level impurities were detected in a modified 21-mer siRNA, with the lowest at 0.05% (UV area%, peak #1 in **Figure 9**).

TRANSLATIONAL INSIGHT

LC–MS has emerged as a powerful and increasingly practical tool for translating oligonucleotide impurity profiling from research settings into routine QC and regulated environments. Its major strength lies

FIGURE 7

Overlay of standard versus harsh ESI spectra illustrating adduct discrimination.



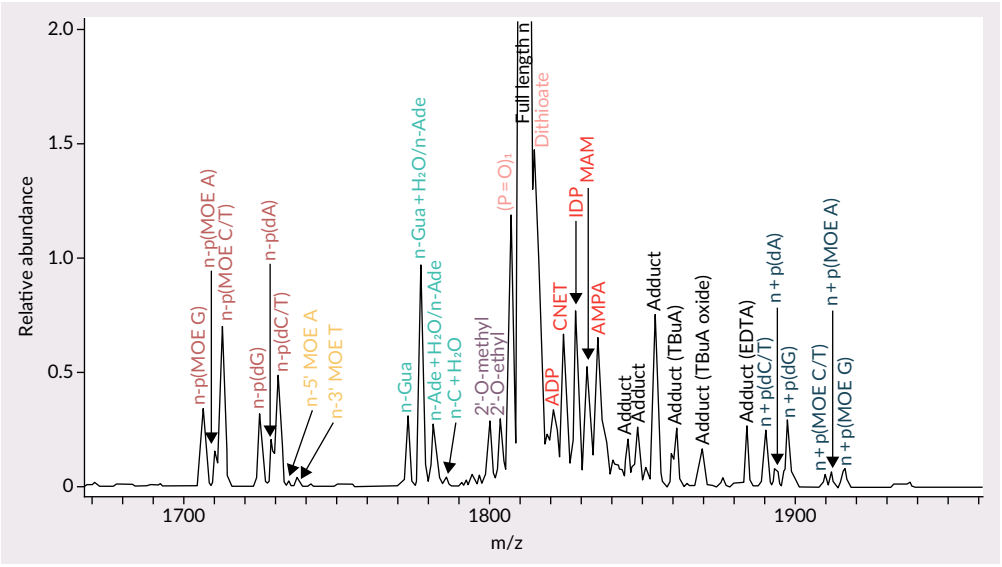
The Oligo Analysis Accelerator software user interface enables direct inspection of the overlaid spectra. Dashed lines indicate the thresholds: 0.2% 'prepeak' (m/z values less than the 4– charge state of the full-length product) and 0.3% 'postpeak' (m/z values greater than the 4– charge state of the full-length product). The software then automatically classifies ions accordingly based on whether the harsh-condition ions are still above the threshold. Republished from Agilent Application Note [26].

in its high sensitivity and specificity, with reported limits of quantitation as low as ~0.2% and the ability to resolve full-length oligonucleotides from truncated, adducted, or chemically modified variants based on molecular weight. Robustness has been demonstrated across laboratories: a multicenter validation of an ion-pairing LC–MS method for mipomersen met key accuracy and precision criteria, although notable carry-over in several labs underscores ongoing practical challenges. Importantly, impurity profiling does not always require high-resolution MS – vendors have shown that nominal-mass, single-quadrupole LC–UV–MS platforms can support accessible and cost-effective impurity analysis, aided by increasingly sophisticated software tools capable of automated deconvolution, impurity classification, and compliance-ready reporting. For structurally challenging or isobaric impurities, more advanced approaches such as LC–ion mobility–MS are being explored, including FDA-led work demonstrating their utility in resolving species that elude conventional LC–MS. Routine QC feasibility has also been demonstrated through 24-hour stability studies on single-quadrupole systems with automated workflows to support high-throughput analysis.

Despite these translational advantages, several limitations temper broad implementation. Oligonucleotide spectra remain

FIGURE 8

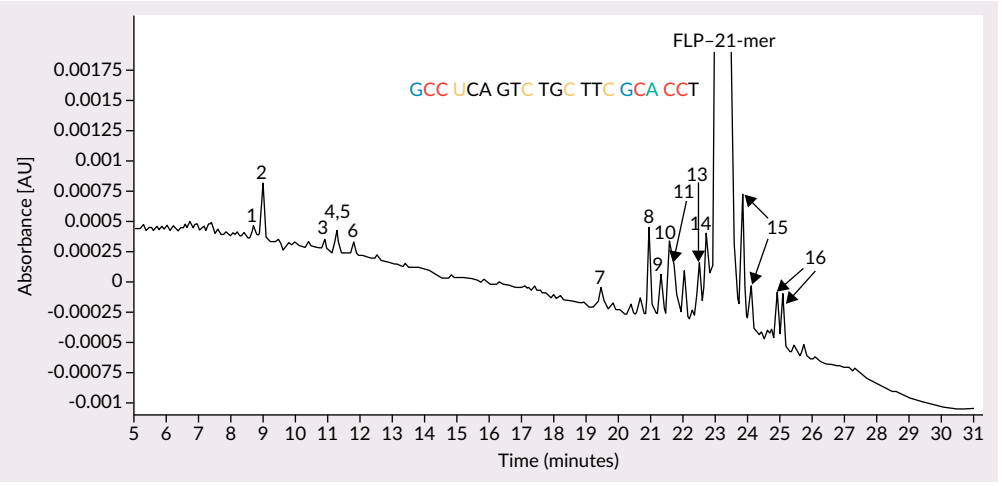
Identification of co-eluting impurities in mass spectrum of IP-LCMS main peak.



Showing species lacking a nucleotide (n−1, brown), a nucleoside (TPT, orange), and a nucleobase (abasic, green); impurities with modifications of the sugar (2¢-O-alkyl, purple), the internucleotide linkage (phosphodiester or [P=O], and dithioate, pink), and the nucleobase (ADP, CNET, IPD, MAM, and AMPA, red); addition of a nucleotide (n+1, blue). ADP: acetyldiaminopurine. AMPA: 3-(3-acetyl-4-methylpyrimidin-2-one-6-yl)-2-aminoimidazole. CNET: N3-(2-cyanoethyl)thymine. IDP: isobutyryldiamino purine. LCMS: liquid chromatography mass spectrometry. MAM: N-methylacetamidomethyl. TPT: terminal thiophosphate. Republished from **[25]**.

FIGURE 9

LC-UV chromatogram showing sixteen low-level impurities.



