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Foreword

We are pleased to present a special Bioanalysis Zone interactive supplement, which has been created to bring you the latest developments and leading opinions from some of the key experts in the oligonucleotide field.

Oligonucleotide therapies have gained increasing interest in recent years, due to their capabilities in targeting a diverse range of drug targets, enabling more sophisticated and specific therapeutics to be developed with novel biological functions.

In this special supplement we review how the field has developed since our last examination of the subject in our 2017 Spotlight focusing on oligonucleotides. We consider how attitudes have since changed in our recent survey and explore important developments with contributions from leading experts including: Johannes Winkler (Medical University of Vienna), Laixin Wang (Chongqing Denali Medpharma Co.), Neil Henderson (AstraZeneca) and Zamas Lam (QPS).

We hope you enjoy exploring the field of oligonucleotides further with us!

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Bioanalysis of oligonucleotides – the journey so far

Neil Henderson; Translational Biomarkers and Bioanalysis, Clinical Pharmacology and Safety Sciences, R&D BioPharmaceuticals, AstraZeneca, Gothenburg, Sweden.

Neil Henderson has an academic background within respiratory and inflammatory disease biology. He has worked for AstraZeneca in the UK and Sweden performing GxP biomarker study analysis predominantly using ligand-binding assays. Since 2015, as part of the Translational Biomarkers and Bioanalysis team, Neil has been developing and applying bioanalytical strategies for preclinical new modality therapeutics and identification of novel safety biomarkers.

Keywords: antisense oligonucleotides • ASOs • modified mRNA • mRNA

Where we were

As with many companies, the historical success of AstraZeneca was primarily based upon developing and marketing small molecule drug moieties. Going into the mid-to-late 2000s, through the partnering and subsequent acquisition of Cambridge Antibody Technology and later purchase of MedImmune, a greater number of large molecules, predominantly in the form of antibodies and biologics, were contributing to the overall pipeline diversity. Then in the last 5 years, through increased partnerships with external companies, there has been an explosion in the types of drug molecules – the so-called 'new modality' drugs – which includes antisense oligonucleotides (ASOs) and modified mRNA. In fact, the inclusion of these molecules led to the creation of a dedicated bioanalysis group within AstraZeneca to provide PK/PD and safety data for investigative projects and further refined the operating model for regulatory-facing bioanalysis, which is now conducted by our preferred providers. Initial preclinical discovery bioanalysis support to these projects were mainly focused upon LC–MS/MS analysis of ASO concentrations in specific target organs and high accumulating tissues, such as the liver, or for tissue/plasma levels of the mRNA-encoded protein by commercial ligand-binding assay (LBA). Then as projects progressed, the level and diversity of bioanalytical support evolved too to provide meaningful data for the project team's novel questions. For example, the team needed more sensitive methods to measure ASOs, to fully track lower concentrations *in vivo* over time, and so have had to add hybridization ELISA (HELISA) and HPLC-fluorescence probe methods (HPLC-FL) to the bioanalytical repertoire for PK and known-metabolite assessments respectively. Good communication with our collaborative partners has helped in the establishment of the many novel assays and workflows both internally within the team and with external vendors who deliver our regulatory facing study support.

One observation at the time was the lack of direct bioanalytical regulatory guidance for the technologies typically applied within the field of oligonucleotides, particularly for the expectations on modified mRNA bioanalysis. It meant that 'we', as part of the wider bioanalytical community, had to plug the gap, so to speak: "what did we think needed to be measured, and how?" especially when the data ultimately may form part of submissions. The encoded protein could be measured by commercially available (or bespoke) LBA or by large molecule LC–MS/MS methods, using established bioanalytical guidance documents to meet investigative and/or GLP requirements. Moreover, the

basic approach to maintaining protein stability in a sample, during processing, was essentially well established. In comparison, for the measurement of the modified mRNA the key questions, at the time for our bioanalytical lab, centered upon identifying a suitable assay, as well as understanding and demonstrating analyte stability during sample processing. Assessment techniques, such as quantitative RT-PCR (qRT-PCR) and branched DNA (bDNA) techniques, although used routinely to measure mRNA in academic evaluations, were less familiar in quantitative bioanalysis labs. For our investigative bioanalysis, albeit keeping an eye on future possible regulatory utility, our group opted for bDNA; a choice partly based upon the techniques' similarities with ELISA ligand-binding assays and hence allowing the possibility to align the method characterization to the LBA bioanalytical guidance documents. A further advantage was that the bDNA assay could work directly with homogenized tissue extracts without the additional necessity to purify the mRNA first, and therefore potentially keeping the bioanalytical complexity to a minimum. The final piece in the puzzle was identifying simple, suitable blood and tissue sampling regimes, such as removing/inactivating blood nucleases during the sample collection and processing so that, most importantly, the potentially liable mRNA were intact when it came to the bioanalysis step.

Where we are today

Today we have routines in place to meet the needs of the diverse molecular portfolio both in-house for investigative support and with our CRO partners to manage regulatory facing and GLP bioanalysis. The interesting thing about these molecules is their constant evolution whereby molecules are modified to have specific-targeting properties, or indeed novel alternative administration strategies are routinely explored. In terms of ASO drugs, through the attachment of a targeting protein to the oligonucleotide via a linker chain, these molecules have a greater propensity to accumulate in specific target organ/cell types. More complex molecules, therefore, may require more complex or detailed bioanalytical strategies to be able to account for all the components *in vivo* (e.g., ASO-targeting protein complex, ASO alone and targeting protein alone).

In fact, for many of the oligonucleotide projects, our appreciation for the number of bioanalytical endpoints required to fully understand the safety, metabolism and PK/PD relationship for these drugs has substantially increased over the last few years. For example, with modified mRNA drugs, most of these projects are exploring lipid nanoparticle (LNP) encapsulation as a strategy to protect the drug entity from nuclease enzymes following 'traditional' administration strategies (e.g., subcutaneous injection or intravenous). The list of bioanalytical endpoints that may be required now include analysis of: (1) the mRNA, (2) the produced protein, (3) the LNP and (4) markers of immune activation in response to 1–3 (including anti-drug antibodies and inflammatory cytokine profiles). To further complicate the bioanalytical challenge, the different analytes can have incompatible sample processing procedures (e.g., extraction of mRNA from tissue uses proteases), which may have consequences on the overall study design and the ability to obtain enough analyzable matrix (especially in small preclinical species). Moreover, it is also important to consider the potential holistic safety impact of these drug components through biodistribution assessments (*in situ* hybridization, immunohistochemistry and histopathology) and quantitative biodistribution in target safety and efficacy tissues. Investigating all these endpoints is of course 'resource intensive', so we have found that it is vital to clarify with preclinical project teams, which data is going to be the most informative and have the most value in progression of the drug through the pipeline.

What the future holds

As of today, more than 30% of the AstraZeneca drug programs are actively exploring new modalities, and this figure will no doubt increase as the company further embraces the philosophy of 'agnostic drug-entity approaches' to find 'the right drug' to ameliorate diseases. Indeed, there is a large drive to expand the array of novel delivery systems and to make drugs 'smarter', which will potentially open up other areas within the body/routes of administration (e.g., overcoming the challenge of dosing modified mRNA across the lung membrane). For the bioanalyst, this means more 'weird and wonderful' candidate drugs coming through the research and development pipeline, many with new analytical challenges that will require us to evolve and explore novel science and potentially more sensitive technologies.

For further reading, please check out:

Hawthorne G, Henderson N, Hölttä M, Stovold C, Wåhlander A, Wilson A. Bioanalysis – but not as we knew it: an AZ perspective of the last 10 years evolution to meet a diversifying portfolio. *Bioanalysis* 11(7), 595–599 (2019).

EDITORIAL

MEDICAL UNIVERSITY OF VIENNA

The era of therapeutic oligonucleotides has just begun

Johannes Winkler; Department of Cardiology, Medical University of Vienna, Vienna, Austria.

