Complex Characters

Oligonucleotides are now being utilised by manufacturers for therapeutics, but there are analytical challenges involved with characterising these molecules and their associated starting materials and intermediates

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Oligonucleotides are biopolymers consisting of modified ribonucleotide or deoxynucleotide monomer units. Since they play such a critical role at the human cellular level, they are being developed by several biopharmaceutical organisations as potential therapies and vaccines and are considered to be their own unique modality, distinct from traditional small molecule drugs and biologics.

The FDA has approved a number of these biopolymers, including fomivirsen, pegaptanib and mipomersen, primarily antisense oligonucleotides (ASOs), with several more on the way (1). In Europe, the Committee for Medicinal Products for Human Use, a division of the EMA, has recently recommended nusinersen for approval. n 100 oligonucleotide drugs progressing through the development pipeline, designed to target diseases and rare genetic disorders, including Huntington's disease, asthma and certain types of cancer, as well as vaccines for the Ebola, Zika and influenza viruses.

Brief Background

Messenger RNA (mRNA) is a type of oligonucleotide that is critical to the translation of genetic sequence information of DNA into the specific amino acid array of proteins manufactured in the ribosomes of a cell's cytoplasm. In eukaryote human cells, mRNA consists of several distinct regions (see Figure 1). This naturally occurring sequence is known as the sense sequence.

ASOs refer to those synthesised in a manner in which their sequences (3'-5') are complementary to those of specific mRNAs. Other oligonucleotide classes include small interfering RNA and aptamers. The mRNA products encode and drive the expression of desired protein or peptide for therapeutic or vaccination purpose, whereas ASOs are used to 'knock down' or turn off certain genes.

Figure 1: Schematic of mRNA molecule. A 5' cap end (blue), coding regions called exons in the middle (orange), and 100-200 adenylic acids attached to the 3' end (blue) referred to as poly(A) tails (2)

Unlike therapeutic proteins that are made in living cells, oligonucleotides are produced using solid-phase synthesis. It is not as simple as fusing native nucleotides building blocks such as nucleoside-3'- or 5'-phosphates to construct the intended sequences due to their inherent low reactivity – therefore, other building blocks, including phosphorothioates and methoxyethyl and deoxy-modified phosphoramidites, must be utilised (see Figure 2) (3). The starting materials, side products, intermediates, impurities and final products are quite complex and thus require a broad range of sophisticated analytical methodologies to fully characterise them.

Analytical Approaches

Reversed-phase high-performance liquid chromatography (RP-HPLC) is one of the most common separation techniques used in biopharmaceutical testing laboratories and is ideal for the separation of non-volatile, high molecular weight organic compounds. Mixtures are separated based on their differing affinities for the liquid mobile and stationary phases of the chromatographic columns. Smaller particle column packing has led to even more efficient separations and is referred to as ultra-HPLC or UHPLC. Analytes are typically detected using ultraviolet (UV), photodiode array or fluorescence detectors, depending on their properties. The addition of ion-pairing (IP) reagents like triethylamine to the mobile phase has allowed RP-IP-HPLC to become one of the most powerful methods for assessing the purity of synthetic oligonucleotides surpassing the more traditional ion exchange chromatography (3).

The synergy of adding the mass selective detection of mass spectrometry (MS) to HPLC provides orders of magnitude-more information, including the ability to detect very slight structural modifications. LC/MS instrumentation comes in an array of configurations, such as single-quad (MS), ion trap, triple-quad (MS/MS), time-of-flight (TOF) and quad/time-of-flight (QTOF), each with its own advantages.