Republished from Waters Application Note **[30]**.

complicated by sodium and potassium adducts, variable ionization efficiency, and multiple charge states, often requiring careful tuning of 'standard' versus 'harsh' ESI conditions to distinguish true analyte signals. Carry-over persists as a notable

obstacle when monitoring low-abundance impurities. The limited resolving power of nominal-mass instruments restricts detailed characterization of isobaric or isomeric species, making high-resolution MS, ion mobility, or multidimensional separations necessary for deep profiling. Data processing remains a bottleneck: without robust software, manual annotation is laborious and error-prone, and the increasing dimensionality of LC-IM-MS datasets only amplifies this challenge. Regulatory expectations for impurity identification and quantification may exceed what many current workflows address, particularly in the absence of widely available impurity reference standards. Further complexity arises in characterizing rare isomers or phosphorothioate stereochemical variants, which often require specialized tools such as ^{31}P NMR or cyclic ion mobility spectrometry. Additionally, developing high-resolution or multidimensional LC–MS methods is more resource-intensive than routine single-quadrupole approaches, and ensuring consistent performance across instruments and sites (CROs/CDMOs) remains a critical need. Collectively, while LC–MS continues to mature as a translational platform for oligonucleotide impurity analysis, substantial technical, analytical, and standardization challenges remain active areas of development. Pros and cons are shown below.

Pros and strengths

- High sensitivity and specificity
  - LC–MS can detect low-level impurities in oligonucleotides very sensitively. For example, in an Agilent application note, a limit of quantitation (LOQ) down to ~0.2% was achieved;
  - Mass spectrometry (MS) also provides the detection of impurities by mass, which helps distinguish full-length oligonucleotides from impurities such as truncated (n−1, n+1) species, adducts, or chemically modified variants.
- Robustness/reproducibility
  - A multicenter validation study showed that an ion-pairing LC–MS bioanalytical method for oligonucleotides (using mipomersen as a model) met accuracy, precision, selectivity criteria in seven different labs **[58]**;
  - That said, they noted some carry-over (>20%) in a few labs, which is a practical challenge **[58]**.
- Use of nominal mass (single-quadrupole) systems
  - It's not always necessary to have high-resolution MS: instrument vendors (Agilent, Waters, Thermo Fisher Scientific and Shimadzu) demonstrated impurity profiling of oligonucleotides using a single-quadrupole LC-UV-MS system (nominal mass), which is more accessible/lower cost.
- Advanced methods for difficult impurities
  - The FDA has investigated high-resolution ion-mobility MS (IM-MS) coupled with LC to separate isomeric or isobaric impurity species that are otherwise hard to resolve **[29]**;
  - This suggests that additional dimensions (e.g., mobility) are being leveraged to improve profiling as oligonucleotide impurities become more structurally complex.
- Practical QC use
  - According to an Agilent application note, a single-quadrupole LC–MS

system could run impurity profiling in a routine/QC environment over 24 hours with stable ion transmission;

- In the application note, they used software (like Oligo Analysis Accelerator) to automatically classify and quantify impurities, which aids in high-throughput/routine QC analysis.

Cons and limitations/criticisms

- Adducts and ionization complexity
  - Oligonucleotides often form adducts (e.g., sodium adducts), which complicates the mass spectra. Indeed, even in routine oligonucleotide LC–MS, distinguishing true product ions versus buffer adducts can require changing conditions ('standard' vs 'harsh' ESI) and careful interpretation **[26]**;
  - Negative ESI mode is typically used for oligonucleotides, but adducting (especially positively charged adducts with positive mode) is a common issue.
- Carry-over
  - As mentioned in a multicenter validation study, carry-over has been a problem in some labs, which is a problem for correct assay determination **[58]**;
  - Carry-over can be particularly problematic for oligonucleotide impurity profiling when samples with different impurity profiles are injected back-to-back, and low-abundance species are to be detected.
- Resolution/instrument requirements
  - While single-quadrupole systems are usable, they are limited in resolving power compared to high-resolution instruments. This could make identification of very closely related impurities more challenging;
  - For especially challenging impurities, more advanced MS (or additional techniques) may be needed (e.g., ion mobility + MS as referenced FDA work suggests) **[29]**.
- Software/data interpretation
  - Because of the complexity of oligonucleotide spectra (many charge states, adducts, fragment ions), robust data-processing tools are needed. Without suitable software, manual annotation could be laborious and error prone;
  - Automated/compliance-friendly data processing: vendors are providing more mature software workflows (e.g., Agilent's Oligo Analysis Accelerator, Waters' waters_connect, Shimadzu's Insight Biologics, BioPharmaFinder by Thermo Fisher Scientific or PMI-Byos Oligo workflow (Protein Metrics Inc.) **[16]**) to automate deconvolution, impurity classification, and reporting.
- Regulatory/method validation
  - Even though methods have been validated (e.g., in GLP environments) for bioanalysis, impurity profiling for regulatory submissions may require more stringent characterization (identification, quantification of all relevant impurities). The literature doesn't always fully address all impurity-related validation parameters (depending on the study).
- Throughput versus depth trade-off
  - In a QC setting, there's often a trade-off between running a fast method

(for routine monitoring) and deeply characterizing small impurities (which might require longer gradients or high-resolution MS).

- Gaps/challenges still being worked on
  - Even with advanced MS or IM-MS, fully characterizing all low-level impurities (especially rare isomers, diastereomers, or positional variants) is non-trivial **[40–42]**;
  - Demonstrating phosphorothioate isomers to be consistent from batch to batch is challenging without using specialized approaches, such as NMR, or cyclic ion mobility spectrometry (cIMS) **[15,42,59]**;
  - Method development for 2D-LC or high-resolution MS is more complex and resource-intensive than 'routine' single-quadrupole methods;
  - Data interpretation remains challenging: the more dimensions (LC, MS, IM-MS **[15]**), the more complex the data, and robust software with automatic data processing and appropriate validation will be critical;
  - Adducts (Na^+, K^+, and others shown in **Table 4**), and ion suppression continue to be practical hurdles;
  - There is a need for more standardized impurity reference materials (impurity standards) to better validate and benchmark impurity profiling methods;
  - Managing the LC–MS method variations on different systems in QC labs is critical to provide consistent results across different sites (CROs/CDMOs).

SUMMARY

LC–MS has become the central analytical platform for impurity profiling of therapeutic oligonucleotides. Because these molecules can generate an extensive array of structurally subtle impurities – including shortmers, oxidation products, backbone variants, and positional isomers – LC–MS provides the necessary combination of chromatographic resolution and mass accuracy to detect and characterize them. Multiple chromatographic modes are now routinely employed, including IP-RP, IP-HILIC, and increasingly 2D-LC methods, each selected based on the chemical class and the specific types of impurities that must be resolved.