Johannes Winkler studied pharmacy at the University of Vienna and completed his PhD in pharmaceutical chemistry in 2003 on development of therapeutic oligonucleotides. He has postdoctoral experience in Austria and Switzerland, investigating nucleic acid chemistry, biology and the development of targeted drug delivery systems. He completed his habilitation (venia docendi) for pharmaceutical chemistry in 2014 and is now a researcher at the Medical University of Vienna, Department of Cardiology (Austria). His research focus is on oligonucleotide chemistry, analysis, biology and pharmacology, and nucleic acids, including non-coding RNA as potential biomarkers and therapeutic targets in cardiovascular diseases.

Keywords: therapeutic oligonucleotides • siRNA • antisense drugs • non-coding RNA

Therapeutic oligonucleotides are already established as a successful technology, with many additional siRNA and antisense drugs in development. New applications for targeting non-coding RNA and gene editing are on the horizon.

The medical use of synthetic oligonucleotides has gained considerable relevance during the last few years. Oligonucleotides are already quite firmly established as a third major group of drug substances, supplementing small molecules and biologics. From a pharmaceutical point of view, nucleic acid-based drugs have the distinct characteristic to act on protein translation or transcription, inhibiting *de novo* protein synthesis. In contrast, small molecules and antibodies target proteins and modulate their functions. In terms of molecular size, oligonucleotides fall between small molecules and therapeutic proteins [1].

The long journey of antisense, splice-correcting oligonucleotides, siRNA and other therapeutic modalities towards use as drugs has seen a lot of ups and downs, illustrating the enormous challenges and regulatory hurdles for any new class of therapeutics. After all, it has taken close to 30 years for antisense and 20 years for siRNA to be used as drugs since their respective discovery. Many traditional processes and development strategies, which had been optimized for small molecules or proteins, have been shown to be not directly applicable to oligonucleotides, and new or adjusted assays had to be developed. Initial development has mainly focused on rare diseases with unmet medical need. In the near future, oligonucleotide drug development companies will try to move to therapeutic areas with more demand and compete with small molecules and monoclonal antibodies.

Since the previous Bioanalysis Zone Spotlight in 2017, two new oligonucleotide drugs have won market approval: Onpattro® (patisiran), the first siRNA therapeutic and Tegsedi™ (inotersen), an antisense oligonucleotide. Both have been evaluated for treating polyneuropathy symptoms of hereditary transthyretin (TTR) amyloidosis caused by mutations in the *TTR* gene, which promote misfolding of the protein, aggregation and subsequent deposition in organs. This in turn can cause polyneuropathy and/or cardiomyopathy. Both oligonucleotide agents degrade *TTR* mRNA irrespective of distinct mutations.

Patisiran marks a breakthrough as the first siRNA available for therapeutic interventions. It was effective in clinical evaluations for alleviating polyneuropathy symptoms, based on patient questionnaires and assessment of medical personnel [2]. Subanalyses of a Phase III trial were recently reported, showing beneficial effects also for TTR-amyloidosis-mediated cardiomyopathy [3]. A senile form of TTR amyloidosis is caused by aggregation of wild-type TTR and has a high prevalence in an aging population. It is thus attractive to expand the use of patisiran to treatment of cardiomyopathy, but a clinical benefit has to be demonstrated in an adequately designed randomized clinical trial.

Similarly to patisiran, inotersen reduced symptoms and improved quality of life of treated polyneuropathy patients [4]. Compared to patisiran concerns for adverse effects are slightly more severe. In particular, there appears to be a risk for thrombocytopenia, which is possibly a class side effect of antisense agents with distinct chemical modifications.

Patisiran is applied as a lipid nanoparticle formulation. Lipid nanoparticles can cause some adverse effects and are more complicated to produce and less efficacious than covalent conjugates of siRNA with receptor ligands. Thus, most other siRNAs, and also several antisense agents, in current clinical trials make use of covalently attached N-acetylgalactosamine (GalNAc) for intracellular delivery to hepatocytes. The therapeutic success of GalNAc oligonucleotide conjugates is explained by the rapid endocytic internalization via the asialoglycoprotein receptor, which is extremely highly expressed on hepatocytes, and recycled back to the cell surface within a short time. This enables quick cellular uptake of a large amount of oligonucleotides into endosomes. Subsequent translocation from endosomal compartments to the cytosol is believed to take place for only a small fraction of internalized compounds [5]. GalNAc oligonucleotide conjugates are thus a major improvement to the long-standing issue of efficient and safe intracellular delivery, which is required for therapeutic applications. However, they are restricted to silence gene targets in hepatocyctes.

One of these GalNAc siRNA conjugates, givosiran, has recently completed its Phase III trial and the developing company, Alnylam Pharmaceuticals (MA, USA), has announced that primary and secondary endpoints were reached, and only minor adverse effects have been reported. Full details on clinical benefits and toxicity have not yet been published. Unless for possible class-related toxicity, GalNAC conjugates are set to enable a range of efficient therapeutic gene-silencing applications in hepatocytes. Inclisiran is an siRNA that targets PCSK9, inhibition of which strongly lowers LDL cholesterol levels. PCSK9 is a promising target for achieving low LDL levels for patients with high risk for cardiovascular events [6]. For this indication and target, oligonucleotide-mediated inhibition would compete with monoclonal antibodies and in the future with small molecule inhibitors, providing a direct comparison of inhibiting *de novo* protein biosynthesis and modulating the protein function.

For enabling more widespread application of medicinal oligonucleotide technologies, similar solutions for other organs and tissues are required [7]. Thus, preclinical research and development focuses on discovering suitable receptors and respective ligands for tumor cells, the blood–brain barrier, endothelium, muscle cells and others. Until now, identification of suitable receptors has remained largely elusive, and a major breakthrough on the delivery issue is essential for realizing the full potential of therapeutic oligonucleotides [8].

Unlike for compounds delivered within nanoparticles, antisense and siRNAs with or without ligand conjugation are required to be resistant against enzymatic degradation [9]. Both for antisense, mainly phosphorothioate backbones and several terminal 2′-O-modifications, and for siRNA, 2′-O-methyl and 2′-fluoro nucleotides with a few terminal phosphorothioate linkages, efficient and stable chemical modifications are established.

In basic research, newly discovered nucleic acid technologies such as CRISPR have transformed scientific methods. Non-coding RNAs are increasingly investigated as potential biomarkers and therapeutic targets, particularly in oncology and cardiology. In addition to the chemical modifications described above, locked nucleic acids (LNA) with full phosphorothioate backbones are frequently used for targeting miRNAs and long non-coding RNAs *in vivo*. Although the phosphorothioate backbone is useful for achieving sufficient cellular uptake in animal models, LNA–phosphorothioates have not yet made it to clinical trials.

Gene editing by CRISPR is a much-hyped technology for precise changes of genomic DNA sequences and has already transformed scientific research [10]. However, gene editing in the medical context is heavily discussed not only for ethical reasons but also for technical limitations, namely off-target effects and efficacy. Realistic and useful applications still need to be defined and proven. The recent controversy involving human germline editing has highlighted for a general public the transformative potential of the technology and also its ethical

dimensions and limitations. First controlled clinical trials for establishing safe use have already been initiated but the issues of delivery and exclusion of off-target effects shed serious doubts on a safe and responsible *in vivo* use in humans for now. *Ex vivo* editing of autologous or allogenic cells (i.e., selection of successfully edited cells followed by re-implantation) appears to be a more promising medical use.

These developments show that the volume of oligonucleotides for therapy will certainly expand during the next few years together with bioanalytical and regulatory demands. Largely uniform chemical modification patterns, sequence lengths and GalNAc conjugations dominate, meaning that bioanalytical methods can be applied for entire classes with only minor optimization [11]. For future applications, targeting non-coding RNAs and for directed gene editing using CRISPR/Cas9, additional formats with shorter (LNA modified) and longer (CRISPR guide RNA) will be used. Particularly for assessing gene editing and potential off-target effects, targeted and unbiased nucleic acid sequencing methods together with efficient data analysis are required.

References

1. Dirin M, Winkler J. Influence of diverse chemical modifications on the ADME characteristics and toxicology of antisense oligonucleotides. *Expert Opin. Biol. Ther.* 13(6), 875–888 (2013).

2. Adams D, Gonzalez-Duarte A, O'Riordan WD *et al*. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl J. Med.* 379(1), 11–21 (2018).

3. Solomon SD, Adams D, Kristen A *et al.* Effects of patisiran, an RNA interference therapeutic, on cardiac parameters in patients with hereditary transthyretin-mediated amyloidosis. *Circulation* 139(4), 431–443 (2019).

