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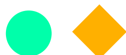

Oligonucleotides:

Where we started, where we stand, and where we would like to go

Common methods to support the bioanalysis of oligonucleotides and future perspective regarding areas for improvement

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OVERVIEW

In October 2022, at American Association of Pharmaceutical Scientists (AAPS) PharmSci 360 in Boston, MA, Director of Laboratory Operations of Aliri, Troy Voelker, Ph.D., was invited as a keynote speaker to present on the current knowledge and future directions of the bioanalysis of oligonucleotides (OGNTs). During this presentation, Dr. Voelker described the pros and cons of various analytical platforms, methods of extraction, and approaches that could improve the bioanalysis of these molecules.

OLIGONUCLEOTIDE BIOANALYSIS BASICS

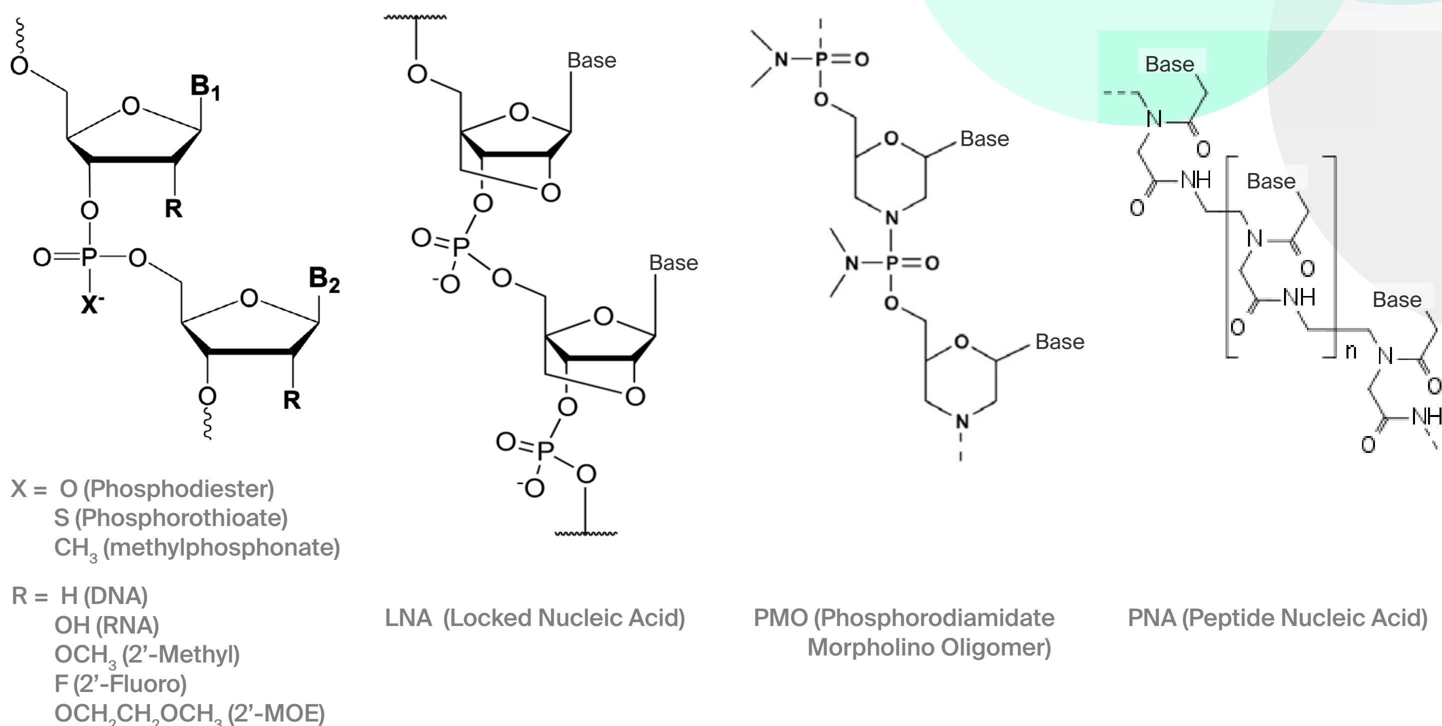
OGNTs are short (10–50 nucleotides) single- or double-stranded DNA or RNA. Some of the most common forms of OGNTs (see Figure 1) include antisense OGNTs, siRNA, miRNA, aptamers, peptide nucleic acids (PNAs), and phosphorodiamidate morpholino oligomers (PMOs). OGNTs can be used to manipulate gene expression as a disease therapy.