High-resolution mass spectrometry, or at minimum high-fidelity deconvolution of multiply charged envelopes, is essential for confident identification of low-abundance impurities and for resolving species that differ by only a few Daltons. Robust method development remains a critical component of any LC–MS workflow, requiring careful optimization of sample preparation, ion-pairing chemistry, chromatographic conditions, and MS parameters. For quality-control laboratories, formal method validation ensures sensitivity, specificity, accuracy, precision, linearity, and robustness across routine testing environments.

Overall, oligonucleotide impurity profiling by LC–MS sits at an intersection of maturity and innovation. On the mature end, well-established IP-RP LC–MS workflows – even on single-quadrupole platforms – provide reliable impurity monitoring for many therapeutic modalities and continue to support routine QC operations. On the evolving end, increasingly complex impurity challenges have accelerated the adoption of high-resolution MS, orthogonal 2D-LC separations, and ion mobility-enabled analyses to address regulatory expectations for structural confirmation. The field is clearly moving toward higher

resolving power, more automated data handling, and the integration of orthogonal separation strategies, driven by both the scientific complexity of modern oligonucleotides and the tightening of regulatory requirements.

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OLIGONUCLEOTIDES
ANALYTICS

Guide RNAs in CRISPR-based therapeutics: addressing CMC and analytical challenges to improve clinical translation

Kok-Seong Lim

VIEWPOINT

"[...] there is a need to identify the key next steps that the industry must take over the coming years to ensure that analytical frameworks keep pace with the increasing complexity of these therapeutics."

On December 19, 2025, **Róisin McGuigan**, Editor, *Nucleic Acid Insights*, spoke to Kok-Seong Lim, Independent Expert in Cell and Gene Therapy Chemistry, Manufacturing and Control (CMC), about the role of guide RNAs (gRNAs) in CRISPR-based therapeutics, focusing on starting material choices, gRNA-specific CMC and analytical challenges, regulatory priorities, and emerging approaches needed to support successful clinical translation. This article has been written based on that interview.

Robust CMC strategies and fit-for-purpose analytical frameworks are essential to the successful development of guide RNA for CRISPR-based therapeutics. In this article, Kok-Seong Lim explores how starting and raw material selection, manufacturing processes and analytical methods evolve from research settings to late-phase development and commercial supply. He discusses how oligonucleotide length and impurity profiles differentiate

guide RNAs from traditional oligonucleotide modalities and highlights current analytical limitations. He also addresses regulatory priorities, including regulatory harmonization, the application of existing guidance and the qualification of emerging technologies, and considers how platform approaches and novel manufacturing strategies may enable scalable and robust development of CRISPR-based therapeutics.

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GUIDE RNA OLIGONUCLEOTIDE: A CRITICAL MODALITY IN CRISPR-BASED THERAPEUTICS

Discussions of oligonucleotides frequently centre on antisense oligonucleotides (ASOs) and small interfering RNA (siRNA), yet oligonucleotides are also a core component of CRISPR-based therapeutics.

In first-generation CRISPR technologies, a guide RNA (gRNA) oligonucleotide directs the Cas nuclease to a specific genomic sequence, serving as a structural scaffold while also allowing chemical modification. Cas nuclease generates a double-strand break at the target site, leading to activation of the DNA repair machinery in the cells. Similarly, in base editing technology, gRNA guides catalytically inactive Cas9 (dCas9)-deaminase complex to specific genomic sequences, converting DNA nucleotides into different bases without requiring DNA breakage or cellular repair machinery. In other next-generation CRISPR technologies, oligonucleotides play an even more complex role. In addition to guiding Cas9 nickase-reverse transcriptase complex to a specific sequence, prime editing gRNAs (pegRNAs) incorporate both a primer binding site and a reverse transcriptase template, encoding the desired genomic edit within the RNA sequence and enabling base conversions, insertions or deletions without creating double-strand breaks. Besides serving as gRNA, oligonucleotides can serve as donor templates for homology-directed repair, most commonly as single-stranded oligodeoxynucleotides or plasmid-based DNA donors, delivered using various different methods. This is outside the scope of this discussion.

With these diverse functions, gRNA development presents distinct CMC and analytical challenges that must be addressed, particularly as more CRISPR programs transition from research settings into clinical development.

CMC STRATEGY FOR OLIGONUCLEOTIDES: FROM R&D THROUGH COMMERCIAL MANUFACTURING

In the early stages of CRISPR therapeutics development, particularly in academic settings, R&D grade oligonucleotides are typically produced at a small scale in a research lab or non-GMP facility. In this early phase, multiple gRNA oligonucleotide candidates are used in *in vitro* and preclinical models, including mice and non-human primates, to support drug candidate screening, optimization and selection process. In contrast, GMP-grade gRNAs, more accurately termed gRNAs manufactured under cGMP conditions and referred to as GMP-manufactured gRNAs in this article, are manufactured at a larger scale in GMP facilities, and process and analytical development efforts generally focus on a single candidate selected for the clinical program. GMP-manufactured gRNAs support human *in vivo* or *ex vivo* use in late-stage clinical trials and commercial supply.

From a manufacturing perspective, control of materials represents a key difference. At early phase, researchers often rely on readily available starting materials

and raw materials that are minimally characterized to produce R&D grade oligonucleotides. These materials often have informal, evolving specifications and are often produced by vendors that lack established quality management systems. These materials may also contain higher amounts of impurities. As programs transition from research to GMP manufacturing, starting materials and raw materials require tighter control, as impurities in the materials can carry through to the final drug product and impact safety and efficacy. For gRNAs (and other oligonucleotides), protected nucleoside phosphoramidites (5′-DMT-protected nucleoside-3′-phosphoramidite) are considered starting materials. Certain impurities in these materials, such as 3′-DMT-5′-phosphoramidite isomer, can react similarly to the intended monomer during coupling and may therefore be incorporated and accumulate in the final drug substance. Due to the length of gRNAs, such incorporated impurities may be difficult to detect, making early detection and control at the starting material stage critical. Accordingly, vendors must be qualified and material specifications justified and established in a phase-appropriate manner, with defined limits for critical impurities in starting and raw materials.

Control of manufacturing process is another key aspect. A significant distinction between R&D-grade and GMP-manufactured gRNA lies in manufacturing scale. R&D-grade gRNA is typically produced at the milligram scale, whereas GMP-manufactured gRNA is usually manufactured at the gram scale or higher. R&D production adjust synthesis and purification parameters empirically whereas GMP manufacturing applies defined, phase-appropriate process controls with in-process analytical testing, supported by design of experiment (DOE) studies and aligned with quality by design (QbD) principles under ICH Q8, Q9 and Q11. In laboratory settings, purification may rely on desalting or simple purification methods, whereas GMP-manufactured gRNAs are more commonly purified using chromatographic methods followed by ultrafiltration and potentially lyophilization. As a result, R&D grade gRNAs tend to have variable purity levels compared to GMP-manufactured gRNAs, depending on the manufacturer.