4. Benson MD, Waddington-Cruz M, Berk JL *et al.* Inotersen treatment for patients with hereditary transthyretin amyloidosis. *N. Engl J. Med.* 379(1), 22–31 (2018).

5. Dowdy SF. Overcoming cellular barriers for RNA therapeutics. *Nat. Biotechnol.* 35(3), 222–229 (2017).

6. Fitzgerald K, White S, Borodovsky A *et al.* A highly durable RNAi therapeutic inhibitor of PCSK9. *N. Engl J. Med.* 376(1), 41–51 (2017).

7. Winkler J. Extrahepatic targeting of oligonucleotides with receptor-binding non-immunoglobulin scaffold proteins. *Nucl. Acids Therap.* 28(3), 137–145 (2018).

8. Lorenzer C, Dirin M, Winkler A-M, Baumann V, Winkler J. Going beyond the liver: progress and challenges of targeted delivery of siRNA therapeutics. *J. Control. Release* 203, 1–15 (2015).

9. Winkler J. Oligonucleotide conjugates for therapeutic applications. *Ther. Deliv.* 4(7), 791–809 (2013).

10. Knott GJ, Doudna JA. CRISPR–Cas guides the future of genetic engineering. *Science* 361(6405), 866–869 (2018).

11. Ku SH, Jo SD, Lee YK, Kim K, Kim SH. Chemical and structural modifications of RNAi therapeutics. *Adv. Drug Deliv. Rev.* 104, 16–28 (2016).

Recent developments in oligonucleotide analysis: an interview with Zamas Lam

Zamas Lam; VP of Preclinical Development at QPS.

Zamas is the senior VP of Preclinical Development at QPS (DE, USA) and trained in mass spectrometry, carbohydrates and glycoproteins. Since graduate school, he keeps thinking that it will be fun to work on a gene level in drug discovery and development versus at the protein level or at the metabolites level!

Q What methods do you use to quantify oligonucleotide-based drugs?

It really depends on the size and the complexity. For the smaller oligonucleotides (the antisense, the sRNAs, the aptamers, the microRNAs and the PEGylated) we use LC–MS and triples. We also use high-resolution, hybridization ELISA, hybridization LC fluorescence and LC-UV. For the bigger mRNAs and for the vectors, we use qPCR.

Q What challenges do you face working with oligonucleotides?

It really depends on the size. The size of the oligonucleotide drives everything. So traditionally people will be thinking that they use mass spectrometry, or they use hybridization ELISA of some kinds and what we do is as the size gets bigger, we go to qPCR. The real difficulty is there are not a lot of people that have been doing this for a long time. There is really no exchange of knowledge in the working levels as the protein area, similar to the small molecule LC–MS area. So essentially what that really means is there is a lot of trial and error, and when people talk about how they worked on oligonucleotides, what they really mean most of the time is they worked on multiple oligonucleotides of the same chemistry versus multiple oligonucleotides of different chemistry, because the methodology is very different. Then depending on how the backbone is modified, depending on the tag that you use to go after the therapeutic targets, the chemistry and the method again is different.

Q What progress has been made to regulate and standardize oligonucleotide analysis?

The first oligonucleotide that we did, validated per FDA guidance, was in 2002 using LC–MS triple quadrupole. It has been 17 years since then and what we have done differently from then to now is, now we use a lot of high resolution for the small oligonucleotides. We progress on to the hybridization ELISA and hybridization fluorescence. About 10 years ago, the guidance changed, the requirement is different, but fundamentally when you're looking at the smaller oligonucleotides, the guidance and the criteria we are using is the small molecule guidance. Now the issue is this, the ISR is fairly easy to deal with for your typical small and mid-size oligonucleotide. When you go after the mRNA level, the guidance using ICH and the ICH guidance do not specify ISR. What that really means is that there is a lot of unclarity when people say, "Do I need to do ISR or don't I need to do ISR? And when I do ISR, what does that really mean?"

Q Where do you hope this field will be in 5–10 years' time?

Things have changed a lot in the last 17 years since we have gone into the oligonucleotides area. My expectation is that oligonucleotides in 5 years will be essentially like the protein of now. What that really means is now there is single patient therapy using oligonucleotides. Generally, oligonucleotides have been in orphan or rare disease areas, however, the first trial in normal, non-orphan diseases is currently being undertaken. In the future, we believe oligonucleotides will be more common, and as more biotech companies out-license oligonucleotides to big companies, knowledge will be passed along better meaning we will be able to discuss oligonucleotide quantitation and metabolism with more clarity.

Watch the full interview here:

Recent developments in oligonucleotide analysis: an interview with Zamas Lam

Recent advances in bioanalysis and metabolite profiling of oligonucleotide therapeutics

Laixin Wang[1], Min Meng[1] & Patrick Bennett[2]

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Laixin Wang, Co-founder and Senior Vice President of Chongqing Denali Medpharma Co., Ltd. (China) obtained his PhD in Medicinal Chemistry from Beijing Medical University (now Peking University, China). He subsequently conducted post-doctoral training in Pharmaceutical Chemistry and Combinatory Chemistry at the University of Utah (UT, USA) and Duke University (NC, USA). Dr Wang started his industry career as a senior research scientist working on formulation and delivery of oligonucleotide therapeutics and hydrophobic drugs in a startup biotech company. After the company was acquired, Dr Wang joined Tandem Labs (now Covance; VA, USA) supporting GLP bioanalysis and dose formulation analysis. He assumed increasing leadership roles for managing scientists and projects in the quantitation of both small and large molecular therapeutics/biomarkers using LC–MS/MS, LC–HRAM, UPLC-UV and hybridization-based LC-fluorescence assays. He was an associate laboratory director at Tandem Labs (UT, USA) prior to joining NovaBioAssay (MA, USA) as the Vice President of bioanalytical in 2015. Dr Wang returned to China to co-start the Chongqing Denali Medpharma Co., and Chongqing Helens Clinical Research Center Co., in 2017.

Keywords: oligonucleotide-based therapeutics • meso scale discovery electrochemiluminescence • MSD-ECL • ELISA

The development of oligonucleotide-based therapeutics has gone through development cycles, ranging from intense excitement to abandonment and then rediscovered enthusiasm as the technology evolved and the previous challenges and resulting failures were overcome. Throughout these cycles, there has been steady progress in advancing the technologies. The recent approvals of several antisense and siRNA provide optimism that oligonucleotide-based therapeutics will continue to emerge as an important class of therapeutics [1,2].

Oligonucleotides can interfere with biomolecules representing the entire extended central dogma. Up to now, seven oligonucleotide-based drugs have received market authorization by regulatory authorities. All of them are chemically modified oligonucleotides containing 18–30 nucleoside units. The chemical modification can affect pairing, stability and conformation of the oligonucleotides and therefore affect their interactions with proteins and either RNA or DNAs. Improved knowledge on nucleic acid chemistry and RNA/protein regulatory mechanisms are leading to the design and development of more potent and convenient oligonucleotide therapeutics. The evolving applications and reduced dosages to be effective inevitably generate an increased demand for sensitive, accurate and reliable methods for qualitative and quantitative analysis of oligonucleotides in a variety of biological matrices.

This editorial will briefly discuss the recent advance in the techniques for bioanalysis of oligonucleotide therapeutics with the focus on novel hybridization ELISA and chromatographic-based assays that are simple and suitable for all chemically modified oligonucleotide therapeutics.

Meso scale discovery electrochemiluminescence (MSD-ECL) ELISA

MSD-ECL ELISA is a method similar to conventional ELISA except that MSD uses ECL as a detection technique as opposed to a colorimetric reaction in plates employed by conventional ELISA. An ECL system, such as the MSD platform, has many advantages over a traditional ELISA system such as: higher sensitivity, increased dynamic range (up to five orders of magnitude), less matrix effects, ability to multiplex and less sample volume required [3,4]. The only drawback is that the assays are still not able to distinguish the parent drugs from their critical metabolites. Therefore, the MSD-ECL ELISA is gaining popularity for quantitative analysis of oligonucleotide therapeutics to support the studies that require very sensitive assays. The use of locked nucleic acid (LNA) modified DNA probes can further improve the assay sensitivity. Combining the LNA probe design and MSD-ECL detection, Thayer *et al.* developed an assay with a dynamic range of 0.1–10,000 pM in serum, liver and kidney homogenates for the detection of a 22-mer modified siRNA [4].

