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Mastering Oligonucleotides: From Prep and Manufacture to Regulatory Compliance

- Confidence in Analytical Characterization
- Challenges and Opportunities in Method Development
- Effective Strategies to Address Compliance

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Oligonucleotide Analysis in Biopharma Manufacturing

Method Development when Scaling up to Prep.

AUTHORS

Connor Flannery

Global Product Manager
HPLC Biocolumns

Agilent Technologies

INTRODUCTION

Thanks to the advancement of nucleic acid research and an increase in investments from the private and public sectors, a number of different oligonucleotide therapeutics have emerged. These include antisense oligonucleotides, which are single-stranded DNA or RNA molecules generally 13 to 25 nucleotides long, that bind to complementary target mRNA molecules; small interfering RNA (siRNA), which are double-stranded RNA molecules 20 to 25 base pairs in length, that interfere with gene expression via the RNA interference (RNAi) pathway; and microRNAs (miRNA), which are small, single-stranded, naturally occurring, non-coding RNAs that are 20 to 25 nucleotides in length and target gene expression by either blocking translation or causing degradation of target mRNAs.

Regardless of the type, separating the full-length target product from impurities can be challenging. By considering the nature of the oligo, it is possible to make informed choices about which approach is most ideal. However, it's important to first understand the scale of these molecules as they exist and are synthesized.

UNDERSTANDING OLIGO-THERAPEUTICS

Many oligo-therapeutics have been designed or synthesized to have a specific sequence and structure to optimize their efficiency. The process by which these oligonucleotides are synthesized is stepwise, meaning that each step introduces the next nucleotide in the sequence. With each addition, sequence failures or impurities can end up in the sample and, as the length of the oligo increases, yield decreases.

In addition to any sequence failure impurities, there may also be acetylation byproducts, truncations, incomplete deprotection, deamination, self-dimerization, and hair pins, where a single-stranded RNA backbites or associates with itself. All of this can lead to impurities in the sample.

Ultimately, liquid chromatography (LC) can play a critical role in analyzing and purifying the target product to improve yield and purity despite these challenges. There are several analytical approaches available.

CHOOSING BETWEEN IP-RP AND AEX

There are two main approaches to consider when it comes to purification: ion-pair reversed-phase chromatography (IP-RP) and anion exchange chromatography (AEX). Many technicians will select their choice based on previous experience and familiarity; however, there are a few factors that can influence the decision in the absence of a strong preference.

The first factor is the size or sequence length of the oligonucleotide. Longer oligos will have more points of ionic interaction and bind more strongly to an anion exchange column; as such, it can be difficult to elute these sequences off the column without using extreme mobile phase conditions, which can ultimately damage the product and lead to poor recovery. Therefore, oligos over 200 nucleotides in length are often better suited for IP-RP.

Other factors to consider are cost and environmental impact, especially if a scale-up is on the horizon. Ion pairing reagents and organic solvents are less environmentally friendly and can become cost prohibitive when working at the prep scale compared to anion exchange mobile phase reagents; this is where AEX can be a better option.

Another consideration is the sample's next step after purification. If IP-RP is chosen, for example, necessary organic solvents

may not be compatible with the formulation buffer; if AEX is selected, excess salts may interfere with the next step.

Finally, detection methods must be considered. AEX chromatography utilizes a high salt concentration and buffers that are not volatile, and thus is not compatible with mass spectrometry (MS); IP-RP is better suited for mass spectrometry.

OLIGONUCLEOTIDE PURIFICATION: AEX CHROMATOGRAPHY

The anionic phosphate backbone of oligonucleotides lend themselves well to AEX retention mechanisms, where interactions between the negatively charged backbone of the oligo and a positively charged anion exchange media would lead to retention on column.

Agilent offers two options to optimize AEX separations. The first is the non-porous Agilent Bio SAX, available in size options from 1.7 to 10 µm. This is ideal for high resolution separations because of improved mass transfer. The second is Agilent PL-SAX, also a polymeric particle, and uniquely suitable for high-pH and high-temperature conditions. It is available in analytical, semi-prep, prep, and bulk media, with size options ranging from 5 to 30 µm for large-scale purification suitability. PL-SAX is a totally porous particle, which provides increased surface area compared to the non-porous Bio SAX, meaning it can accommodate a range of oligo sizes and sequence lengths.

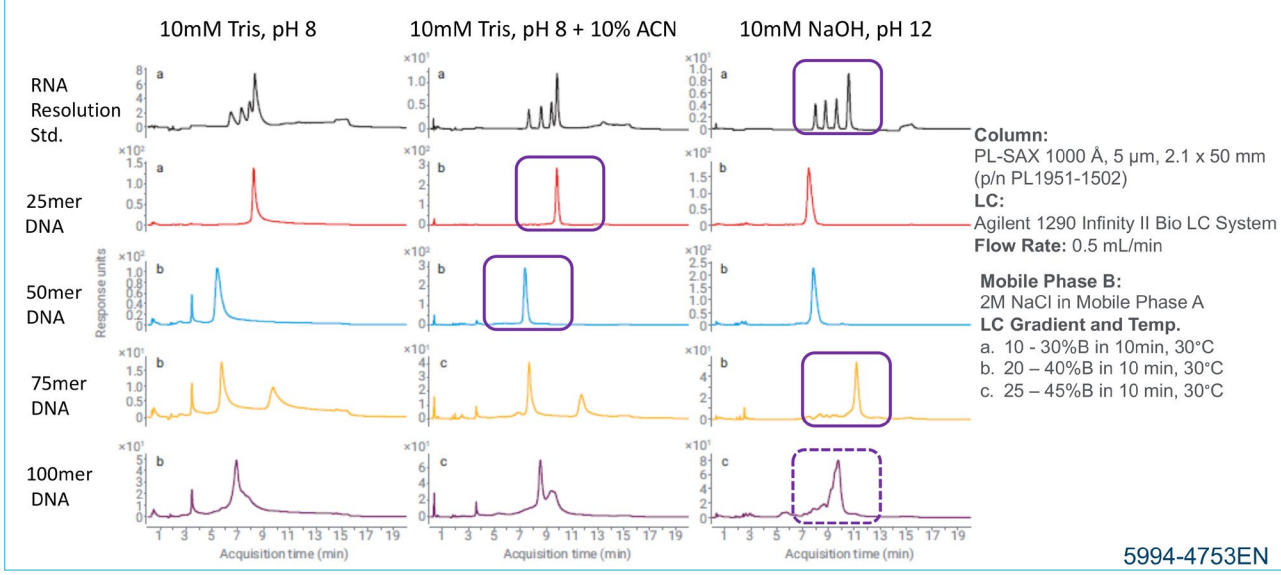
There are several variables that can be tweaked to optimize AEX separations. Typical method conditions are as follows:

- Buffer type: suitable options include phosphate and Tris
- Buffer concentration: about 20 mM
- Buffer pH: 7 or 8
- Eluting salt: sodium chloride
- Temperature: elevated temperatures can improve peak shape, but may compromise column life
- Organic modifiers: secondary interactions with certain stationary phases can be mitigated by the addition of acetonitrile up to 10-15%

That said, it is recommended to take a systematic approach to method development and screen modifiers as needed. While the above represents typical method conditions, every oligonucleotide is different, and the optimum conditions will be different for each one. For example, it's possible to change selectivity with a pH gradient. A very high pH may induce additional negative charges on some side chains of the bases, leading to different selectivity and a breaking up of some of the secondary structures.

An example test run by Agilent Technologies utilizing its Bio SAX column revealed that oligos with longer sequence lengths had more retention on column. If it's desired that those longer oligos elute earlier off the column, the gradient must be changed and the salt concentration in the mobile

FIGURE 1: Oligo Separation with a PL-SAX Column: pH and Organic Modifiers



phase must be increased. Indeed, one benefit of the non-porous Bio SAX media is that it minimizes the diffusion path that analytes must traverse between the stationary phase and the mobile phase, allowing for column efficiency to essentially become independent of the flow rate. This is also advantageous because the same particle can be utilized across a range of oligonucleotide sizes, and a salt gradient can be used to adjust overall retention. Adding an organic modifier like acetonitrile may also improve peak shape by reducing secondary interactions. Ultimately, there are many method tweaks and options available to optimize separation.