Analytical requirements also diverge substantially. R&D grade gRNAs are often characterized using only a small set of unqualified assays, including limited purity assessment. In contrast, GMP-manufactured gRNAs undergo about 10-20 release tests, which are qualified or validated based on ICH Q2(R2). Furthermore, purity data often lack continuity across stages. For example, ion-pair reverse-phase (IPRP) HPLC is often used in both R&D and GMP settings to measure oligonucleotide purity and impurities. However, differential method resolution between R&D and GMP settings affects the separation and quantification of oligonucleotide impurities (particularly the truncated species) and therefore affect reported purity values. Improved chromatographic resolution in the GMP method often reveals impurities not detected by the R&D method, resulting in lower measured purity value. Lastly, although gRNA purity of around 80% may be acceptable in early phase clinical development, the regulatory expectations would generally target greater than 90%, as analytical methods move from qualification in early phases to full validation in late and commercial phases.

gRNA-SPECIFIC CMC CONSIDERATIONS RELATIVE TO TRADITIONAL OLIGONUCLEOTIDES

The intrinsic properties of gRNAs introduce CMC challenges that differ from those associated with traditional oligonucleotide drugs such as ASOs and siRNAs. One of the key differences between

traditional oligonucleotides and gRNAs lies in their length.

Firstly, oligonucleotide length directly affects both manufacturing complexity and product yield during solid-phase phosphoramidite chemical synthesis. For typical siRNA (19–23 nucleotides) or ASO (15–25 nucleotides), crude synthesis yield is often in the range of 40–80%. However, when oligonucleotide length increases to approximately 100 nucleotides, as is common for CRISPR-Cas9 gRNAs, crude yield can drop substantially, often to around 30–50%. The drop in crude yield is primarily driven by cumulative losses in coupling efficiency with increasing oligonucleotide length during solid-phase synthesis. Even with a per-cycle coupling efficiency of 99%, a synthesis involving around 100 cycles will result in a significant reduction in overall yield. While Cas9 gRNAs are close to 100 nucleotides, gRNA length can vary widely depending on the Cas enzyme and editing strategy employed. For example, Cas12a gRNAs may be as short as 40–50 nucleotides, whereas prime editing gRNAs can be longer at 120–200 nucleotides. Longer gRNAs are inherently more susceptible to degradation, which adds another layer of complexity compared with traditional oligonucleotides. While chemical modifications to gRNA have been used to improve stability and potentially reduce immunogenicity, they also increase manufacturing cost and complexity.

Oligonucleotide length also affects crude purity, which usually falls between 30% and 80%. Similar to crude yield, longer oligonucleotides are associated with lower cumulative coupling efficiency and lower crude purity due to the accumulation of truncations and other types of modifications. After purification, the oligonucleotide purity can usually reach >80%, depending on the purification method employed during manufacturing. The final purity of ASO and siRNA is often higher at 95% or above, whereas the final purity of gRNAs typically falls within the range of 80%–95%, depending on their length.

Impurity profiles are also impacted by oligonucleotide length. ASOs and siRNAs typically show fewer impurity species than longer gRNAs. As length increases, impurity profiles become considerably more complex, with increased contributions from truncations, insertions, and, in the case of phosphorothioate chemistry, stereoisomers. In manufacturing, the commonly used purification method, anion exchange chromatography (AEX), lacks the resolution to separate truncated impurities, especially in long gRNAs such as 100-mers. As a result, hundreds or thousands of impurities are detected. Analysts would then need to decide which impurities require identification or qualification based on regulatory expectations.

Lastly, unlike ASO and siRNAs that bind to target RNA to cause degradation or steric blocking leading to reversible and transient effect, gRNA guides the CRISPR enzyme by hybridizing to DNA sequence, leading to genome editing that is permanent and heritable (DNA targeting remains the primary approach in gene editing while RNA targeting is an emerging one). For this reason, assessing off-target specificity and associated toxicity is a key regulatory requirement for gRNA due to the irreversible consequences.

All things considered, there is a need to identify the key next steps that the industry must take over the coming years to ensure that analytical frameworks keep pace with the increasing complexity of these therapeutics.

INDUSTRY & REGULATORY PRIORITIES FOR ADVANCING gRNA ANALYTICAL FRAMEWORKS

In order to ensure that analytical frameworks keep pace with the complexity of CRISPR-based therapeutics, there are

three aspects to consider. The first is harmonization of guidance across regulatory agencies [1,2,3], the second is the application of ICH (International Council for Harmonisation) guidelines, and the third is the standardization and qualification of analytical technologies.

Firstly, regulatory harmonization is particularly important in areas such as impurity characterization and analytical control. Most existing oligonucleotide guidance is focused on traditional oligonucleotide therapeutics and does not explicitly address gene editing gRNAs. So additional clarification is needed on how current guidance should be applied to gRNAs used in CRISPR applications.

The industry needs clearer alignment on how to characterize gRNA impurities and how to link them to nonclinical and clinical attributes. A central issue is the depth of impurity characterization across development stages. Full separation of individual impurities is not feasible, and impurities with similar molecular weights often co-elute during purification. Therefore, robust analytical tools are needed to resolve and identify these closely related impurities.

At present, no single technology can fully sequence long gRNA molecules while simultaneously mapping chemical modifications in the oligonucleotide and its impurities. Liquid chromatography-mass spectrometry (LC-MS) often requires enzymatic digestion of gRNAs to generate smaller fragments suitable for analysis and modification mapping, while next generation sequencing (NGS) provides complementary sequence information. Open questions remain regarding validation of NGS as a quantitative analytical method. Current industry experience supports NGS as a reliable qualitative tool for characterization of sequence variants in impurities due to high sensitivity. FDA recommends the use of NGS in combination with mass spectrometry for impurity characterization and identification of any impurities present at levels of 1% or higher. In this context, NGS extends beyond an gRNA identity test and serves as an orthogonal purity assay for identification (and potentially quantitation) of sequence variants. Establishing a clear relationship between variant levels measured by NGS and those reported by mass spectrometry remains an area for further development. The next issue is clinical relevance. We need to understand and align on how these impurities, impurity-driven off-target effects, and potency and safety are connected, as this should guide control strategies and specifications throughout development.

The EMA and FDA have slightly different terminologies depending on whether gRNAs are delivered *in vivo* or used *ex vivo*. For *in vivo* gene editing applications, gRNA is classified as an active substance by the EMA and as an active pharmaceutical ingredient (API) by the FDA. In contrast, when used in *ex vivo* cell therapy manufacturing, gRNA is considered a starting material by the EMA and a critical component by the FDA. Despite these differences in classification and delivery context, the level of characterization, control and supporting data expected for gRNA is equivalent to that required for a chemical medicinal product.