Enzyme-linked oligonucleotide hybridization assay (ELOHA)

ELOHAs are based on the competition of analytes with a detection oligo for hybridization to a capture oligo covalently linked to a solid substrate. The versatility of ELOHA was recently demonstrated by Lorenson *et al.* using the measurement of three oligonucleotides, including two 25-mer morpholino-oligomers and an unmodified 23-mer DNA oligonucleotide [5]. The assays have sensitivity as low as 0.28 pmol/sample reaction at 50% hybridization. Unlike the MSD-ECL ELISA, which requires a proprietary instrument, these assays only require a basic 96-well plate colorimetric reader, which is standard in clinical and research laboratories.

Triplex-forming oligonucleotide (TFO) ELISA for quantitation of siRNA–protein conjugates

TFO-ELISA was developed to quantify intact double-stranded siRNA complexes. It is well known that appropriately designed LNA or peptide nucleic acid (PNA)-containing probes can form stable triplexes with their target DNA or RNA duplexes for a variety of biomedical applications [6]. Based on this mechanism, Humphreys *et al.* developed a TFO-ELISA to quantify intact siRNA–mAb conjugates in biological matrices [7]. In their preliminary studies, a lower LOD of 120 pg/ml was achieved to detect intact siRNA–protein conjugates in serum or plasma. With further characterization and optimization of the LNA modification pattern in the TFO, it is believed that the assay sensitivity could be further improved. Although this is the first study providing a proof-of-concept for the application of TFOs to detect intact siRNA–mAb conjugates, this technology can be readily applied to other modalities, including siRNA conjugated to peptides or other non-mAb proteins, unconjugated siRNA, or polynucleotides in bioanalytical samples.

Liquid chromatography coupled high resolution accuracy mass spectrometry (LC-HRAM) for sensitive and accurate quantitation and metabolite profiling

LC–MS has become an important analytical tool for the analysis of oligonucleotides because of its capabilities for both accurate identification and quantification. Many attempts have been devoted to improve the assay sensitivity, specificity and throughput by improving sample preparation, LC separation and MS technologies.

1. Sample extractions

Sample cleanup is a critical step to removing most of the lipids and proteins in biological matrices for LC–MS analysis of oligonucleotides. While liquid–liquid extraction (LLE) is still proven to be the most effective extraction method for plasma and urine assays, several solid phase extraction (SPE) methods or the combination of LLE and SPE methods have been developed for various structures of oligonucleotides in a variety of biological matrices [8–13]. Unlike LLE, which is simple and works for almost all classes (except for the short hydrophobic

oligomers) of oligonucleotides, SPE is very sensitive to the sequences and chemical modifications of the target oligonucleotides. Therefore, the SPE sorbents and/or extraction conditions need to be carefully selected and optimized for each individual class of oligonucleotide therapeutics. We found it is particularly challenging to extract hydrophobic group modified oligonucleotides from biological matrices. Therefore, the probe hybridization extraction method is probably the preferred option for LC–MS analysis [9].

2. Liquid chromatographic separation

Many chromatographic separation methods have been used for the analysis of oligonucleotides including anion-exchange chromatography, hydrophilic interaction chromatography (HILIC) and ion-pair reverse phase liquid chromatography (IP-LC). However, IP-LC is still the major technique used for LC–MS analysis of oligonucleotide therapeutics [14]. The ion pairing reagents and the mobile phase compositions for the analysis of a variety of oligonucleotides were extensively studied by different research groups over the last few years [15–17]. The 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and trimethylamine (TEA) or N,N-Diisopropylethylamine (DIPEA) together with methanol remain the most effective mobile phases for a majority of the therapeutic oligonucleotides. However, in some cases, other fluoroalcohol and/or alkylamine combinations may provide better sensitivity and/or separation depending on the length, sequence and chemical modifications of the oligonucleotides. The advances in column chemistry and packing techniques are also providing more durable and higher resolution LC columns for IP separation of oligonucleotides. One example is the new wide-pore hydrophobic polymer-based DNAPac RP column [18]. These columns can tolerate high pH and/or high temperature conditions and provide excellent separation for a variety of single- and double-stranded oligonucleotides under MS compatible conditions. More importantly it has an increased lifetime and lower carry over, which are critical for bioanalysis of oligonucleotide therapeutics.

In addition to IP-LC, HILIC is advancing to a promising alternative for the analysis of oligonucleotides [19]. Instead of the hydrophobic stationary phase employed in IP-LC, HILIC stationary phases consist of polar (hydrophilic) stationary phases where compounds are eluted in order of increasing polarity. Like RP-LC columns, most of the stationary phases in HILIC columns are silica based. These include those as simple as bare silica or bonded phases that contain neutral or ionizable functional groups. But unlike most RP separations, there is no single HILIC stationary phase that can be universally applied to the separation of polar compounds. Without the use of ion-pairing reagents, MS ionization of oligonucleotides in HILIC conditions is proven to be more efficient compared to IP-RPLC conditions. However, early studies were not able to achieve satisfactory LC separations for complex mixtures of oligonucleotides [17]. More recently, a new polymer-based diol HILIC column (Shodex VN-520) was reported to produce good separation and sensitivity for LC–MS/MS analysis of a variety of oligonucleotides in biological matrices under carefully optimized mobile phase compositions and gradient conditions [19]. Additionally, a direct comparison experiment demonstrates that the HILIC-based LC–MS methods yielded comparable sensitivities and separations to existing IP-RP-LC methods for various classes of oligonucleotide samples, providing the basis for alternative options for sensitive, reproducible and quantitative analysis of oligonucleotides without the need for ion-pair reagents.

3. Mass spectrometry detection

The development of sensitive LC–MS/MS assays remains challenging for oligonucleotides despite the substantial improvements recently identified in sample extraction, liquid chromatographic separations and mass spectrometry detection methodologies. Assays with sub-ng/ml LLOQ were only reported for measuring some short oligonucleotides in plasma with well-designed sample extraction and LC–MS analysis conditions [13,20]. Notably, high-resolution accurate-mass (HRAM) mass spectrometry appears to achieve comparable or better sensitivity for most of the evaluated therapeutic oligonucleotides and therefore are gaining increasing applications in their bioanalysis, especially for the non-phosphorothioate-containing oligonucleotides [12]. For the quantitation of phosphorothioate-modified oligonucleotides, LC–MS/MS methods are sometimes advantageous on a triple quadrupole MS as the major and unique fragmentation PO2S- (m/z = 94.9) ion provides sensitive and adequately specific quantitation [17,21]. However, to enable the distinction between PO2S- (m/z = 94.9362) and PO4- (m/z = 94.9540) ions, a minimum resolving power of 5500 would be required. This resolution is not feasible for current triple quadrupole MS systems but can be easily achieved using either Orbitrap or TOF mass analyzers. Actually, by monitoring the PO2S- product ion of a 22-mer phosphorothioate oligonucleotide

using high-resolution multiple reaction monitoring (MRMHR) on a TripleTOF® 6600 System, Tozaki *et al.* were able to develop an assay to successfully quantify a 22-mer phosphorothioate oligonucleotide in plasma with a dynamic range of 0.1–200 ng/ml in horse plasma [20]. Another big advantage for LC-HRAM-MS analysis is that typical m/z peaks or MS/MS transitions for particular analytes are not required to be selected prior to the data acquisition, making it possible for simultaneously quantitative and qualitative analysis of oligonucleotide therapeutics and their metabolites in biological matrices [22]. Additionally, the LC-HRAM-MS utility for the rapid determination of the relative amounts of coeluting impurities in oligonucleotide product samples has been demonstrated [23].