The PL-SAX column can withstand a variety of mobile phase conditions; however, because it's a fully porous particle, diffusion will occur as the oligo moves in and out of the pores of the particle. An experiment by Agilent Technologies examined three different mobile phase conditions on the same column at the same temperatures (see FIGURE 1). In this example, all the samples separated but were tailing, and the peaks were broadened. This can all be caused by secondary interactions. It's possible to sharpen the peaks by adding a small amount of organic modifier (in this case, 10% ACN), especially for the 25 and 50 mer DNA samples. By elevating the pH to 12 and changing the mobile phase to a 10 millimolar NaOH, sharper peaks and the disruption of secondary interactions is possible.

The same methodology can be applied to other, more complex molecules–for example, a single guide RNA with 105 base pairs (see FIGURE 2). As shown on the left of the figure, an increase in column temperature alone didn't seem to greatly improve the results. However, with the addition of an organic

FIGURE 2: Effect of Organic Solvent and Temperature on a Single Guide RNA with PL-SAX

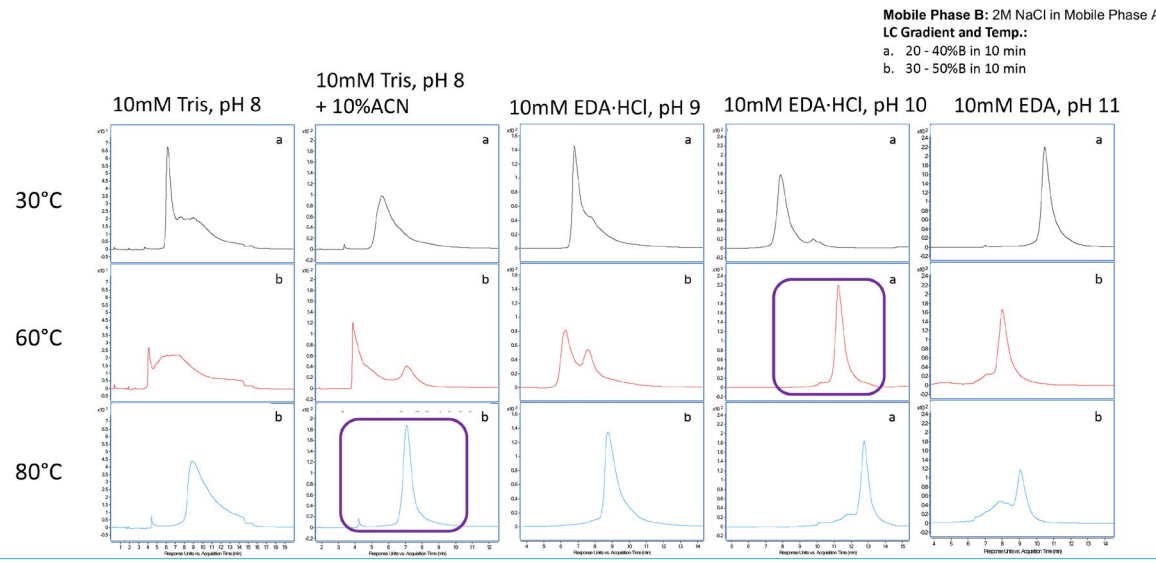
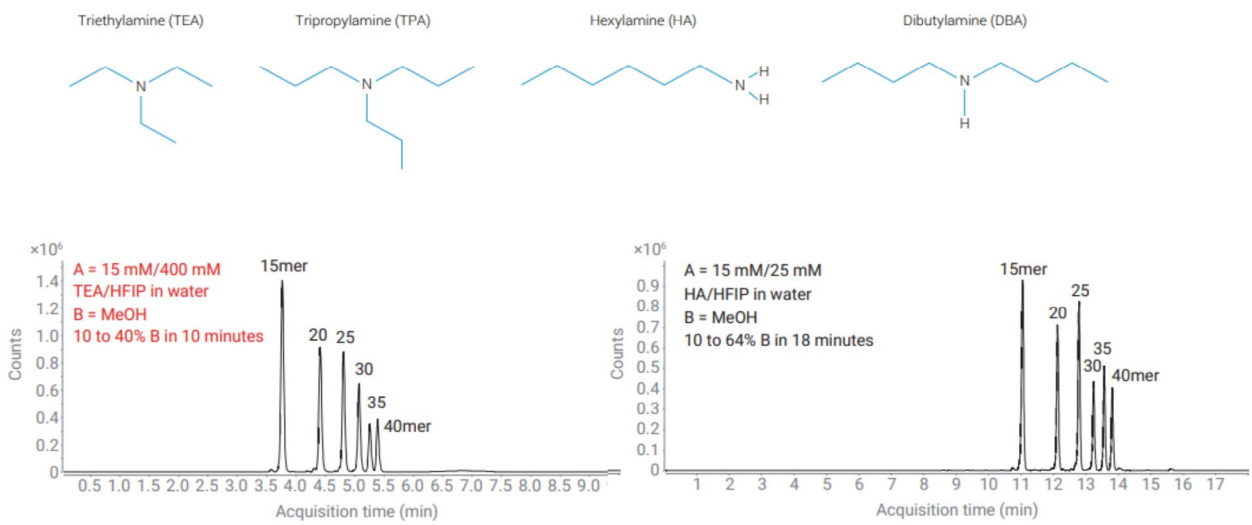


FIGURE 3: Ion-Pairing Effect on Oligonucleotide Resolution/Retention/Signal Response



modifier (in this case, 10% acetonitrile) at an elevated temperature, peak shape was improved. If an increase in temperature is not ideal, it's possible to change the mobile phase modifier and increase the overall pH, as shown in the next three columns of the figure. Similar results were achieved with a pH of 10 and a temperature of 60°C as with the addition of an organic modifier at 80°C. For this reason, it's important to screen all of these different modifiers to see which condition will yield the best results.

OLIGONUCLEOTIDE PURIFICATION: IP-RP CHROMATOGRAPHY

Oligonucleotides are polar and have an anionic or negatively charged backbone. In RP chromatography, the hydrophobic interactions between the nonpolar stationary phase and the sample are leveraged to create retention. In other words, the more hydrophobic the analyte, the more retention there will be on column. As such, oligos on their own will have little to no interaction or retention on a standard C18 column. By introducing an ion pairing agent into the mobile phase, however, it's possible to facilitate that interaction.

How does IP-RP work on a reversed-phase stationary phase? Most ion pairing agents are alkylamines. The positively charged nitrogen group interacts with the negatively charged phosphate backbone to create a pair. Then, the hydrophobic ethyl groups can interact with the hydrophobic stationary phase to create retention and separation on column.

The advantages of IP-RP include:

- The ability to provide high resolution separation of fail sequences and other impurities.
- Compatibility with MS and UV detection
- Compatibility with longer oligo sequences (greater than 200 base pairs)
- Sharper peaks: denaturing conditions, elevated pH, and temperature break up secondary interactions.