Secondly, ICH Q2(R2) focuses on analytical validation whereas ICH Q14 addresses analytical procedure development. Together, these guidelines should be applied across the product lifecycle. A key concept introduced in ICH Q14 is the analytical target profile, which can help streamline analytical development and support more effective lifecycle management. Orthogonal methods such as IPRP-HPLC, AEX-HPLC and LC-MS should be used to analyse impurities. Although ICH Q3A explicitly excludes oligonucleotides from its scope, its impurity guidelines are commonly used as a reference for underlying principles. Impurities that are

highly similar, of identical sequence length and not well resolved should be characterized and controlled as groups, based on their chemical class and/or chromatographic retention time.

Thirdly, the standardization and qualification of analytical technologies are crucial. HPLC and mass spectrometry are commonly used in the identity and purity assessment; UV absorbance at 260nm is routine for concentration measurement. Newer methods like NGS are emerging as a powerful tool for comprehensive gRNA impurity characterization. However, the industry is still working to define best practices for qualifying NGS as a quantitative method, despite its widespread use as a qualitative tool in other applications.

LOOKING AHEAD: PLATFORM APPROACHES & OTHER EMERGING APPROACHES

As CRISPR-based therapeutics continue to mature, innovative manufacturing and analytical approaches will be crucial in order to improve efficiency, consistency and scalability across development programs.

Firstly, platform approaches have the potential to streamline the development of CRISPR-based therapeutics while maintaining consistency across programs. In 2024, the US FDA published its Platform Technology Designation Program draft guidance [4], marking the first time the agency has addressed in details platform concepts that have been discussed within the FDA and industry for some time. This program is intended to improve efficiencies in drug development, manufacturing and review processes for drug product applications that incorporate designated platform technologies. The FDA also acknowledges that the FDA and industry have often used the term 'platform technology' in ways that differ from the definition in the FDA guidance.

In the context of CMC, two platform approaches are commonly used: platform manufacturing and platform analytical methods. For *in vivo* CRISPR-based therapeutics, which are administered directly to patients, delivery most often relies on lipid nanoparticles (LNPs). When targeting the same organ, the LNP formulation (i.e. composition, amount and manufacturing process) can remain the same, while elements such as the gRNA backbone, scaffold, or modification chemistry can be treated as a platform, with only the targeting sequence varied. Therefore, the same manufacturing and purification approach can be applied to multiple products.

Beyond traditional solid-phase synthesis, alternative platform manufacturing approaches are also emerging, including liquid-phase and enzymatic oligonucleotide synthesis. These methods may support larger production volumes, reduce reliance on organic solvents, and offer improved scalability compared with solid-phase synthesis.

Platform concepts also extend to analytical methods. At the GMP stage, drug substance characterization typically involves 10–20 analytical assays. Compendial methods such as endotoxin, bioburden, moisture, appearance, and other commonly used methods such as content and identity naturally lend themselves to platform-based approaches as they can be applied across multiple products.

Finally, the emergence of N-of-1 clinical trials underscores the importance of platform development from a CMC perspective. In 2025, the first personalized CRISPR therapy was administered to Baby KJ under an N-of-1 clinical trial framework, with the therapy designed, manufactured, and delivered within six months through a collaboration led by Children's Hospital of Philadelphia (CHOP), PENN Medicine, Innovative Genomics Institute (IGI) and Danaher. This milestone demonstrated both the technical and regulatory feasibility of rapid, patient-specific gene

editing interventions. However, the scalability of such approaches depends on the establishment of robust CMC platforms in which core elements, such as gRNA design, manufacturing process, analytical methods and control strategies, are standardized, while patient-specific components are treated as configurable variables. The field must now define how to operationalize these platform principles to enable reproducible, cost-effective and timely delivery of personalized therapies, while maintaining consistent quality, efficacy and safety.

Overall, I am optimistic that CRISPR-based therapeutics will continue to advance over the coming years, supported by innovative manufacturing and analytical technologies and greater harmonization of regulatory guidance among different agencies, ultimately increasing patient accessibility.

BIOGRAPHY

Kok-Seong Lim is a pharmaceutical leader with over 20 years of experience in biological research and development, specializing in analytical sciences, quality control, and CMC strategy for advanced therapy platforms. He has held management roles at Metagenomi, Aura Biosciences, Editas Medicine and Thermo Fisher Scientific. In these roles, he supported the development of more than 10 gene therapies and CRISPR-based medicines across rare disease and oncology. His work includes building GMP-compliant facilities and analytical infrastructures to support early- and late-stage development. He has contributed to regulatory submissions in both the US and EU. An active industry contributor, Kok-Seong presents regularly at conferences and serves as Chair of the RAPS San Francisco Chapter and as a member of the US Pharmacopeia Biologics – Cell and Gene Therapy Expert Committee where he contributes to the development of industry standards and best practices for cell and gene therapy. He earned his PhD in Biochemistry from the National University of Singapore and a BSc in Pharmacy from the University of Strathclyde, Scotland, UK.

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INDUSTRY INSIGHTS • JANUARY 2026

Advances in enzymatic manufacturing, therapeutic pipelines, and regulatory pathways for nucleic acid therapeutics

Jokūbas Leikauskas

With a background in science communication and digital publishing, Jokūbas focuses on advancing the nucleic acid therapeutics field by commissioning and shaping high-impact, open access content for *Nucleic Acid Insights*. As Commissioning Editor, he leads the development of interviews, expert articles, and industry perspectives that highlight emerging advances across mRNA, DNA, oligonucleotide, and drug delivery modalities. Jokūbas is driven to translate complex scientific topics into engaging, accessible content while maintaining strong connections across the nucleic acids community.

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SUMMARY

Across December 2025 and mid-January 2026, activity in the nucleic acid therapeutics landscape reflected continued investment in manufacturing, expanding clinical pipelines, and multiple regulatory milestones. Alnylam announced a $250 million expansion of its RNA interference (RNAi) manufacturing capacity using enzymatic ligation technology, while GSK entered a partnership with CAMP4 worth up to $458 million to advance antisense oligonucleotide (ASO) therapies targeting regulatory RNAs. Furthermore, several companies reported progress across preclinical and clinical programs, including ASO and small interfering RNA (siRNA) candidates for amyotrophic lateral sclerosis (ALS), cardiovascular disease, and rare neurological disorders, alongside new clinical data presentations at major scientific meetings. Regulatory momentum was also evident, with Breakthrough Therapy designations and new approvals granted for RNA-based medicines across the USA and Canada.