Hybridization LC–fluorescence assay for sensitive and specific quantitation of modified oligonucleotides

Hybridization LC–fluorescence assays are the combination of probe hybridization and LC–fluorescence analysis. These hybrid assays maintain the advantages of both types of assays. Similar to hybridization ELISA assays, hybridization LC–fluorescence assays can easily achieve a 1.0 ng/ml or lower LLOQ to quantify oligonucleotides in plasma or tissue homogenate without the use of special sample extractions [24,25]. Parent drugs can be separated adequately via liquid chromatography to distinguish them from their critical metabolites, including the separation between 5´-phosphorylated and non-phosphorylated guide strands of the siRNA [26]. Additionally, since no enzyme is involved in the entire process, the assays are not affected by oligonucleotide structural modifications like lipid group conjugations [25]. The only challenge is the requirement of an appropriate probe design, which is critical for assay sensitivity, specificity and dynamic range. This leads to longer method development times in addition to increased cost [27]. Therefore, these assays are usually used to support clinical or late stage preclinical studies, while the LC-HRAM-MS assays are more commonly used in drug discovery or early development stages.

Discussion and future perspectives

As non-modified DNA or RNA oligonucleotides are not stable in biological matrices and cannot adequately penetrate into living cells, the therapeutic oligonucleotides are usually chemically modified to reach their target and produce the desired pharmacological effects. Although qPCR is a golden standard for sensitive quantitation of DNA and mRNA molecules, the assays usually have insufficient sensitivity for short or heavily modified oligonucleotide therapeutics because qPCR bioanalysis of therapeutic oligonucleotides involves sample extraction and PCR amplification. Additionally, they require expensive reagents, complex instrumentation and careful assay optimization [5,28]. MSD-ECL-ELISA and ELOHAs do not require extensive plasma/tissue extractions, they have better sensitivity compared to conventional hybridization ELISA and their assay dynamic ranges are significantly improved. The only drawback is that these assays are being unable to distinguish the full-length analytes from their close metabolites. As a complementary technique, the LC–MS assay can clearly distinguish the analyte oligonucleotides from their metabolites. However, the development of sensitive LC–MS/MS assays remains challenging. Lowering the limit of quantification to 1 ng/ml or below still appears difficult with the exception of some short oligonucleotides. Given they are easy to use and usually more sensitive compare to conventional LC–MS/MS assays, LC-HRAM-MS assays have shown many advantages in supporting research and development of oligonucleotide therapeutics. More importantly, they can be used to conduct quantitative analysis and metabolite profiling simultaneously. Hybridization LC–fluorescence assays are good compromises between the ECL-ELISA and LC–MS methods. They are adequately sensitive and specific for most of the oligonucleotide therapeutics. As the fluorescent probes need to be carefully designed and optimized for each specific oligonucleotide analyte, they are often used to support late stage preclinical research of clinical studies.

References

1. Smith CIE, Zain R. Therapeutic oligonucleotides: state of the art. *Annu. Rev. Pharmacol. Toxicol.* 59, 605–630 (2019).

2. Huang Y. Preclinical and clinical advances of GalNAc decorated nucleic acid therapeutics. *Mol. Ther. Nucleic Acids* 17(6), 116–132 (2017).

3. Shemesh CS, Yu RZ, Gaus HJ *et al.* Pharmacokinetic and pharmacodynamic investigations of ION-353382, a model antisense oligonucleotide: using alpha-2-macroglobulin and murinoglobulin double-knockout mice. *Nucleic Acid Ther.* 26(4), 223–235 (2016).

4. Thayer MB, Lade JM, Doherty D *et al.* Application of locked nucleic acid oligonucleotides for siRNA preclinical bioanalytics. *Sci. Rep.* 9(1), 3566 (2019).

5. Lorenson MY, Chen KHE, Walker AM. Enzyme-linked oligonucleotide hybridization assay for direct oligo measurement in blood. *Biol. Methods Protoc.* 4(1), 1–8 (2019).

6. Gupta A, Mishra A, Puri N. Peptide nucleic acids: advanced tools for biomedical applications. *J. Biotechnol.* 259, 148–159 (2017).

7. Humphreys SC, Thayer MB, Campuzano IDG, Netirojjanakul C, Rock BM. Quantification of siRNA–antibody conjugates in biological matrices by triplex-forming oligonucleotide ELISA. *Nucleic Acid Ther.* 29(3), 161–166 (2019).

8. Franzoni S, Vezzelli A, Turtoro A *et al.* Development and validation of a bioanalytical method for quantification of LNA-i-miR-u221, a 13-mer oligonucleotide, in rat plasma using LC–MS/MS. *J. Pharm. Biomed. Anal.* 150, 300–307 (2018).

9. Dillen L, Sips L, Greway T, Verhaeghe T. Quantitative analysis of imetelstat in plasma with LC–MS/MS using solid-phase or hybridization extraction. *Bioanalysis* 9(23), 1859–1872 (2017).

10. Meng M, Zhang J, Liu A, Reuschel S, Sazani P, Wong M. Quantitative determination of AVI-7100 (Radavirsen), a phosphorodiamidate morpholino oligomer (PMOplus®), in human plasma using LC–MS/MS. *Bioanalysis* 9(10), 827–839 (2017).

11. Ewles M, Goodwin L, Schneider A, Rothhammer-Hampl T. Quantification of oligonucleotides by LC–MS/MS: the challenges of quantifying a phosphorothioate oligonucleotide and multiple metabolites. *Bioanalysis* 6(4),447–464 (2014).

12. Wheller R, Summerfield S, Barfield M. Comparison of accurate mass LC–MS and MRM LC–MS/MS for the quantification of a therapeutic small interfering RNA. *Int. J. Mass Spectrom.* 345–347, 45–53 (2013).

13. Hemsley M, Ewles M, Goodwin L. Development of a bioanalytical method for quantification of a 15-mer oligonucleotide at sub-ng/ml concentrations using LC–MS/MS. *Bioanalysis* 4(12), 1457–69 (2012).

14. Kaczmarkiewicz A, Nuckowski L, Studzinska S, Buszewski B. Analysis of antisense oligonucleotides and their metabolites with the use of ion pair reversed-phase liquid chromatography coupled with mass spectrometry. *Crit. Rev. Anal. Chem.* 49(3), 256–270 (2019).

15. Liu R, Ruan Y, Liu Z, Gong L. The role of fluoroalcohols as counter anions for ion-pairing reversed-phase liquid chromatography/high-resolution electrospray ionization mass spectrometry analysis of oligonucleotides. *Rapid Commun. Mass Spectrom.* 33(7), 697–709 (2019).

16. Li N, El Zahar NM, Saad JG, van der Hage ERE, Bartlett MG. Alkylamine ion-pairing reagents and the chromatographic separation of oligonucleotides. *J. Chromatogr. A.* 1580, 110–119 (2018).

17. Studzinka S, Rola R, Buszewski B. The impact of ion-pairing reagents on the selectivity and sensitivity in the analysis of modified oligonucleotides in serum samples by liquid chromatography coupled with tandem mass spectrometry. *J. Pharm. Biomed. Anal.* 138, 146–152 (2017).

18. Baek J, Thayer J, Lin SH, Birznieks I, D'Silva K, Cubbon S. Chromatography for the analysis of oligonucleotides. *Chromatography Today,* 10(2), 26–29 (2017).

19. Lobue PA, Jora M, Addepalli B, Limbach PA. Oligonucleotide analysis by hydrophilic interaction liquid chromatography-mass spectrometry in the absence of ion-pair reagents. *J. Chromatogr. A.* 1595, 39–48(2019).

20. Tozaki T, Karasawa K, Minamijima Y *et al.* Detection of phosphorothioated (PS) oligonucleotides in horse plasma using a product ion (m/z 94.9362) derived from the PS moiety for doping control. *BMC Res. Notes* 11(1), 770 (2018).

21. Husser C, Brink A, Zell M, Müller MB, Koller E, Schadt S. Identification of GalNAc-conjugated antisense oligonucleotide metabolites using an untargeted and generic approach based on high resolution mass spectrometry. *Anal. Chem.* 89(12), 6821–6826 (2017).

22. Friese OV, Sperry JB, He Y, Joseph L, Carroll JA, Rouse JC. Characterization of apolipoprotein C3 (Apo C3) LNA/DNA impurities and degradation products by LC–MS/MS. *AAPS J.* 19(6), 1735–1744 (2017).

23. Roussis SG, Cedillo I, Rentel C. Semi-quantitative determination of co-eluting impurities in oligonucleotide drugs using ion-pair reversed-phase liquid chromatography mass spectrometry. *J. Chromatogr. A.* 1584, 106–114 (2019).