Agilent offers two different particle chemistries to support IP-RP analysis of oligos. First is the Agilent AdvanceBio Oligonucleotide column, which offers:

- High efficiency, high resolution, superficially porous particle chemistry available in two particle sizes: 2.7 and 4 µm
- Analytical dimensions through 21.2 mm ID prep
- Analytical characterization post-purification
- Silica-based C18 with novel high-pH resistant modification
- UHPLC resolution at HPLC pressure

Second, the Agilent PLRP-S is a polymeric particle offering:

- A variety of pore sizes for all sizes and types of oligos—100 Å to 4000 Å
- Scalability for purification, including bulk media
- An inherently hydrophobic surface
- Stability up to pH 13
- High binding capacity

When optimizing IP-RP, there are several variables to consider. One of the advantages of IP-RP is its compatibility with both MS and UV detectors; however, depending on the detector used, the ion pairing agent options may be limited since the acetate counterion in triethylamine acetate will cause signal suppression and a loss of ionization efficiency. Hexafluoroisopropanol (HFIP) is a good replacement for mass spec, but is not soluble

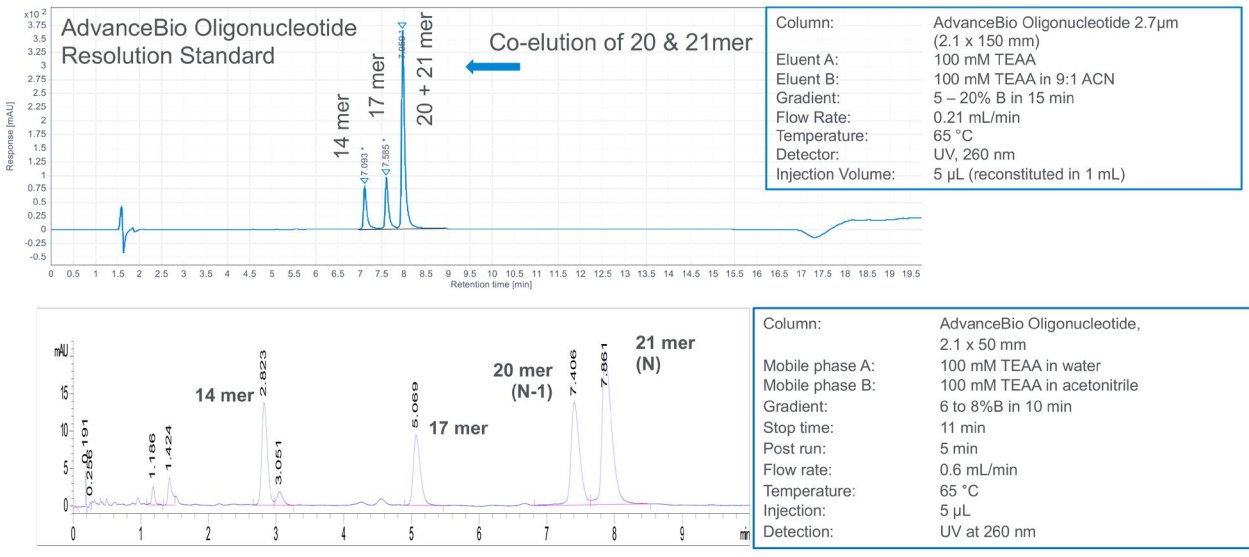
in acetonitrile; as such, it's recommended to use methanol as the organic modifier.

Further, since oligos come in different sizes and forms, choosing the right ion pairing agent is important, especially because mobile phase composition will affect MS sensitivity and mobile phase pH. While 50 to 60°C is most commonly used, the temperature can also be increased to denature the oligo and reduce secondary interactions.

Finally, and most importantly, is the particle support and column chemistry. Ion pairing reagents and elevated temperatures require a particle support that's stable under high pH and temperature; for this reason, a traditional silica particle is not recommended, and a particle support that can handle these harsh mobile phase conditions is necessary. Labs can also choose between superficially porous (offering increased resolution at higher flow rates and lower back pressures) and fully porous particles. Ultimately, significant improvements in resolution and sensitivity can be obtained by careful method development and optimization, especially since there won't be one single set of conditions that perform optimally for all oligos.

Once the detector, particle support, and column chemistry are chosen, labs can begin to determine which ion pairing agent is best for the sample. Higher concentrations of ion pairing agents in the mobile phase will lead to increased retention, but can also affect signal response; as such, it's important to monitor both when evaluating the method. Concentration of HFIP is also important to consider, especially when considering scale-up—HFIP is expensive. As shown in FIGURE 3, there can be significant differences

FIGURE 4: Gradient Optimizations to Improve N and (N-1) Separation



in chromatography depending on which ion pairing agent is chosen.

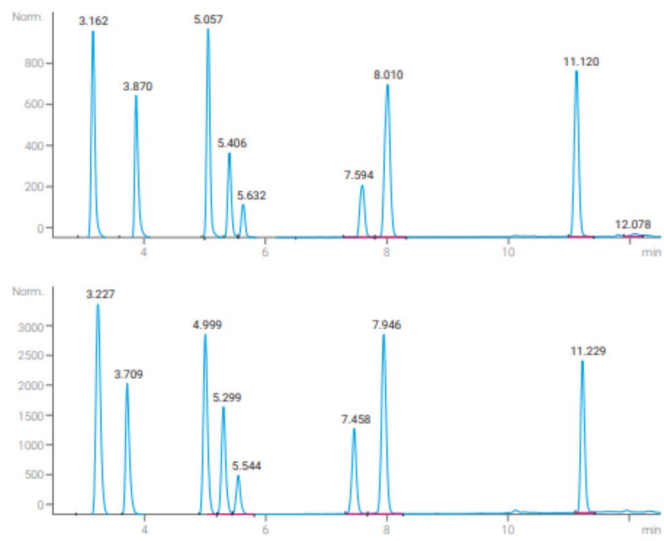
After evaluating the ion pairing options, labs can begin to make gradient optimizations to improve resolution. FIGURE 4 shows two different separations using the same column chemistry, particle size, and mobile phase. However, it's clear that a shallower gradient can optimize the resolution.

SCALING-UP TO PREP

While the goal of a chromatographic separation at the analytical scale is the generation of data, success is ultimately defined by high yield and purity at the preparative scale. Particle size, pore size, analyte type and size, and column chemistry can all impact loadability and, for this reason, it's recommended to evaluate loadability on the same particle size and column chemistry as prep. However, to reduce sample consumption, column loading studies should be done at the analytical scale. Once the maximum load has been established on a small volume analytical column, it can be scaled to a larger column capable of purifying larger sample volumes. FIGURE 5 shows an example of a successful method that has been scaled up from an analytical column to a prep column, and FIGURE 6 shows the reproducibility that can be achieved in scale-up from smaller to larger particle sizes.

To demonstrate that the method scale-up was successful, a sample of two of the fractions collected were reinjected on an analytical column to check purity. As shown in FIGURE 7, both fractions reanalyzed had excellent purity results. To achieve optimal results, it's important to match the column chosen to the right instrument type by flow rate and back pressure.

FIGURE 5: Prep Purification: Scaling Up



Scale-up from 4.6 x 150mm analytical column to 21.2 x 150mm Prep column

$$V_{inj,P} = V_{inj,A} \frac{d_P^2}{d_A^2} \frac{L_P}{L_A}$$

Equation 6. Scale-up calculation for column load.

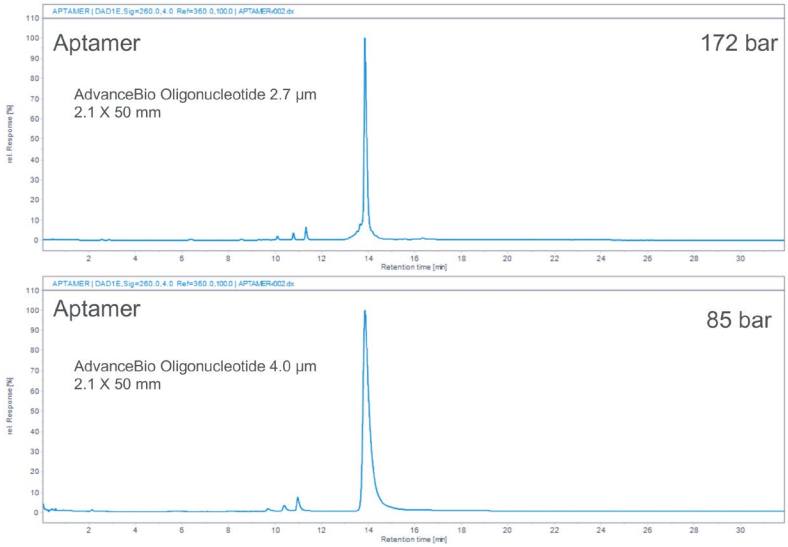
d_A Diameter of analytical column
d_P Diameter of preparative column
L_A Length of analytical column
L_P Length of preparative column
$V_{inj,P}$ Injection volume for analytical system
$V_{inj,A}$ Injection volume for preparative system

$$f_{p,P} = f_{a,A} \frac{d_P^2}{d_A^2} \frac{p_A}{p_P}$$

Equation 3. Calculation of flow rate for analytical to preparative scale-up.