A variety of techniques are available to help support scientists during the analysis of OGNTs, including LC/UV, LC/MS-MS, hybridization LC/FD, LC/HRMS, and hybridization ELISA. Although LC/UV might not be a common approach, it is still used. Most of the analysis, however, relies on LC/MS-MS, hybridization LC/FD, and LC/HRMS.

LC/UV bioanalysis of OGNTs is simple, requires no extraction, the equipment—a UV detector and binary HPLC or UPLC pumps with an autosampler—is relatively inexpensive, and multiple analytes can be detected. Although there is no internal standard, reference standards are required for identification. On the downside, LC/UV is extremely nonselective and detection limits are usually only micrograms per milliliter.

Conversely, LC-MS is very selective and provides nanogram-per-milliliter detection of multiple analytes. It should be noted that the sensitivity decreases with longer OGNTs. With this approach, sample extraction allows sample concentration, if needed. Plus, LC-MS is much faster than LC/UV, but the equipment is also more expensive, requires an internal standard, and must be carefully maintained. Even the auto-sampler impacts the signal from a sample. With LC/MS-MS, the detection limit drops to about 10–20 ng/ml, although lower limits may be achieved depending on the chemical properties of the oligonucleotide.

FIGURE 1. Common structures of OGNTs.



LC-FD hybridizes the sample with a PNA probe, works very well with small sample aliquots, such as 30 microliters, and the raw sample can be injected directly onto the system after going through the necessary hybridization step. With reference standards, a range of metabolites can be separated to avoid interference. This technique takes longer than LC/MS—about 20 minutes [as compared to 6–7 minutes].

LC-HRMS is a high-resolution, accurate-mass approach that is very selective and analyzes either intact OGNTs or fragments depending on the preferred approach. This method provides much lower limits of quantitation compared to LC/UV, with the potential for sample concentration and faster run times versus LC/UV. The sensitivity decreases, however, with the length of the OGNTs. This method can also be applied with no intended target, and post-analysis can be used to mine the data. Nonetheless, the equipment is much more expensive to purchase and maintain.

Hybridization ELISA provides fast sample preparation and run analysis, which can be completed in a day. This method also works well with modified OGNTs, and the length of the OGNTs does not impact the sensitivity. In fact, Dr. Voelker called these "the most sensitive systems out there, in terms of limits of quantitation." However, hybridization ELISA is less selective than MS-based methods, only detects single strands, can be difficult troubleshooting, and can require critical reagents.

SELECTING AN APPROACH

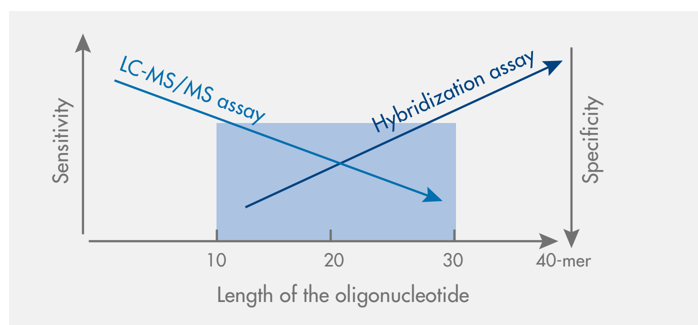
Given the variety of bioanalytical options, customers often ask for advice when selecting a method. "Typically, the more specific your assay can be for your compound, the better the data is going to be for regulatory submission," Dr. Voelker said.

Customers should also consider the desired detection range. For example, detection below 5ng/ml will typically require some form of hybridization. As shown in Figure 2, scientists need to use a method that fits their purpose—finding the right balance of sensitivity and specificity.

With an LC-MS approach, for example, the need for MS-MS or HRMS must be considered, as well as peak shape, retention, and carryover. Based on experience, Dr. Voelker gets better sensitivity with MS-MS, and the platform is easier to maintain than an HRMS-based system. The dwell time can also be optimized to improve sensitivity. As Dr. Voelker added, "During development, you certainly want to monitor multiple charge states and determine which one is going to afford your best selectivity and sensitivity." The ion-pairing reagents should also be selected to adjust the charge envelope.

With LC/HRMS, analysis can be either be targeted or full scan. A targeted approach, for example, can improve the signal-to-noise ratio, and it's easier to optimize the automated gain control. Nonetheless, data can only be mined with a full-scan approach. From a chromatographic perspective, optimization is required for various parameters, including the peak shape, LC retention, and carryover. "You can get a greater peak shape on a lot of columns, and you get absolutely terrible carryover," Dr. Voelker said.

FIGURE 2. Right method for the right purpose.