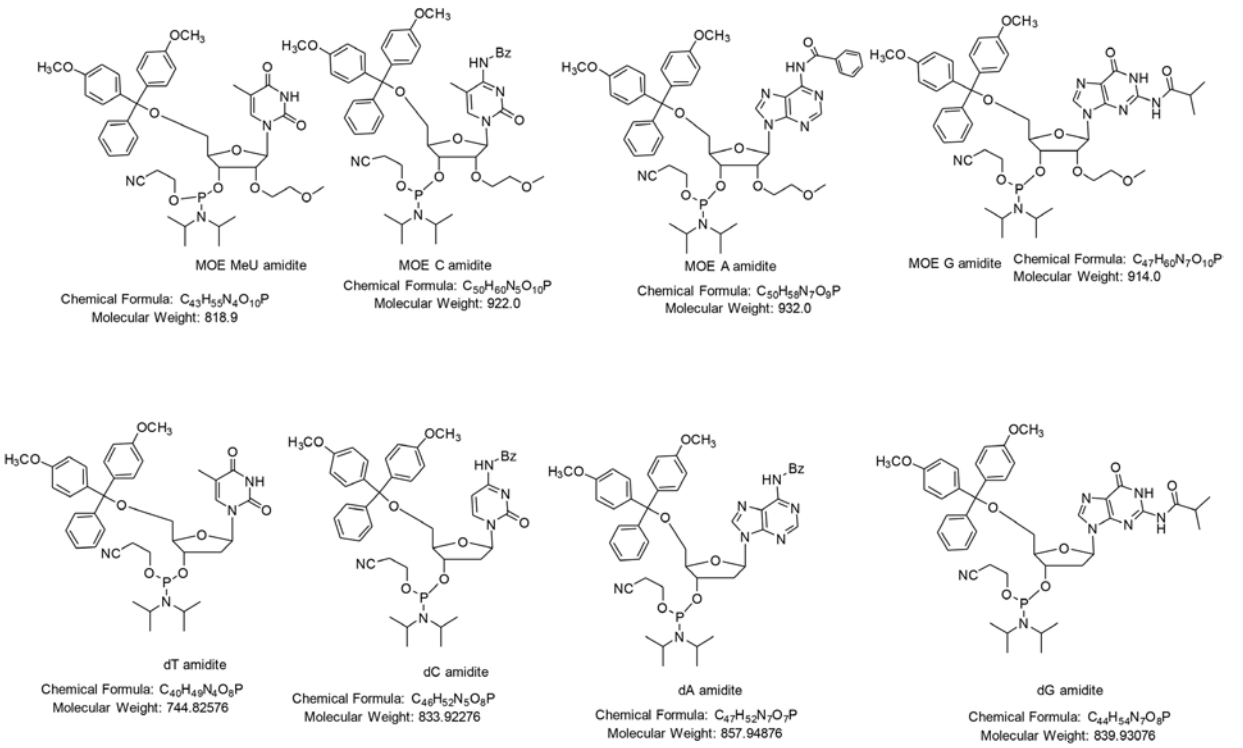


Figure 2: Structures of common methoxy-ethyl (MOE) and deoxy-modified phosphoramidites

In particular, the TOF and Orbitrap detectors provide accurate mass measurement. This means, instead of determining the mass of 3423 specific structure, the resolution of a TOF can provide an 'accurate mass' measurement of 3423.5548. The fraction of mass after the decimal provides a wealth of information to increase the confidence in the identification and narrow down the possible other molecular candidates with similar masses.

Synthetic oligonucleotides tend to exhibit quite a range of potential impurities, including uncapped 5' ends, tailless 3' ends, oxidation impurities, n-1 and n+1 species, to name a few. RP-IP-HPLC-UV-MS has become one of the most effective and widely-used approaches for characterising these impurities. The process can be quite tedious and labour-intensive due to the number of impurities and amount of data produced by an analysis (see Figure 3). The first step is to integrate the LC/UV chromatogram of the oligonucleotide, then the mass spectrum of the main peak is evaluated. Next is the most time-consuming step: evaluating all of the extracted ion chromatograms. Figure 3C conveys the complexity of the analysis with the early eluting (n-1) and late eluting (n+1) impurities overlaid on top of the full length oligonucleotide. There may be as many as 30 different impurities under the main peak.

Capillary electrophoresis (CE) is a separation technique. Traditional gel methods, such as sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western Blot,

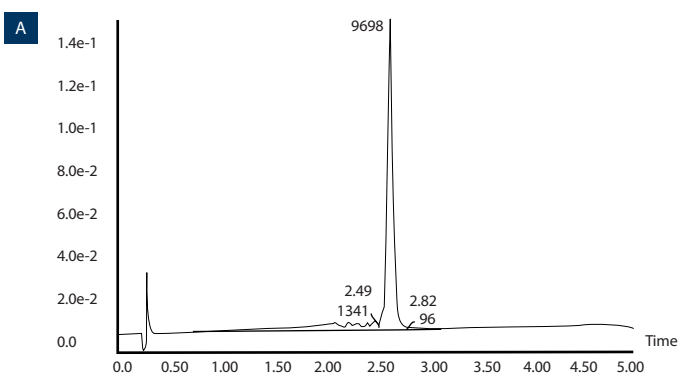


Figure 3A: RP-IP-LC-UV-MS work flow for impurity determination (A) LC/UV chromatogram