24. Tian Q, Rogness J, Meng M, Li Z. Quantitative determination of a siRNA (AD00370) in rat plasma using peptide nucleic acid probe and HPLC with fluorescence detection. *Bioanalysis* 9(11), 861–872 (2017).

25. Godinho BMDC, Gilbert JW, Haraszti RA *et al*. Pharmacokinetic profiling of conjugated therapeutic oligonucleotides: a high-throughput method based upon serial blood microsampling coupled to peptide nucleic acid hybridization assay. *Nucleic Acid Ther.* 27(6), 323–334 (2017).

26. Trubetskoy VS, Griffin JB, Nicholas AL *et al.* Phosphorylation-specific status of RNAi triggers in pharmacokinetic and biodistribution analyses. *Nucleic Acids Res.* 45(3), 1469–1478 (2017).

27. Ji YH, Liu YJ, Xia WH, Behling A, Bennett P, Wang L. Probe design is critical for the sensitivity and specificity of the bioanalysis of oligonucleotide using hybridization based LC–fluorescence assays. *Bioanalysis* (2019) [manuscript in preparation].

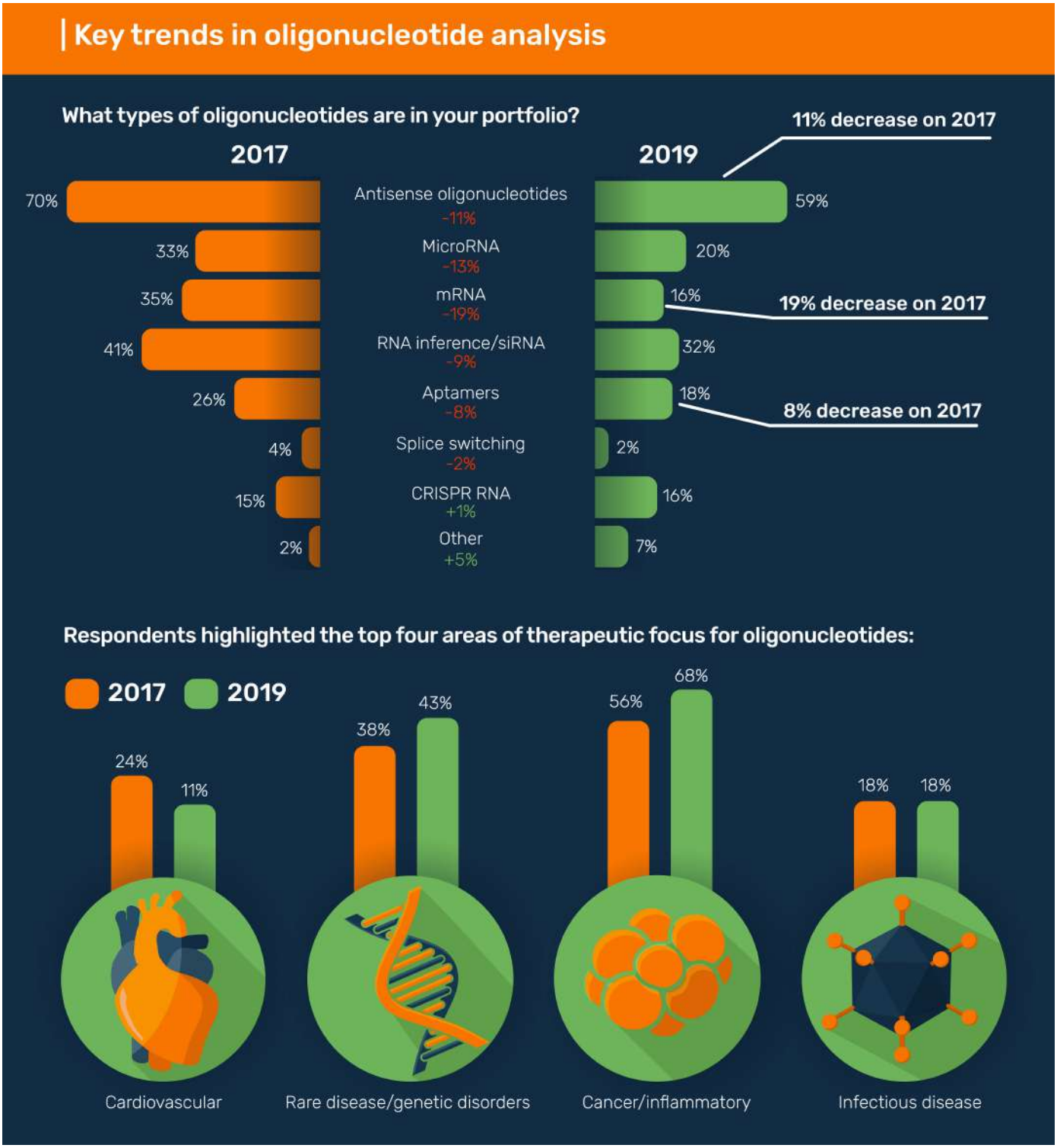
28. Wang L, Ji C. Advances in quantitative bioanalysis of oligonucleotide biomarkers and therapeutics. *Bioanalysis* 8(2), 143–155 (2016).

The exciting world of oligonucleotides – how far have we come?

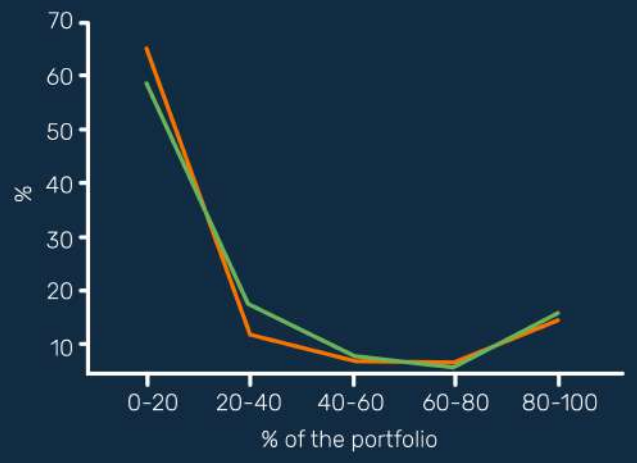
Over 2 years ago, Bioanalysis Zone hosted an engaging Spotlight on oligonucleotides. This brought together leading experts within the bioanalytical community to discuss their experience with oligonucleotides and the current challenges facing the field, in addition to looking at what the future could hold.

The 2017 Spotlight survey highlighted key trends regarding what types of oligonucleotides are studied, assay formats used to analyze oligonucleotides and opinions on methodology and guidance for oligonucleotide analysis. To explore how this field has changed over the last 2 years, a second survey was conducted in 2019, enabling key variations in data to be analyzed.

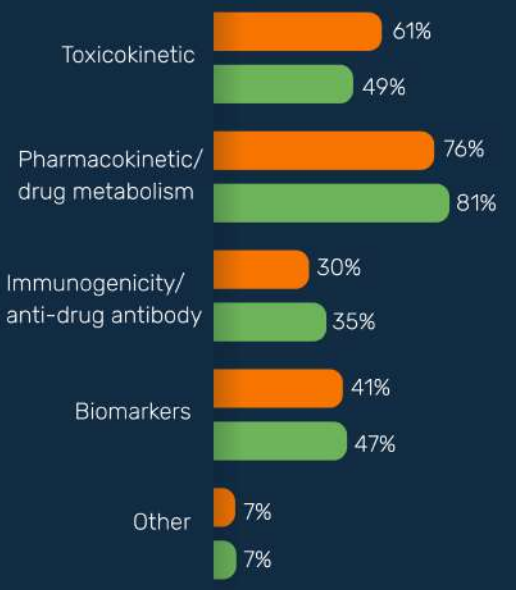
A full comparison of the statistical data can be seen below.