- LC-MS/MS (HRAM) assays are good for small oligonucleotides. Typical LLOQ is ~5–20 ng/mL
- Hybridization-based assays are good for large oligonucleotides. Typical LLOQ is ~0.5–1 ng/mL

The use of hexaflouroisopropanol (HFIP) (see Figure 3)[1] can be used to overcome surface tension of droplets and increase ionization of the oligonucleotide. "If you don't have enough HFIP, the oligonucleotide is a lover of aqueous matter," Dr. Voelker said. "Fluorinated alcohols help break down that surface tension of your droplet." On the other hand, too much fluorinated alcohol can suppress the signal. So, this process requires a careful balance.

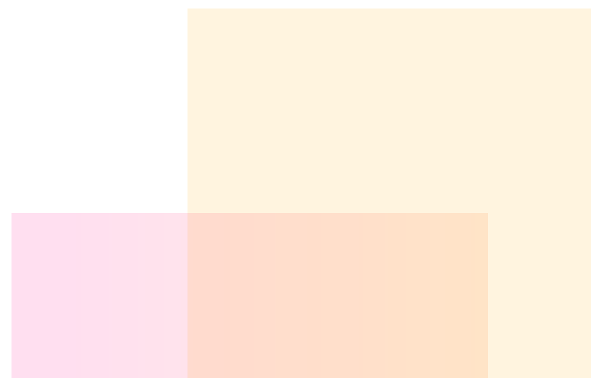
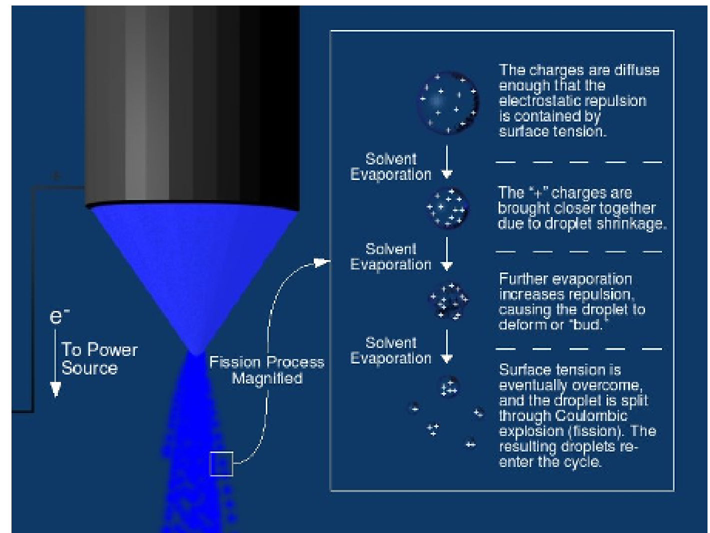


FIGURE 3. Why do we need HFIP?[1]



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METHODS OF EXTRACTION

When analyzing OGNTs with LC/MS, the common forms of extraction are liquid/liquid exchange with phenol/chloroform or solid phase.

A liquid/liquid exchange with phenol/chloroform works well with 25–50 microliter aliquots, because large aliquots can gel the sample due to residual proteins left behind in the extracts. Still, thorough mixing is required to avoid post-extraction gelling. When using automated liquid handling, the pipette should not reach the protein layer in a sample. If the tip does reach that layer, "You pretty much lost your extracts, and you have to go do the run again." For final sample clean-up, Dr. Voelker recommended back extraction of the aqueous layer with methylene chloride.

As a key precaution in any oligonucleotide extraction, Dr. Voelker emphasized: "Do not lose your sample before you actually get to inject it." As he noted, the sample can be lost in various ways, including the nonspecific binding properties of OGNTs, including absorption in stock and spiking solutions, to extraction plates, or to the LC system. Oxidation of your phosphorothioate backbone can be a serious problem.

For solid-phase extraction, Dr. Voelker highlighted the Clarity OTX as "the go-to, at this point in time." This four-step process is a high-throughput method, but it does take a long time to wash and dry down the samples.

In describing an upward quadratic response in solid-phase extraction, Dr. Voelker noted that it can show a decrease in extracts after extraction, a loss of OGNTs during the preparation of samples, or oxidation. In the case of a decrease in extracts after extraction, for instance, he suggested potential sources, such as the extraction plate, which might necessitate using a surfactant or changing the final extraction plate.

For solid-phase extraction with the Clarity OTX, Dr. Voelker noted that a sample-to-buffer ratio of at least 1:5 can often improve the recovery. For the elution step, oxidation can be reduced by stabilizing tetrahydrofuran with butyl hydroxylated toluene.