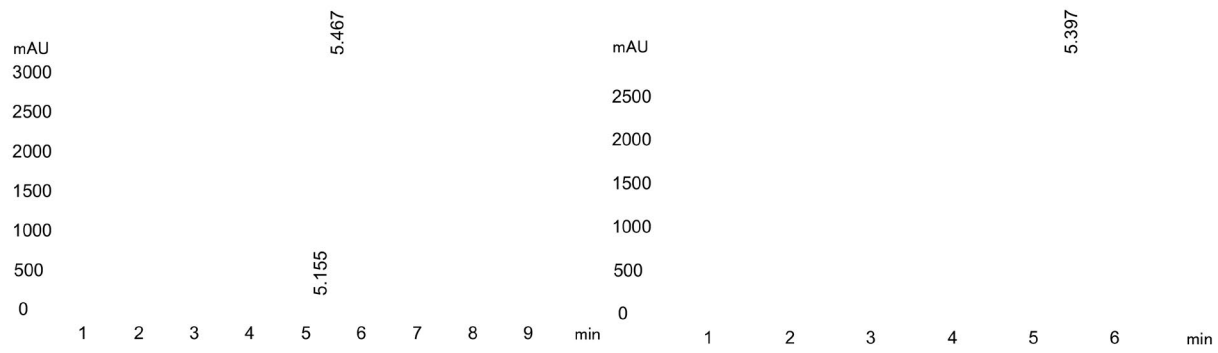
d_A Diameter of analytical column
d_P Diameter of preparative column
$f_{a,A}$ Actual flow in analytical system
$f_{p,P}$ Proposed flow in preparative system
p_A Column particle size in analytical system
p_P Column particle size in preparative system

FIGURE 6: AdvanceBio Oligonucleotide 2.7 µm vs 4 µm



Aptamer RNA (w/ Fluoro modifications) 27mer
/52FC/mGmGrArA/i2FU//i2FC/mAm G/i2FU/mGmAmA/i2FU/mG/i2FC//i 2FU//i2FU/mA/i2FU/mA/i2FC/mA/i 2FU//i2FC//i2FC/mG/3InvdT/

FIGURE 7: Analytical Characterization to Prep Purification for Oligonucleotides



CONCLUSION

Therapeutic oligonucleotides have emerged as an area of promise in biopharmaceutical research because of their ability to target different biological pathways and treat a wide range of diseases. But since they range in size, sequence complexity, and overall modifications, their analysis and purification by liquid chromatography is complex.

Agilent Technologies offers a myriad of solutions for method development optimization when scaling up from analytical characterization to preparative purification and manufacturing.

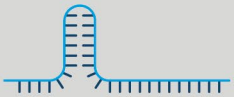
Separation Your Way: siRNA to mRNA

Agilent column chemistries for diverse oligonucleotides

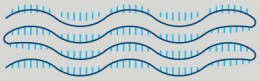
Agilent Oligonucleotide Separation and Purification Guide

Oligo therapeutic classes

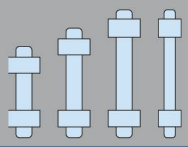
18-30 bases
siRNA/ASO



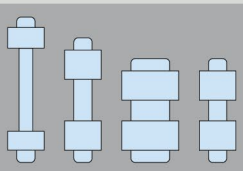
Up to 200 bases
gRNA

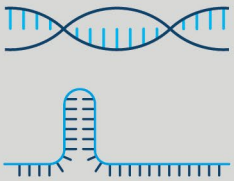
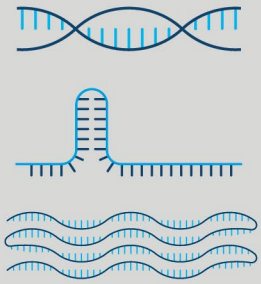
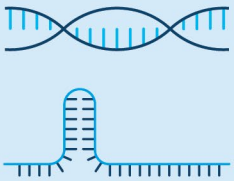
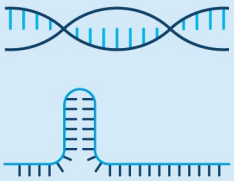


1000s of bases
mRNA

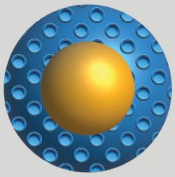
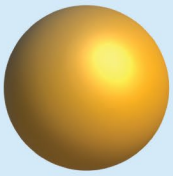
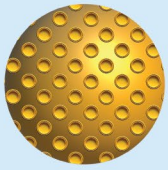


Columns for oligonucleotide chromatography



Ion paired reversed-phase (IP-RP)		Anion exchange (AEX)	
Agilent AdvanceBio Oligonucleotide columns	**Agilent PLRP-S columns**	**Agilent Bio SAX columns**	**Agilent PL-SAX columns**
Suitable for siRNA/ASO and gRNA	Suitable for siRNA/ASO, gRNA and mRNA	Suitable for siRNA/ASO and gRNA	Suitable for siRNA/ASO and gRNA
			

High pH and temperature stability

MS Compatible		MS Compatible with desalting	
• High efficiency particles in 2.7 and 4 µm • Available from analytical to 21.2 mm id columns • Agilent Poroshell technology • Optimized 120 Å pore size for targeted, high resolution IP-RP separations	• Particle sizes 3 to 50 µm • Available from analytical to 100 mm id preparative columns and bulk media • Fully porous polymeric particle • Available in 4 pore sizes: 100, 300, 1000 & 4000 Å	• Particle sizes from 1.7 to 10 µm • Available in analytical to 21.2 mm id columns • Nonporous particle for high resolution IEX separations	• Particle sizes 5 to 30 µm • Available from analytical to 100 mm id preparative columns and bulk media • Fully porous polymeric particle • Available in 1000 & 4000 Å pore sizes
			

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Oligo Analytical Method Development - Optimization to Species

Practical advice for selecting the best chromatographic technique based on the type of oligo of interest. *By LCGC Staff*

The therapeutic oligonucleotide landscape has been growing extensively over the last few years, because of their potential to treat a wide range of diseases. Oligos, as they are called, comprise a large family of molecules approved for therapeutic use. They include small interfering RNA (siRNA), antisense (ASO), splice switching (SSO), aptamers, and messenger RNA (mRNA). The most common manufacturing methods for oligos are solid phase synthesis, for oligos with up to 50 nucleotides, and in-vitro transcription for mRNA, which has very many sequences of nucleotides. There are close to 20 drugs on the market based on these types of oligos, with more than 100 clinical trials in progress and an estimated market value of over $100 billion by 2026.

A key challenge in this area is the development of analytical methods to identify and eliminate the many types of impurities that result from the complex synthesis used to make oligos. In addition, analytical methods are essential to the Chemistry, Manufacturing, and Controls (CMC)

FIGURE 1: In-Vitro Transcription

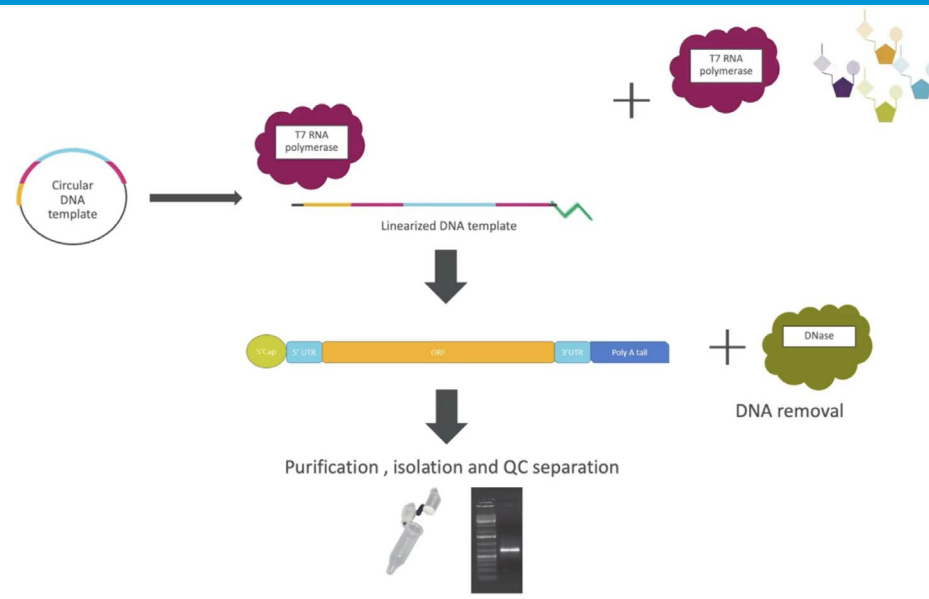


Image courtesy of: Dr Christina Vanhinsbergh, Senior Scientist, AstraZeneca, New Modalities and Parenteral Development, Pharmaceutical Technology & Development, Operations, Macclesfield UK