MARKET TRENDS

Alnylam announced a $250 million expansion of RNAi manufacturing using enzymatic ligation technology [1]

Alnylam Pharmaceuticals announced a $250 million investment to expand its RNAi manufacturing facility in Norton, Massachusetts, incorporating its enzymatic ligation–based siRELIS platform to increase siRNA production efficiency. The platform, which has been accepted into the US FDA Emerging Technology Program, assembles siRNA from pre-synthesized nucleotide blocks rather than traditional stepwise synthesis. The expanded site, expected to be operational by late 2027, will support scaling of Alnylam's pipeline, including candidates such as zilebesiran and nucresiran, amid broader efforts to strengthen US-based pharmaceutical manufacturing.

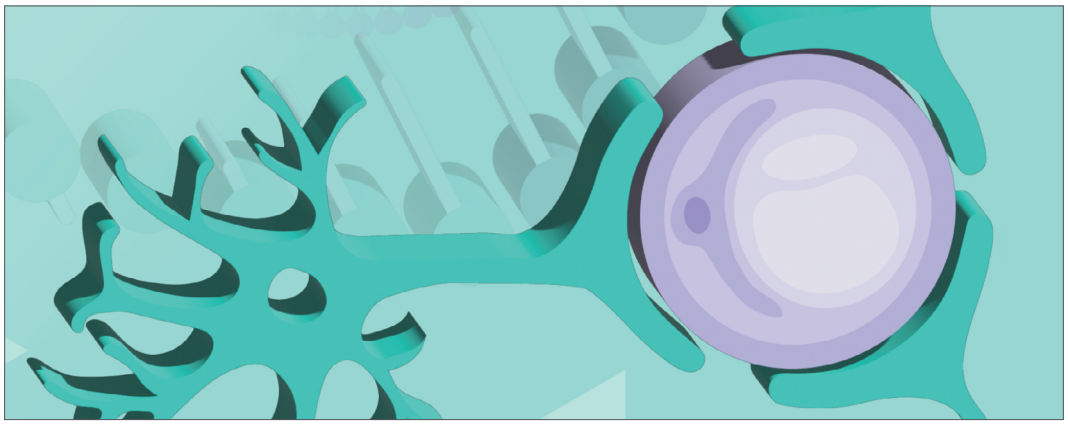
GSK partnered with CAMP4 on antisense RNA therapies for neuro and kidney diseases [2]

GSK announced a collaboration with CAMP4 Therapeutics focused on developing ASOs targeting regulatory RNAs implicated in neurodegenerative and renal diseases. Under the agreement, CAMP4 will receive $17.5 million upfront and is eligible for up to $440 million in development and commercial milestones, along with tiered royalties. CAMP4 will apply its RAP Platform to identify and validate ASO candidates designed to increase protein expression by modulating regulatory RNAs, while GSK will take responsibility for global development, manufacturing, and commercialization. The deal expands GSK's growing portfolio of RNA-focused partnerships.

RESEARCH AND DEVELOPMENT HIGHLIGHTS

Aperture advanced an MMP9 (ASO) program for ALS [3]

Aperture Therapeutics announced progress on APRTX-003, a first-in-class ASO targeting matrix metalloproteinase-9 (MMP9) for the treatment of ALS. The program aims to suppress MMP9 at the RNA level to address chronic neuroinflammation and neurodegeneration, key



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drivers of motor neuron loss in ALS. Using its proprietary platform combining human genetic evidence and machine learning–guided oligonucleotide design, Aperture identified MMP9-targeting ASOs that achieved robust knockdown of MMP9 mRNA and protein and reduced inflammatory biomarkers in human iPSC-derived microglia. The company has also developed a humanized MMP9 knock-in mouse model to support translational studies.

Geron reported new imetelstat data in lower-risk MDS and myelofibrosis at ASH 2025 [4]

Geron Corporation presented new oral and poster data at the American Society of Hematology 2025 Annual Meeting, highlighting clinical and mechanistic findings for imetelstat in lower-risk myelodysplastic syndromes (MDS) and myelofibrosis. Pooled analyses from the Phase 3 IMerge trial suggested early treatment-emergent cytopenias were associated with greater hemoglobin increases and higher rates of red blood cell transfusion independence, supporting an on-target effect of telomerase inhibition. Long-term follow-up showed favorable trends in overall survival and progression-related endpoints, although the study was not powered for statistical significance.

CLINICAL TRIALS AND RESEARCH

Rona advanced a dual-target siRNA into Phase 1 development [5]

Rona Therapeutics announced the submission of RN5681, a GalNAc-conjugated bi-valent siRNA targeting both PCSK9 and LPA, to the Australian Human Research Ethics Committee, marking its entry into clinical development. The Phase 1 study is expected to begin dosing in Q1 2026. RN5681 is designed to simultaneously lower LDL cholesterol and lipoprotein(a) by silencing two genetically validated cardiovascular risk drivers within a single molecule. The program represents the company's first clinical candidate from its bi- and multi-target siRNA platform and is intended to address residual cardiovascular risk not adequately managed by existing lipid-lowering therapies.

Amylyx reported early Phase 1 safety data for a novel ASO in ALS [6]

Amylyx Pharmaceuticals presented early safety and tolerability data from cohort 1 of the first-in-human Phase 1 LUMINA trial evaluating AMX0114, an ASO targeting calpain-2, in people with ALS. In cohort 1 (n=12), AMX0114 was generally well tolerated, with no treatment-related serious adverse events or dose-limiting toxicities reported. Based on these findings, the company plans to open enrollment for the second cohort in Canada and the USA. The randomized, double-blind, placebo-controlled trial is assessing safety, pharmacokinetics, pharmacodynamics, and biomarker changes, including neurofilament light chain levels, with initial biomarker data from cohort 1 expected in the first half of 2026.

Biogen and Stoke reported new zorevunersen data in Dravet syndrome [7]

Biogen and Stoke Therapeutics presented new clinical and mechanistic data for zorevunersen, an investigational ASO for Dravet syndrome, at the 2025 American Epilepsy

Society Annual Meeting. Long-term Phase 1/2a and open-label extension data has shown durable reductions in seizure frequency, an increase in seizure-free days, and improvements in cognition, behavior, and quality of life when added to standard anti-seizure medicines. A propensity score weighted analysis comparing treated patients with a natural history cohort demonstrated statistically significant seizure reductions and cognitive and behavioral improvements at timepoints aligned with the ongoing Phase 3 EMPEROR study. EEG analyses supported a disease-modifying mechanism of action, alongside accumulating long-term safety data.