Key trends in oligonucleotide analysis
What types of oligonucleotides are in your portfolio?
2017
2019
11% decrease on 2017
19% decrease on 2017
8% decrease on 2017
70%
Antisense oligonucleotides
-11%
59%
33%
MicroRNA
-13%
20%
35%
mRNA
-19%
16%
41%
RNA inference/siRNA
-9%
32%
26%
Aptamers
-8%
18%
4%
Splice switching
-2%
2%
15%
CRISPR RNA
+1%
16%
2%
Other
+5%
7%
Respondents highlighted the top four areas of therapeutic focus for oligonucleotides:
2017
2019
24%
11%
38%
43%
56%
68%
18%
18%
Cardiovascular
Rare disease/genetic disorders
Cancer/inflammatory
Infectious disease

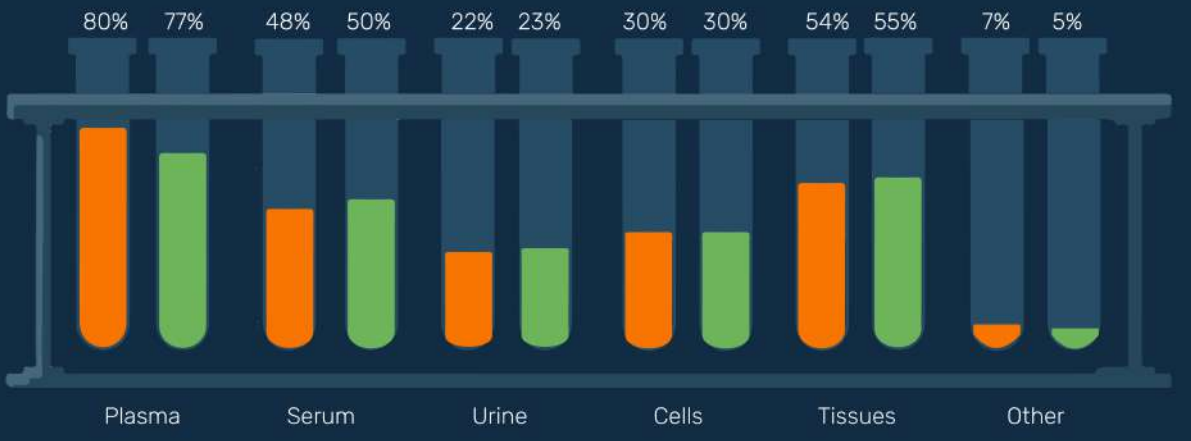
What percentage of the portfolio is oligonucleotides compared to traditional small molecules and large molecules?



What type of oligonucleotide analysis are you conducting?

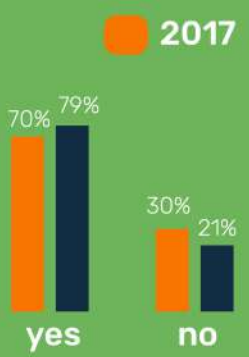


Types of matrices that are used for analyzing oligonucleotides:

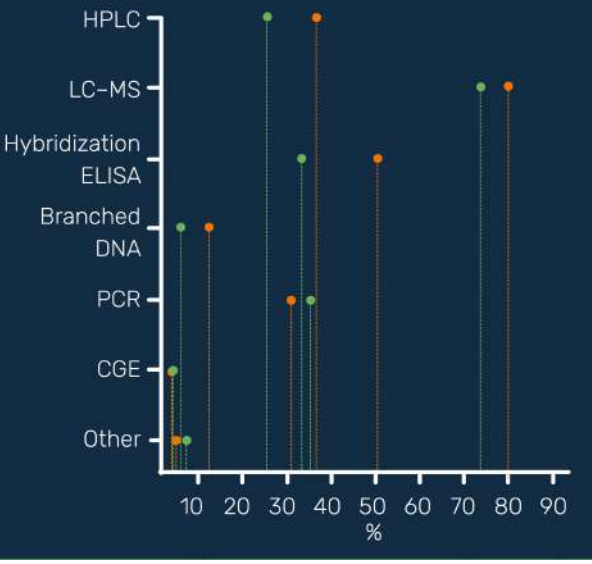


Methodology and guidance for oligonucleotide analysis

For the majority of the time, does existing methodology meet the project needs?



Assay formats used to analyze oligonucleotides:




Where are the gaps in oligonucleotide research?
Specificity/ selectivity
2017 2019
71% 40%
Sensitivity
2017 2019
79% 60%
Robustness
2017 2019
64% 30%
Regulation of tissue analysis
2017 2019
29% 30%
Other
2017 2019
7% 10%
Are the current regulatory guidance's satisfactory for the analysis of oligonucleotides?
YES
2017 2019
52% 48%
NO
2017 2019
48% 52%
What do you think are the main challenges of bringing oligonucleotides to market?
2017
2019
Cost
28%
44%
Production time
7%
21%
Facility capacity
11%
28%
Issues with technology
35%
36%
Requiring highly skilled experts
23%
26%
Issues with off target toxicity
19%
26%
Competition from other companies
2%
3%
Regulatory acceptance
32%
23%
Future trends in oligonucleotide analysis
In the next 5 years, do you think the percentage of oligonucleotides in your company portfolio will...
2017
2019
Increase
52%
71%
Stay the same
44%
24%
Decrease
4%
5%

What new methodologies/instrumentation do you think will improve current analysis?

2017

- Better cleaning of instruments
- New PCR
- Microflow LC-HRMS, qPCR
- One that offers more sensitive assays

2019

- HPLC
- HRMS
- Hybrid LC–MS assays
- More sensitive detection

About the respondents

Location:

	2017	2019
North America	52%	58%
Europe	22%	25%
Asia	21%	13%
Rest of World	5%	4%

Job title:

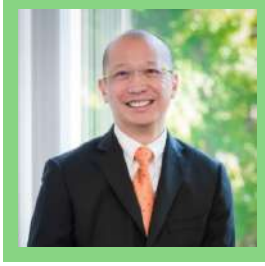
	2017	2019
Technician	7%	8%
Chemist/Scientist	34%	34%
Manager/Group Leader	31%	29%
Director/CEO/VP	19%	26%
Other	9%	3%

Bioanalysis ZONE

QPS CUSTOM-BUILT RESEARCH™

Infographic commentary: oligonucleotide trends by Zamas Lam

Zamas Lam; VP of Preclinical Development at QPS.



Zamas is the senior VP of Preclinical Development at QPS (DE, USA) and trained in mass spectrometry, carbohydrates and glycoproteins. Since graduate school, he keeps thinking that it will be fun to work on a gene level in drug discovery and development versus at the protein level or at the metabolites level!

QPS (DE, USA) validated our first ASO plasma PK assay in 2002 using LC–MS/MS on a triple quadrupole. The 'state-of-the-art' technology at that time was hybridization-ELISA while LC–MS was an unknown 'possibility'. Fast forward 17 years and we have worked on over 50 ASO, siRNA, PEGylated aptamers, mRNA and vectors. The lesson learned is there is no one best method for bioanalysis, as hybridization ELISA, UPLC–HRMS, UPLC–MS/MS, hybridization-LC-FLD, LC UV and qPCR are all viable techniques. It is the physicochemical property of the oligonucleotides, mRNA and vectors that determines the methodologies. This specific commentary addresses the changes in bioanalysis of oligonucleotides over the past few years.

Although the bioanalytical technology has improved dramatically over the past 17 years, the fundamental differences between hybridization-ELISA verses chromatography has not changed. Hybridization ELISA generally has better sensitivity while chromatography methods have better selectivity. Hybridization ELISA using electrochemiluminescence detection can regularly achieve low pg/mL range. Although the LLOQ is at the low ng/mL for the previous generation of UPLC–MS, the newer UPLC–MS, that have been commercialized in the last 2 years, can reach down to mid to low pg/mL range and thus negating the previous sensitivity advantage of hybridization-ELISA.

Hybridization ELISA assays consist of many formats, such as sandwich, competitive, ligation and dual ligation assays. They all take advantage of a complementarily to capture the oligonucleotides and have another complementary strand with a probe. The sandwich and the competitive hybridization formats are more general and can capture parent and potential metabolites. The hybridization-ligation format was developed to target the 3'-end of the oligonucleotides, which means the assays can be distinguished from the parent oligonucleotides from the 3'-end N 1, N 2, N 3… metabolites. The main drawback with this format is it cannot be used with any oligonucleotide chemistry that modify the 3'-end. The dual ligation hybridization format targets both the 3'-end and the 5'-end of the oligonucleotides and therefore have even more specificity than the hybridization-ligation format.