HANDLING HYBRIDIZATION METHODS

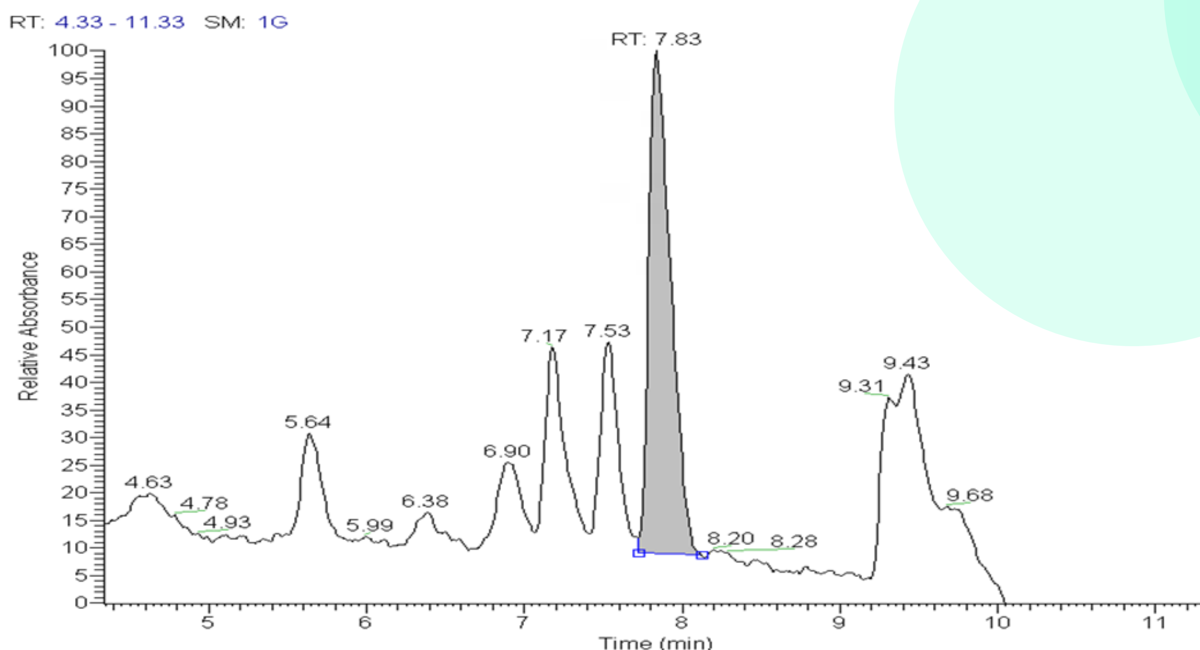
Hybridization-based fluorescence assays provide lower limits of quantification—not as low as ELISA hybridization, but enough for most programs. As Dr. Voelker says, “It’s very simple.” The sample, such as an anti-sense OGNT, is heated, hybridized with a PNA probe, and cooled followed with anion exchange and analysis. Extraction is not required, but can be used when lower limits of quantification are required. Without an internal standard, however, extraction can impact accuracy and precision.

Figure 4 demonstrates an example chromatogram for an HPLC–hybridization-based fluorescence assay. Hybridization is very straightforward, but the separation on the back end can be time-consuming.

With hybridization-based ELISA assays, scientists can use a sandwich-complex or ligation-assay approach. The sandwich-complex approach is simple, straightforward, and may detect the parent OGNT and metabolites. In addition, it can provide increased specificity with larger therapeutic OGNTs.

With a ligation assay, the 3'-hydroxyl end of the OGNT must be accessible to ligate to the detection probe. “The main advantage of this is the 3'-metabolites typically do not have that and therefore go undetected,” Dr. Voelker said. “You’re still going to detect the 5', but there’s much less of those 5's in the matrix than the 3's.”

FIGURE 4. Hybridization-based HPLC-fluorescence assay.



WHAT'S AHEAD?

Dr. Voelker concluded his presentation with a discussion regarding future analysis. He noted six goals:

- Improving specificity and selectivity in LC/MS extraction and basing it more on structure than charge
- Simpler LC conditions
- Reduction of multiple charge states
- More bioinert consumables and instrumentation
- Increased sensitivity of mass spectrometers
- 3D spatial tissue analysis

Dr. Voelker noted that 3D spatial analysis of tissue might address where drugs are penetrating and how far they are getting into the tissues.

Overall, Dr. Voelker's presentation explored a range of analytical techniques for OGNTs. A scientist must consider the complexity of an approach and its strong and weak points. Only then can the best approach be selected for a specific application.

References

[1] LibreTexts™ Physics. 6.3: Electrospray ionization (ESI) mass spectrometry. (2022, November 8). https://phys.libretexts.org/@go/page/14603.

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