TOOLS AND TECHNOLOGIES

Mount Sinai researchers reported a cell-selective mRNA therapy platform [8]

Researchers at the Icahn School of Medicine at Mount Sinai reported the development of a cell-selective modified mRNA translation system (cSMRTS) designed to activate therapeutic gene expression preferentially in targeted cells. Described in *Molecular Therapy* and demonstrated in mouse cancer models, the platform uses microRNA-responsive control elements to switch mRNA activity on in tumor cells while suppressing expression in healthy tissues. When delivered systemically in lipid nanoparticles, cSMRTS showed markedly higher gene expression in tumors and substantially reduced off-target activity in major organs, leading to significant tumor growth reduction. The approach shifts selectivity from delivery vehicles to the mRNA payload itself and is being explored for broader preclinical development and commercialization.

AI-driven approaches for RNA drug development were outlined in a new review article [9]

A peer-reviewed article recently published in *Engineering* reviewed how AI could accelerate and reshape RNA drug development. The authors outlined the advantages of RNA-based therapeutics, including shorter development timelines and higher clinical success rates compared with traditional drugs, while highlighting current experimental and computational limitations. The review described three AI-driven strategies – data-driven, learning-strategy-driven, and deep-learning-driven approaches – and discussed their application to RNA design, target identification, and optimization. The authors also proposed an integrated, software-based workflow combining AI model feedback with real-world data to enable personalized RNA drug discovery, automated synthesis, and early biological validation, positioning AI as a key enabler of future RNA therapeutics.

REGULATORY CHANGES AND UPDATES

Ionis received the FDA Breakthrough Therapy designation for zilganersen in Alexander disease [10]

Ionis Pharmaceuticals announced that the FDA granted Breakthrough Therapy designation to zilganersen, an investigational ASO for the treatment of Alexander disease, a rare and often fatal neurological disorder. The designation was supported by topline results from a pivotal Phase 1–3 study showing statistically significant stabilization of gait speed versus control at week 61, with favorable safety and tolerability. Zilganersen also demonstrated consistent benefit across key secondary endpoints. Ionis plans to submit a new drug application in Q1 2026 and is preparing to initiate an Expanded Access Program in the USA. Zilganersen is designed to reduce excess glial fibrillary acidic protein produced by disease-causing variants in the GFAP gene.

Arrowhead received FDA Breakthrough Therapy designation for plozasiran in severe hypertriglyceridemia [11]

Arrowhead Pharmaceuticals announced that the FDA granted Breakthrough Therapy designation to plozasiran, an investigational RNAi therapeutic, as an adjunct to diet for adults with severe hypertriglyceridemia. The designation is based on preliminary clinical evidence suggesting substantial improvement over existing therapies. Plozasiran targets apolipoprotein C-III to reduce triglyceride-rich lipoproteins and lower serum triglyceride levels. Arrowhead is conducting multiple Phase 3 trials, including SHASTA-3, SHASTA-4, and MUIR-3, with completion expected in mid-2026, and plans to submit a supplemental new drug application to the FDA by the end of 2026, followed by additional global regulatory filings.

Alnylam received Health Canada approval for vutrisiran in ATTR cardiomyopathy [12]

Alnylam announced that Health Canada granted a Notice of Compliance for AMVUTTRA® (vutrisiran) for the treatment of cardiomyopathy in adult patients with wild-type or hereditary transthyretin-mediated amyloidosis. The approval expands the drug's existing indication in Canada, making vutrisiran the first RNAi therapeutic authorized for both ATTR cardiomyopathy and polyneuropathy. The decision was based on results from the Phase 3 HELIOS-B trial, which showed significant reductions in all-cause mortality and recurrent cardiovascular events compared with placebo, with a safety profile similar to placebo. Vutrisiran targets transthyretin mRNA to reduce production of both variant and wild-type TTR and limit amyloid deposition.

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EVENT PREVIEW

RNAi-Based Therapeutics Summit 2026

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As part of our ongoing coverage of major gatherings in the nucleic acid therapeutics space, *Nucleic Acid Insights* presents a preview of the RNAi-Based Therapeutics Summit 2026. Taking place January 27–29, 2026, in Boston, Massachusetts, the summit will convene stakeholders from across the RNA interference (RNAi) ecosystem to examine how small interfering RNA (siRNA) and microRNA therapeutics are progressing beyond established hepatic indications toward broader systemic and extrahepatic applications.

Against a backdrop of recent regulatory approvals, late-stage clinical advances, and major strategic partnerships, the meeting will focus on translating RNAi innovation into durable clinical and commercial success. With a strong emphasis on delivery technologies, novel chemistries, and emerging disease areas, the summit provides a forum to address the scientific and operational challenges that continue to shape RNAi development.

EXTRAHEPATIC DELIVERY: EXPANDING RNAI BEYOND THE LIVER

A central theme of the 2026 agenda is the advancement of RNAi delivery strategies capable of targeting tissues beyond the liver, including the kidney, muscle, adipose tissue, and central nervous system (CNS). Sessions will examine how conjugates, ligand-based targeting, and non-lipid nanoparticle (LNP) approaches are redefining the therapeutic reach of RNAi.

David Jackson (CEO, Ceria Therapeutics) will present a novel RNAi delivery platform designed for extrahepatic targeting, outlining how unique uptake and release kinetics can enable acute RNAi therapeutics in previously inaccessible tissues. He will also discuss the use of selective translational models to de-risk development and accelerate pipeline progression. Alfica Sehgal (CSO, Judo Biosciences) will highlight the STRIKE™ platform, which employs proprietary ligand–siRNA conjugates to achieve receptor-mediated uptake in kidney cells.

Her presentation will explore how targeted delivery minimizes systemic exposure while enabling potent and durable gene silencing for renal and systemic diseases. Audrey Bernstein (CSO, DUB Therapeutics) will focus on self-delivering siRNA technologies for fibrotic disease, describing how targeting integrin pathways can reverse pathological scarring and promote regenerative healing in chronic fibrosis models.

RNAi ACROSS DIVERSE DISEASE INDICATIONS

The summit will showcase how RNAi is being applied across a widening range of disease areas, from metabolic disorders to immunology, fibrosis, and neurodegeneration. Andrew Coles (Senior Scientist, AbbVie) will discuss the application of RNAi in complex immunological and fibrotic conditions, examining how targeted LNP engineering and optimized siRNA design can improve biodistribution and therapeutic response. Uyanga Tsedev (CSO, Gensaic) will present an AI-designed, dual-targeting siRNA therapeutic for sarcopenia. Weimin Wang (Founder and CEO, Sangene Bio) will share insights into the LEAD™ RNAi technology platform for obesity and metabolic disease. His session will explore ligand-enhanced delivery strategies designed to achieve durable, tissue-specific silencing across metabolic compartments. Yacoub Habib (CEO, Ophidion Inc.) will address systemic RNAi delivery to the brain, describing Trojan Horse formulations that enhance blood–brain barrier penetration and enable the treatment of neurodegenerative diseases associated with gain-of-function mutations.