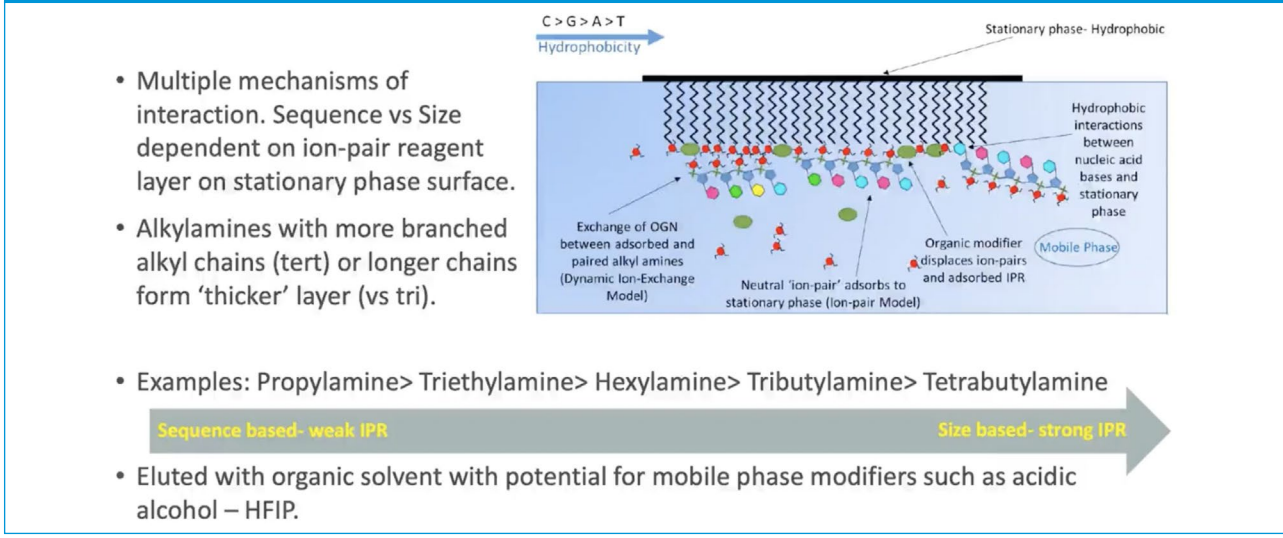
regulatory process that applies to these drugs. In general, chromatography methods are useful for many key steps of the oligo manufacturing process, which include process control, purification, yield and quality analysis, identity, assay, and stability and release. This summary discusses the challenges and opportunities that chromatographic methods present in this area, covering the best techniques and detection methods for the CMC testing required for stability and release, and process control.

THE NEED FOR SEPARATION TECHNIQUES

The synthesis of oligos, be it solid phase or in-vitro transcription, is a complex, multi-step process that can generate unwanted molecules that would be detrimental to their efficacy or safety. Shorter than target oligos, or oligos with the wrong nuclear base sequence, are among the most common impurities when using solid phase synthesis methods. For oligos with from 20 to 50 nucleotides the synthesis yield drops from 90% to 80%, which means that up to 20% of the product will be a mixture of impurities.

In the case of mRNA, where the desired length can reach thousands of nucleotides, solid phase synthesis is not an option. Instead, the preferred method entails in-vitro transcription by first forming a plasmid DNA template, then linearizing it and transcribing it into a mRNA sequence using RNA polymerase and ribonucleoside triphosphate (FIGURE 1). Further chemical modifications improve the efficacy of this method but also

FIGURE 2: Ion-Pair Reversed Phase LC: Mechanisms of Action

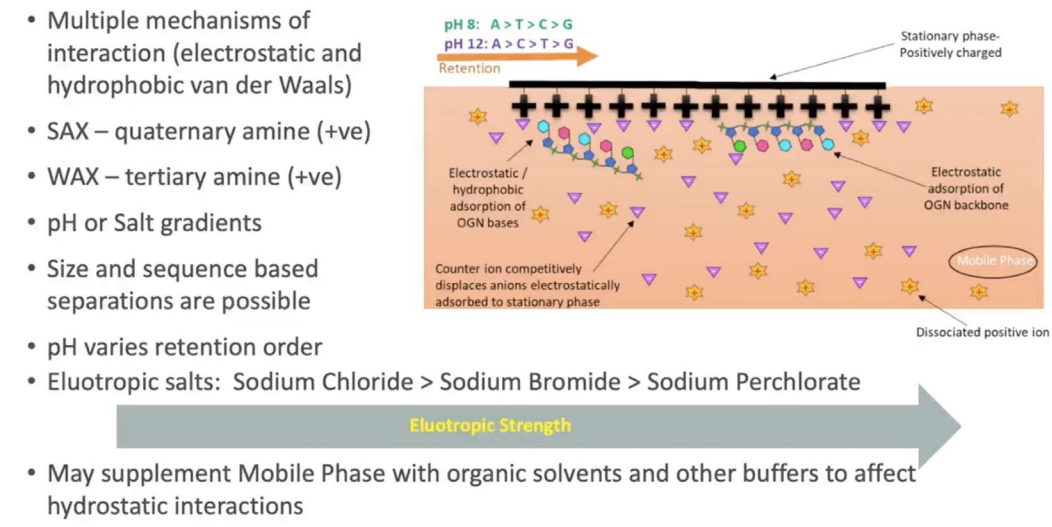


can generate impurities. In addition, as they degrade in the body, oligos can generate other impurities, whose characterization and identification present key challenges within the drug development process. Finally, there are process-related impurities that arise from the starting materials or from unwanted side reactions, which the standard filtration or purification steps do not fully remove. This is why chromatography and electro separations are essential to ensure the safety and efficacy of therapeutic oligonucleotides.

ION-PAIR REVERSED-PHASE LC (IP RP-LC)

This type of separation involves several mechanisms of action when it comes to oligos, which have a negatively charged phosphate backbone (FIGURE 2). They associate with the amine component of an alkyl amine ion pair agent (IPR), which in turn interacts hydrophobically with the stationary phase through its free alkyl chain. In addition, the alkyl amine self-associates with the stationary phase, creating a surface layer that dynamically ion-exchanges the oligo at the surface of the column. Furthermore, the nucleic bases engage in hydrophobic interactions with the stationary phase with differential retentive strength, resulting in a sequence-based separation. IPRs with highly hydrophobic alkyl chains, such as tert-butylamine, form such a strongly partitioned layer that it inhibits the nucleic base interaction. Less hydrophobic IPRs, such as propylamine do

FIGURE 3: Anion Exchange LC: Mechanisms of Action



not partition as strongly and form a weaker surface layer that does allow nucleic base interaction. The choice of IPR depends on the objective of the separation. To achieve high selectivity and resolve more impurities, it is best to try a sequence-based weak IPR. To separate isomers, use more hydrophobic IPRs, but bear in mind that there will be a reduction in selectivity.

Mobile phase additives, such as hexafluoroisopropanol (HFIP) increase the ionization efficiency and in turn resolution and detection sensitivity. However, some combinations of HFIP and IPRs can cause a basic degradation during the analysis. In addition, as some IPRs can oxidize and cause ghost peaks, it is recommended that analysts prepare their mobile phases under inert atmosphere conditions (N_2). Other recommendations include the use of long, shallow gradients, and, if the LC equipment can tolerate higher back-pressure, the use of methanol with the HFIP. MeCN, if used, should be at very low concentrations.