UPLC–MS oligonucleotide quantitation has undergone a dramatic change over the past 5 years. In the early 2000s, the only viable quantitative mass spectrometers were unit resolution triple quadrupole; however, with the advent of faster electronics, high-resolution mass spectrometers, such as ion-traps and quadrupole time-of-flight (qToF), seem to be the mass analyzers of choice. The advantages of triple quadrupole are lower instrumentation cost, lower operating cost, ease of use, higher availability and more trained personnel; while the two main disadvantages are the difficulties in trouble shooting a failing method and the simultaneous quantitation of parent and metabolites. The main advantages of HRMS is the

≥35,000 resolution to resolve the isotopic pattern, which easily affords the simultaneous quantitation of parent and metabolites and in separating the various cationized species of the same oligonucleotides; while the disadvantages are high operating cost, difficulties in training qualified personnel and the high data density associated with full scan quantitation.

There is an interesting divergence of opinion on whether ion-traps or qToF mass spectrometers are better for oligonucleotides quantitation. The resolving power of an ion-trap is a function of mass and scan speed and the most common 18 Hz instruments have 120K resolving power at 200 amu. However, the resolution goes down as the mass goes up. On the other hand, qToFs regularly function at 40,000 resolving power and have uniform resolution across all masses. As most chromatographic separations are performed by UPLC, and the peak width is ~2 seconds, therefore, the working resolution for both ion-traps and qToF is effectively at ~35,000 resolving power. There is a misconception that higher resolution is better, in fact, there is major expense associated with running HRMS on a full scan mode. The data file size for a batch of three 96 well plates from a triple quadrupole is ~2–3 MB, while it is ~4–5 GB for a HRMS running at 35,000 resolving power. The higher the resolution the bigger the data file size, and the logistics and infrastructure necessary for uploading the files, data processing, archiving and downloading back to the instrument for regulatory audits needs to be considered as part of the decision process.

The choice of LBA or chromatography for bioanalysis is really based on the sensitivity requirement, the potential off-target toxicity in different organs, which need quantitation and an understanding of metabolism, along with familiarity of the techniques employed. There have been reciprocal arguments that transferring validated serum hybridization-ELISA methods to various tissues homogenates is less demanding than transferring validated plasma LC–MS methods. However, in our hands we found as long as the appropriate attention has been paid to homogenization, sample preparation and method development transferring hybridization-ELISA method or LC–MS method between different matrices takes about the same amount of time and the quality of the data for both techniques is well within regulatory requirement. What is important is to plan and to have adequate well-trained personnel and appropriate instrumentation, as often oligonucleotides and their metabolites are highly distributed across all matrices. We have worked on up to 20 different tissues during preclinical and nonclinical studies.

With each new generation of oligonucleotides, endo- and exo-nucleases metabolism has contributed less and less to the clearance mechanism and the latest generation of oligonucleotide drug candidates generally show fewer N 1, N 2, N 3, … metabolites. Therefore, some believe it is best to just analyze the parent for IND-enabling and Phase I/II studies and leave metabolite profiling and quantitation until after positive Phase II PoC studies. Chromatography methods can simultaneous detect and quantitate the parent and each individual metabolites while hybridization ELISA cannot. If the oligonucleotides potentially have a fair amount of metabolism, it is more straightforward to develop and validate LC–MS methods to capture the full PK profile of the parent and metabolites. If hybridization ELISA is the bioanalytical platform, then after Phase II PoC, additional chromatographic methods may be needed.

In the early 2000s, it was uncertain if oligonucleotides trigger immune response and there was uniform rule for anti-drug antibody (ADA) assays. However, it is now clear that oligonucleotide drugs do solicit immune response. Therefore, it is highly advisable to initiate scientific discussions with the regulatory agencies on the merit of potentially developing and validating ADA assay in parallel to PK assay.

As oligonucleotides are becoming a more common drug modality, there is some ambiguity on what criteria should be used for method validation. Within QPS, we use the LBA criteria for hybridization ELISA methods and the chromatography criteria for LC–HRMS or LC–MS/MS or hybridization LC-FLD or LC-UV assays.

With six oligonucleotides that have been approved, at least another 20 in various stages of preclinical and clinical development, and the increasing interest in gene therapy and rare disease, we should expect to see corresponding improvement in bioanalytical platforms, the surrounding supporting reagents and more appropriate regulatory guidance on method validation in the next few years. Maybe by 2025, oligonucleotides will be just another common drug modality that will solicit less excitement in the pharmaceutical industry.

JOURNAL ARTICLES

Analytical techniques for characterization of biological molecules – proteins and aptamers/oligonucleotides

With the advent of the high-throughput technologies and exciting times for biology, the discipline of analytical methodology is experiencing a surge in the growth and the scope. Over the years, a multitude of analytical techniques have evolved from a work-intensive, low sensitivity and high volume of reagent and sample consumption endeavor to automated, better selectivity, lower limit of quantification and cost-effective techniques for biological research. In this review, we give an overview of the currently available wide range of cell-based, non-cell-based and structural-based analytical techniques, their principle and biological applications. The analytical techniques discussed in this paper includes surface plasmon resonance, electrophoresis, enzyme-linked immunosorbent assay, Western blotting, flow cytometry, fluorescence activated cell sorting, mass spectrometry, nuclear magnetic resonance and x-ray crystallography.

Read the full article in *Bioanalysis* 11(2), 103–117 (2019).

Development of SPE method for the extraction of phosphorothioate oligonucleotides from serum samples

Aim: Comprehensive development of a method for SPE extraction of antisense phosphorothioate oligonucleotide and its metabolites and their determination with the use of UHPLC. **Results:** Polymer-based adsorbent and high percentage of methanol in elution solvent provided high recoveries compared with silica-based octadecyl cartridge. As to the type and concentration of ion pair reagent and organic solvent, the mixture of 5 mM of N,N-dimethylbutylamine/150 mM of 1,1,1,3,3,3-hexafluoroisopropanol and methanol was selected. Relatively high recoveries in the range of 79.2–81.2% with the SDs of 3.4–6.2% were obtained for the oligonucleotide and its metabolites extracted from human serum. **Conclusion:** The developed method may be successfully applied for routine analysis of antisense oligonucleotides in serum since it is relatively easy, quick and reliable.

Read the full article in *Bioanalysis* 10(20), 1667–1677 (2018).

Quantitative analysis of imetelstat in plasma with LC–MS/MS using solid-phase or hybridization extraction

Aim: Imetelstat, a 13-mer oligonucleotide with a lipid tail is being evaluated for treating hematologic myeloid malignancies. This report describes the development of extraction and quantification methods for imetelstat. **Methodology & results:** Imetelstat was extracted using SPE (rat plasma) or by hybridization using a biotinylated capture probe (human plasma) and was quantified by LC–MS/MS. Calibration curves were established (0.1–50 µg/ml). Stability of imetelstat in plasma was demonstrated. Concentrations of imetelstat extracted using either of the methods and quantified with LC–MS/MS were comparable with a validated ELISA. **Conclusion:** Two extraction methods (solid phase and hybridization) were developed for quantifying imetelstat in plasma using LC–MS/MS. The hybridization extraction in combination with LC–MS/MS is a novel extraction approach.

Read the full article in *Bioanalysis* 9(23), 1859–1872 (2017).

Challenges and opportunities in bioanalytical support for gene therapy medicinal product development

Gene and nucleic acid therapies have demonstrated patient benefits to address unmet medical needs. Beside considerations regarding the biological nature of the gene therapy, the quality of bioanalytical methods plays an important role in ensuring the success of these novel therapies. Inconsistent approaches among bioanalytical labs during preclinical and clinical phases have been observed. There are many underlying reasons for this inconsistency. Various platforms and reagents used in quantitative methods, lack of detailed regulatory guidance on method validation and uncertainty of immunogenicity strategy in supporting gene therapy may all be influential. This review summarizes recent practices and considerations in bioanalytical support of pharmacokinetics/pharmacodynamics and immunogenicity evaluations in gene therapy development with insight into method design, development and validations.

Read the full article in *Bioanalysis* 9(18), 1423–1430 (2017).

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