NOVEL DELIVERY ARCHITECTURES: RNA & DNA ORIGAMI

Emerging delivery vehicles based on programmable nucleic acid architectures will also feature prominently, reflecting growing interest in non-traditional RNAi carriers. Claire Zeng (Chief Executive and Technology Officer, DoriNano) will explore DNA origami-assisted siRNA delivery, detailing how multivalent, folded DNA nanostructures can be engineered to optimize cellular uptake and endosomal escape. Her presentation will demonstrate how modular nanoscale design enables precision delivery while overcoming limitations associated with conventional lipid-based systems. James Carroll (President and CEO, RNA NanoBiotics) will discuss self-assembling RNA nanostructures, highlighting how RNA origami can function as a programmable nanocarrier to improve tissue specificity and payload delivery efficiency.

INVESTMENT, INTEGRATION, & THE FUTURE OF RNAi

In addition to presentations, the summit will feature dedicated panel discussions addressing the evolving RNAi investment landscape and the integration of emerging technologies. A panel focused on investor perspectives will examine how delivery platform versatility, clinical differentiation, and manufacturability influence asset valuation and partnership potential. Another forward-looking discussion will explore how RNAi pipelines can be future-proofed through AI-enabled target discovery,

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multi-target silencing strategies, and combination approaches with other therapeutic modalities. Together, these sessions will provide strategic context for aligning scientific innovation with long-term development and commercialization goals.

As the premier industry-led forum dedicated to translating RNAi beyond the liver, the 7th RNAi-Based Therapeutics Summit is the definitive meeting for 80+ senior leaders from large pharma, innovative biotech, and investment firms driving the next wave of siRNA and microRNA drugs for neurology, cardiometabolic, oncology, and rare diseases. This year's conversation goes beyond the liver, focusing on the novel strategies turning extra-hepatic delivery into a clinical reality, from next-generation LNPs and conjugates to emerging platforms targeting the lungs, CNS, and skeletal muscle. You can find out more about the RNAi-Based Therapeutics Summit events **here**.

Want to keep up to date with all of the latest nucleic acid events you might want to attend or exhibit at? Explore our free online Events Calendar **here**.

EVENT PREVIEW

NextGen Biomed 2026

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As part of our ongoing coverage of key gatherings in life sciences, BioInsights presents a preview of NextGen Biomed 2026. Scheduled for March 24–25, 2026, in London, UK, this event will unite the brightest scientific minds and the most disruptive innovations in biomedicine under one roof. The agenda spans advances in biologics, peptide and oligonucleotide therapeutics, immunotherapy strategies, vaccine research and development, and sustainable bioprocessing, featuring a keynote presentation from Andreas Plückthun (Professor of Biochemistry, University of Zurich), who will discuss molecular engineering for the future across therapeutic modalities, alongside contributions from a broad range of academic and industry leaders.

EXPLORING ADVANCES IN ADC ENGINEERING

The Proteins, Antibodies and ADCs program will explore topics ranging from protein and antibody engineering through to advanced bioanalytics, real-world case studies, and innovations in upstream and downstream processing. Senior leaders from pharmaceutical companies, biotechnology firms, and research institutions will share perspectives on antibody discovery, analytical development, and protein purification strategies. Charlotte Deane (Professor, University of Oxford) will examine the application of AI in protein design, while Dan Bach Kristensen (Scientific Director, Servier) will present on characterization of ADCs and next-generation biologics in biological matrices using affinity capture liquid chromatography–mass spectrometry.

OLIGONUCLEOTIDE DESIGN, CMC, & SCALE-UP STRATEGIES

Beyond ADCs, the conference will also include presentations on how computational design approaches, CMC strategies, sustainable process improvements, and advanced analytical controls are being applied to accelerate oligonucleotide-based drug development timelines. A thought leadership panel featuring Anna Perdrix (Chief Executive Officer, Sixfold Bioscience), Sritama Bose (Associate Director of Chemistry, Orfonyx Bio), and Sandor Batkai (Life Science Consultant, formerly Head of Medical Research and Intelligence, Cardior Pharmaceuticals) will explore regulatory considerations, novel technologies, and

scale-up challenges in oligonucleotide drug development.

NOVEL IMMUNOTHERAPY & IMMUNO-ONCOLOGY APPROACHES

Immunotherapy for cancer and autoimmune diseases will be another central theme, with sessions focused on biomarker-guided strategies, next-generation cellular and antibody-based approaches, and personalized treatment paradigms. Alexander Eggermont (Professor, University Medical Center Utrecht) will present on the neoadjuvant immunotherapy revolution across multiple tumor types, discussing why neoadjuvant immuno-oncology strategies may offer advantages over adjuvant approaches. Callum Scott (Senior Vice President and Head of Development, Scancell) will address the role of potency testing across the cancer vaccine development lifecycle, highlighting the intersection of technical feasibility, regulatory expectations, and commercial considerations.

INNOVATIONS IN VACCINE DESIGN & DEVELOPMENT

The vaccines program will examine advances in nucleotide-based vaccine platforms for infectious diseases, AI-driven antigen design, and emerging delivery systems. Daniel Larocque (Innovation Leader, Sanofi) will present a historical and forward-looking perspective in a session titled 'Tracing 200 years of vaccine innovation: from prophylactic to therapeutic vaccines and AI-driven vaccines'. Supriyadi Hafiz (Senior Scientist, Merck) will discuss the growing demand for responsibly sourced alternatives to animal-derived ingredients, with a focus on fermentation-derived, non-animal-origin squalene. Across the program, attendees will gain insights into adjuvant production, clinical development strategies, and translational approaches that support the progression of vaccine candidates from research to real-world application.

WOMEN IN NEXTGEN BIOMED PANEL DISCUSSION

The two-day event will also feature a Women in NextGen Biomed panel discussion. Building on the success of the previous year, this session will bring together leading female scientists and industry professionals for a discussion focused on innovation, career pathways, and addressing barriers in STEM. The panel will highlight achievements, ongoing challenges, and future opportunities for women contributing to the next generation of biomedical research and development.

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NextGen Biomed 2026 will bring together innovators across biologics, tides, and immunotherapy fields to share real-world case studies spanning novel target discovery and clinical validation, as well as biologics engineering and therapeutic development. Together, these discussions will offer a deep dive into the science and strategy behind the field's most promising breakthroughs.

You can find out more about the NextGen Biomed 2026 events **here**.

To learn about other events coming up in your field, you can find our online Events Calendars here: ***Bioconjugate Insights***, ***Nucleic Acid Insights***, and ***Vaccine Insights***.