ANION EXCHANGE LC (AEX)

This technique is a form of ion exchange chromatography (IEC) that relies on electrostatic and hydrophobic van der Waals interactions to separate oligos. Here, the negatively charged backbone of the oligos adsorbs onto the positively charged stationary phase (FIGURE 3). Since the oligo charge is proportional to the number of its phosphate bonds and therefore to its length, this results in a size-based separation. In addition, the polar nucleic bases also

adsorb onto the stationary phase and may participate in hydrophobic interactions, which makes them amenable to separation using a pH or salt gradient. Analysts can vary the retention order either by controlling the pH gradient or the eluotropic strength of the salts. Furthermore, they can supplement the mobile phase with organic solvents and other buffers to affect hydrostatic interactions. As a result, this technique enables tunable size- and sequence-based separations.

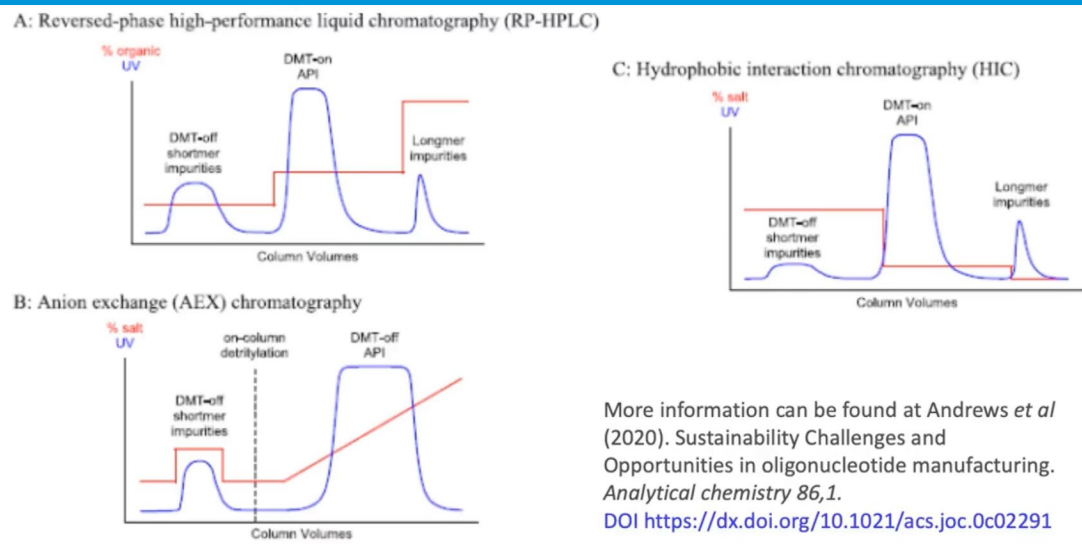
Some disadvantages of this technique include lower sensitivity and peak broadening, and more importantly, incompatibility with MS detection because of the use of salts. A desalting step can overcome this limitation, but it increases the complexity, cost, and length of the analysis. Moreover, stainless steel systems can suffer from salt corrosion. On the other hand, because the salts are non-denaturing this technique is perfect for purification. Another advantage is that the polymeric stationary phases available for this chromatography are resistant to high temperatures. To optimize this technique, analysts should use guanidine hydrochloride (GuHCL) to control electrostatic interactions and hydrogen bonding but avoid sodium thiocyanate (NaSCN), which is highly toxic under certain conditions, and bromide compounds, which are highly corrosive to stainless steel. For modifying the mobile phase, methanol is better than acetonitrile to avoid salt precipitation, but it creates higher backpressure.

ALTERNATIVE MODES OF LC

Analysts can consider other chromatographic methods for oligo analysis but should be aware of their challenges and disadvantages. Hydrophilic interaction LC (HILIC), for example, is useful for P=O impurity separation and is MS friendly, but a full understanding of its retention mechanism when it comes to oligos is not yet available. In some cases, depending on the chosen stationary phase, retention is too strong, to the point of having to use high concentrations of mobile phase buffer to allow the oligo to elute. These buffers are typically ammonium acetate or ammonium formate, which can cause peak broadening and tailing. Despite these drawbacks, HILIC can at times match the selectivity of reverse phase C18 columns and resolve key impurities.

Mixed-mode (multimodal) chromatography expands the range of chromatographic modes applied in oligo purification. Mixed-mode media combine ionic and hydrophobic interactions and offer different selectivity and a higher salt tolerance than traditional ion exchange media. However, the multiple retention mechanisms at work in this method reduce its predictability and control of selectivity. On the other hand, it offers the potential for two-dimensional LC applications. Finally, size exclusion chromatography (SEC) is always worth considering because of its non-denaturing approach, even though it provides lower resolution than an ion exchange separation.

FIGURE 4: Purification Approaches



More information can be found at Andrews *et al* (2020). Sustainability Challenges and Opportunities in oligonucleotide manufacturing. *Analytical chemistry 86,1.* DOI https://dx.doi.org/10.1021/acs.joc.0c02291

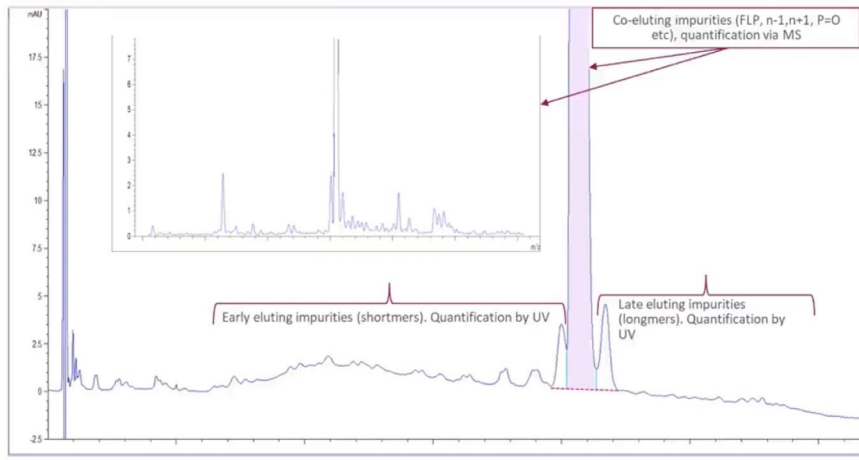
J. Org. Chem. 2021, 86, 1, 49–61. Publication Date: November 30, 2020. https://doi.org/10.1021/acs.joc.0c02291.

Size exclusion is not retentive, so you are not separating based on a chemical interaction, but on the size or hydrodynamic radius of the oligos. In this separation, the column pore size is extremely important when optimizing resolution and flow rate. Accordingly, it may be necessary to modify the mobile phase with organic solvents. In the case of highly chemically modified oligos, such as ASOs, there might be many secondary interactions occurring between them and the stationary phase itself. This mode of analysis, however, is very useful for characterizing polydispersity when there are many isoforms present, as in the case of polyethylene glycol conjugates.

DETECTION METHODS

The most common detection method is MS, as it provides characterization of the oligos by their mass and their retention times. Within MS, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the best detection techniques for oligos, as both help overcome their propensity for fragmentation during the ionization step. Ionization of oligos for negative MS detection results in multiply charged anions, which make the mass spectrum complex and technically challenging. On the other hand, because of the high resolution of this technique, characterization of oligos that coelute in the chromatograph is not difficult. A caveat mentioned previously is the reduction in ionization efficiency and thus sensitivity of detection due to ion pairing reagents. Method developers must manage this across the coupling of LC when using collision-induced dissociation for fragmentation. UV detection is simple, sensitive, quantitative, and compatible

FIGURE 5: Industrial Standard for Assay, Identity, and Impurities



More information about this method can be found at Rentel *et al* (2022) Assay, Purity and Impurity profile of phosphorothioate oligonucleotide therapeutics by ion-pair HPLC-MS. *Nucleic acid therapeutics*, 32, 3.

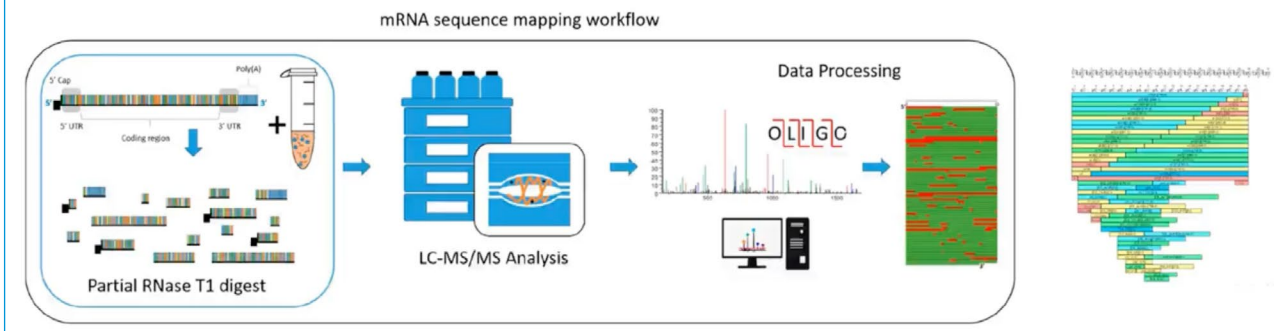
Image courtesy of: Dr Christina Vanhinsbergh, Senior Scientist, AstraZeneca, New Modalities and Parenteral Development, Pharmaceutical Technology & Development, Operations, Macclesfield UK

with most mobile phases, but it cannot differentiate between co-eluting oligos.

APPLICATION EXAMPLES

For purification purposes in methods involving ion pairing agents, analysts have developed specific gradients to use with RP-HILIC, hydrophobic interaction chromatography (HIC), and AEX (FIGURE 4). In some cases, they can use UV detection for the characterization of early and late eluting impurities (shortmers and longmers, respectively), but must resort to MS for co-eluting impurities (FIGURE 5). Sequencing mRNA is very important to identifying and characterizing the active pharmaceutical ingredient (API). For this application, reverse phase LC following partial RNA digestion is a useful separation technique. An mRNA sample consists of a complex mixture of oligos of varying size, from two to 60 nucleotides long and these may be difficult to resolve in one analytical separation. Analysts have accomplished this with the use of a polymeric stationary phase and the combination of triethylamine and HFIP. They analyzed all the oligo digest fragments by tandem MS and used a bespoke data processing software tool. This tool stitches up all the fragments of each digested oligo and matches each sequence to an mRNA sequence to map to the original mRNA sequence. The software creates a sequence coverage across the entire chromatograph separation, delivering in minutes what would have taken weeks to process without it (FIGURE 6). The scientific literature has many more examples of successful application of a variety of chromatographic methods for oligo analysis. Vanhinsbergh mentions several of them and directs the audience to

FIGURE 6: High Resolution Sequencing: Identity Analysis



Anal. Chem. 2022, 94, 20, 7339–7349. Publication Date:May 12, 2022. https://doi.org/10.1021/acs.analchem.2c00765

the appropriate source. Additional examples include LC-MS metabolite profiling[1], novel methods for impurity analysis[2], stability indicating methods[3], and duplex oligo analysis for quality control[4].

CONCLUSION

There are many chromatographic techniques available for developing oligo analytical methods, and selecting the best one requires a case-by-case approach. Given the high number of oligos involved and the different ways to synthesize them, method developers need to tune the separation technique according to the oligo chemistry. In addition, they must consider the CMC requirements for their specific case. In general, they will find that selectivity and efficiency are in competition with sensitivity, and that it is always a fine balance. Finally, they must keep in mind that there are always multiple interactions occurring at any one time and consider all possible implications.

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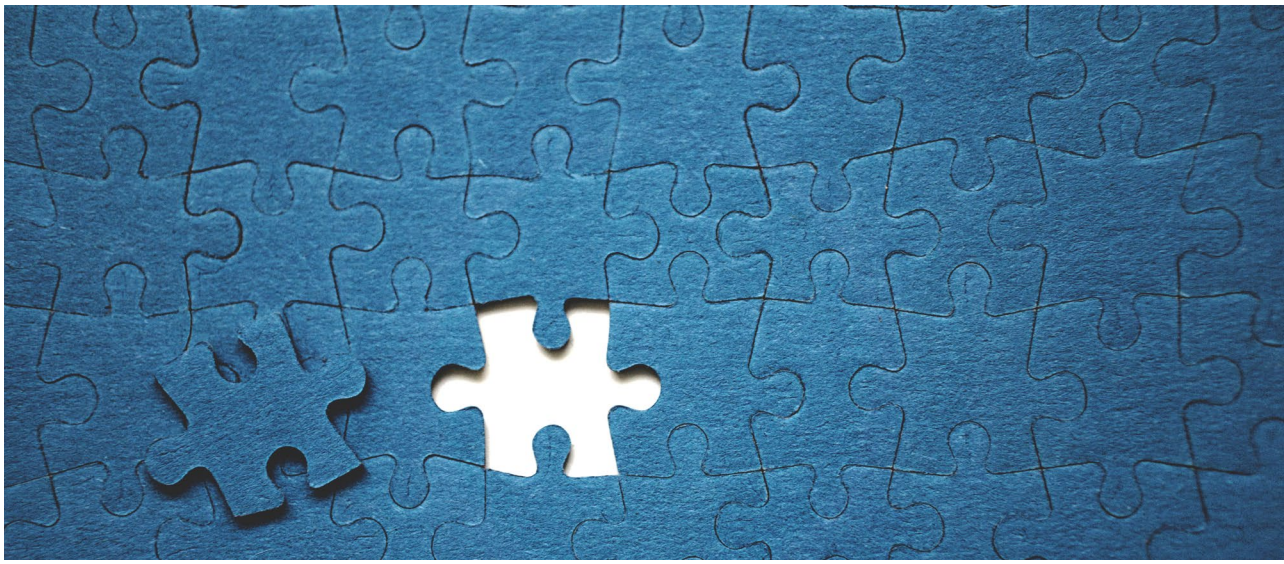


Oligo/Nucleic Acid Regulatory Compliance Considerations

Effective strategies to deal with multi-geography compliance issues related to oligonucleotides and nucleic acid therapeutics

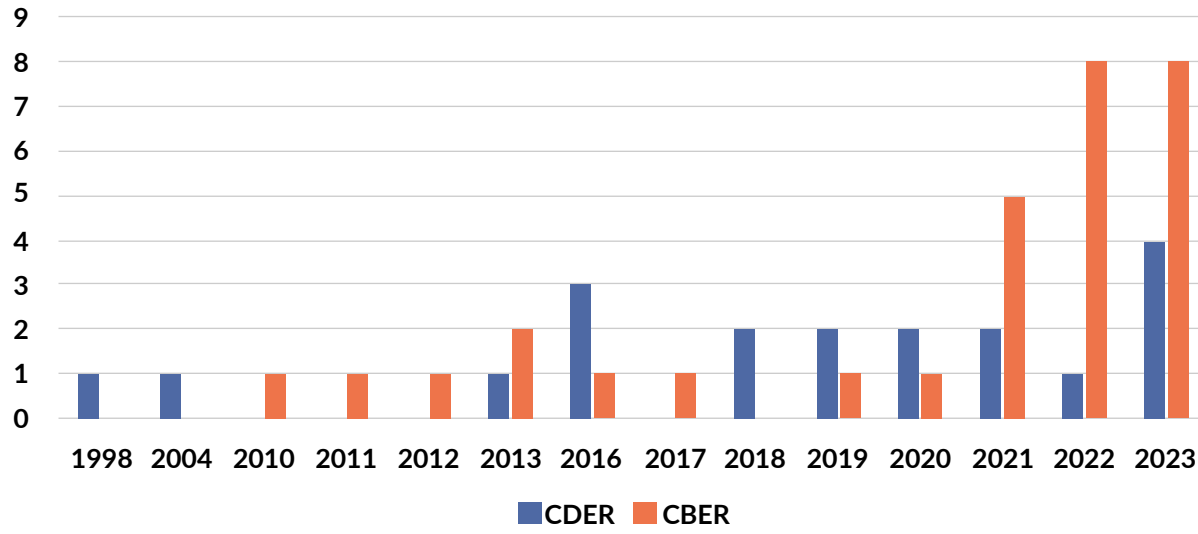
By LCGC Staff

Oligonucleotides are small nucleic acid polymers that can act in sequence-specific ways to control gene expression.[1] They exert their effects through suppression of, or interference with, messenger RNA (mRNA), immune stimulation, or protein binding. Therapeutics based on oligonucleotides can target a broad range of conditions. These materials are highly specific, have functional diversity, and, in general, have limited toxicity; these attributes make them ideal in the development of treatments for several pathologies.

Arguably the most well-known applications of these technologies are the mRNA vaccines used to prevent SARS-CoV-2.[2] As a category, these therapeutics have a broad range of targets, particularly those that have been considered "undruggable" by either small molecules or protein therapeutics. However, from a regulatory standpoint, these materials fall into a gray area; they are not small-molecule therapeutics nor are they biological agents.

These oligonucleotide-based therapeutics can incorporate a variety of materials, including antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), anti-micro-RNAs (anti-mRNAs), aptamers, and other types of mRNAs.

FIGURE 1: Growth of oligonucleotide-based therapeutic approvals at both CDER and CBER.[3-5]



APPLICATIONS AND PROCESSES

Some of these nucleic acid-based therapeutics regulate the expression of mRNA that are their targets, resulting in blocking, binding, cleavage, or repair. Aptamers will target proteins and inhibit their function, and all approaches can be effective in targeting various pathologies.

The regulatory climate for these materials is not straightforward. If it is a synthetic oligonucleotide, it is regulated as a drug through the United States Food and Drug Administration (US FDA) Center for Drug Evaluation and Research (CDER). If, on the other hand, the oligonucleotides are cell or gene therapies, they are regulated by the Center for Biologics Evaluation and Research (CBER) at the FDA. Essentially, there are two arms of the same agency that regulate the materials, which reinforces the concept that these materials fall into a gray area in regulation based on the application. The first regulatory approval of this type of therapeutic agent was Vitravene, approved by the FDA in 1998. Since then, CDER has approved four other therapeutics including Nusinersen and Eteplirsen in 2016 and Inotersen and Patisiran in 2018.

Some therapeutics approved under the auspices of CBER include Kymriah and Breyanzi, which are both cell therapies, and Roctavian and Casgevy, which are gene therapies. Of course, CBER issued the emergency use authorization (EUA) and later approval of the three vaccines from Pfizer-BioNTech, Moderna, and Johnson and Johnson to prevent SARS CoV-2. FIGURE 1 shows the growth of oligonucleotide-based therapeutic approvals from the FDA.[3-5] As the graphic indicates, this class of therapeutics is

increasing and adding to the workload of the regulatory agencies.

Research by therapeutic developers has grown and the number of applications and approvals in the US alone has risen dramatically.[4] This puts a strain on the regulatory resources for approvals at both CBER and CDER.

A major concept that is associated with biopharmaceutical manufacturing is "the process is the product." This means that the quality and consistency of the manufacturing process are just as important as the final product. For many nucleic acid-based therapeutics, the manufacturing process can significantly impact the characteristics of the product and possibly impact the efficacy, safety, and quality. Each step in the manufacturing process—synthesis, cleavage and deprotonation, purification, concentration, isolation, and lyophilization—can impact the active ingredient. Incorporating the active pharmaceutical ingredient (API) into a formulation could also impact the chemistry and therefore efficacy. This is a key point that must be considered in the regulations of new materials.

These constraints are part of the evaluation for a new therapeutic entity, so it is essential to have a well-defined and tightly controlled manufacturing process that meets the required standards of the therapeutic batches. Recognizing these constraints from the first stages of product development is key to product consistency, efficacy, and safety. Of course, the ultimate target for the therapeutic is the patient, so ensuring quality is a must.

GLOBALIZATION AND REGULATION

Globalization has impacted the pharmaceutical industry as much as any other industry. Drug companies operate in many parts of the world and utilize global supply chains. Clinical trials are performed in multiple countries and drug companies desire approval worldwide, but each nation has its own submission and review processes and, unfortunately, the reviews may be conducted without knowledge of a review in another country. Reviewing organizations mainly focus on the primary aim of ensuring that the drug authorized in the country is safe and efficacious.

In well-resourced countries, regulatory agencies such as Health Canada, the European Medicines Agency, Swissmedic, and the US FDA have similar if not identical approaches and requirements for approval. When applications exist in multiple geographies, the result is massive duplication of efforts which does not benefit the pharmaceutical company, the country, or the patient.

With such a large number of drug approvals, there is a potential strain on the resources at regulatory agencies. A strategy to target multiple stakeholders in this equation focuses on harmonization, convergence, and reliance in the approval process. A recent report published by the National Academies of Science Engineering

and Medicine examined the concept of reliance and harmonization for the good of all involved parties.[6]

In the regulatory space, evaluations and approvals are founded on a science-based risk approach. The most effective tactic may be the concept of regulatory convergence. Using the guidelines and guidance documents from regulatory bodies, the evaluations are based on merging the best practices from each of these bodies without any one agency giving up its sovereignty. All cooperating organizations are conducting evaluations in a very similar manner.

Another concept is regulatory reliance, in which regulators from one system can rely on trusted authorities that have approved the drug in the past. It is a recognition of the products of the other organization and using them in an informed manner to support the decision of the authority in question. This type of approach functions well when guidance documents from internationally recognized organizations like the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) or the World Health Organization (WHO) are used as the benchmark in evaluations.

A QUALITY-BY-DESIGN APPROACH

In general, the foundation of these regulatory frameworks is based on the idea that the product is the process. No matter who evaluates a product or how this evaluation is performed, if it is done improperly or carelessly, it may not function as intended. This leads to the concept of Quality by Design (QbD). QbD is a systematic approach to the development of a drug material that begins with predefined objectives and emphasizes understanding the process and controlling it; it is rooted in science- and quality-based risk assessment.[7,8]

Some of the key principles of the QbD approach involve monitoring those variables, called Critical Process Parameters (CPP), that have an impact on the quality attributes of the therapeutic. The Critical Quality Attributes (CQA) are physical, chemical, biological, or microbiological characteristics that have well-defined limits to produce the product as desired. Because of the nature of oligonucleotide therapeutics, they pose some challenges that small molecule therapeutics do not.

Unfortunately, there are no clear guidelines from ICH or FDA addressing the quality aspects of oligonucleotide therapeutics. There is no consensus on reporting impurities and thresholds, partly because it is a challenge to separate and quantify the impurities. One of the analytical challenges includes impurities that will coelute from chromatographic systems due to the very similar chemistries as the molecule of interest. This can occur due to failures in the synthesis process, resulting in longer and shorter molecules or diastereoisomers.[9] These types of impurities require multiple analytical approaches to adequately characterize the quality attribute.

Whether the oligonucleotide drug product manufacturing is outsourced or kept in-house, understanding the key parameters of the product and the process—the Chemistry, Manufacturing, and Controls (CMC)—is critical in drug development. These can include information on the API, analytical methods, their development and validation, stability testing, raw materials specifications, and other tests. All these pieces of information are necessary to understand and document in a Good Manufacturing Practice (GMP) environment, which is necessary for understanding all the implications of product quality.

CONCLUSION

Understanding the process of development and production is key to pulling all the components of QbD together and therefore being able to address the regulatory approval process. Regardless of which agency is approving the product, having all the pieces that define the product—including the API information, the drug formulation information, key process parameters to make a quality product, and the risk/benefit comparisons—is required. Cooperation amongst agencies can ensure that their requirements are mostly compatible, approvals will have minimum duplicative efforts, and crucial drugs will arrive to the patient in a timelier manner.

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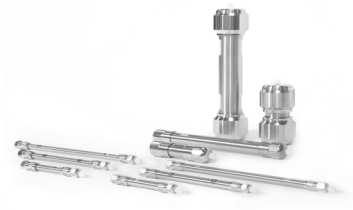

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