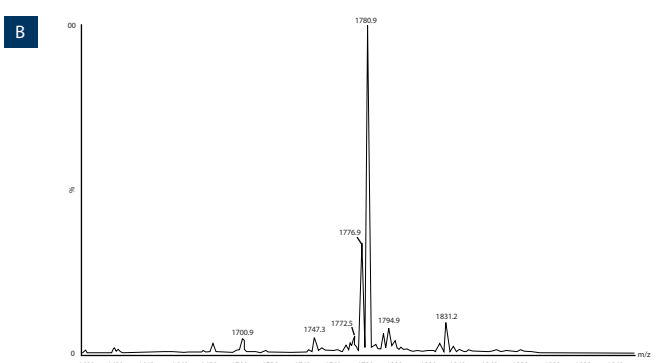


Figure 3B: MS scan of main peak

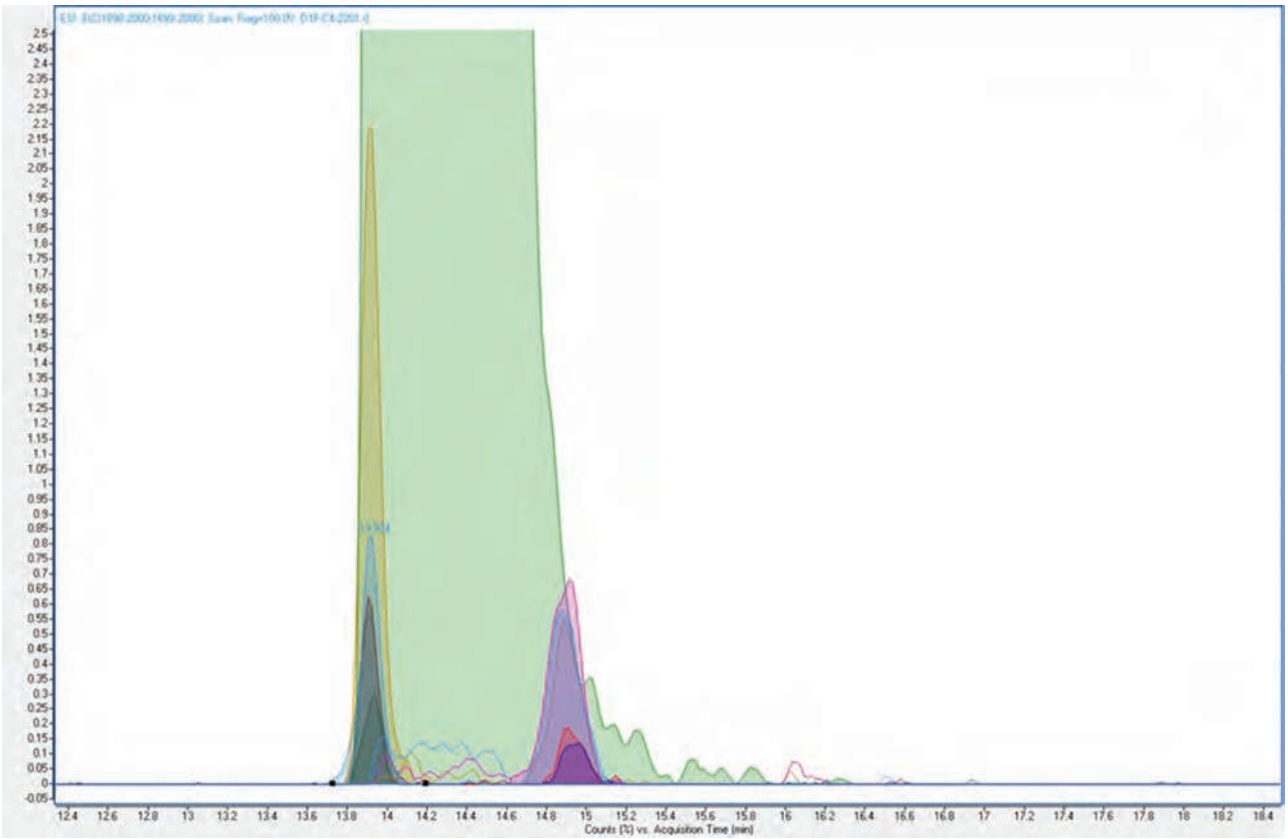


Figure 3C: Extracted ion profiles

can be performed in a more automated, high-throughput and robust capillary approach with CE. Large molecules like proteins or nucleic acids are exposed to an electric field and migrate through an electrolytic solution while analytes are detected by UV or fluorescence detectors.

The various DNA/RNA species present in an oligonucleotide are separated by size in a narrow bore-fused silica capillary filled with a gel matrix. This approach can therefore be used to determine purity and monitor related impurities, including fragments of the oligonucleotides. In the last few years, interfaces have been developed for some CE instruments allowing coupling with MS. This is still in its early stages, but offers great potential for providing additional, orthogonal information to LC/MS.

Potency

This can be measured using cell-based bioassays. The oligonucleotide product is transfected into an indicator cell line and either the reduction/elimination of the target protein in the case of an ASO or the induction/expression of the target protein in the case of an mRNA is subsequently measured.

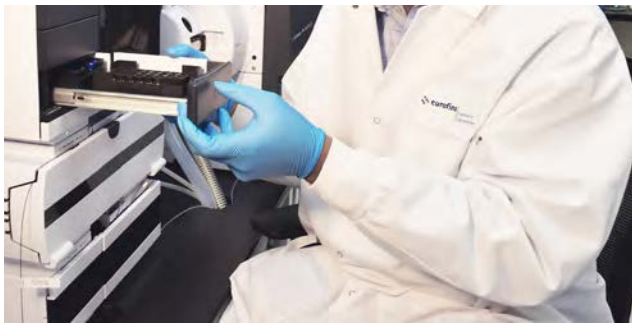
Residual Impurities

Gas chromatography (GC) is another traditional separation technique used to analyse semi-volatile and volatile organic compounds of lower molecular weight. Components of a mixture are separated based on their differing affinities for the gas mobile phase and the liquid stationary phase. They are typically configured with a flame-ionisation detector and, in some cases, GC/MS when additional identification is required. This method is not as common in the biopharma laboratory, but is very useful for monitoring the presence and concentration of residual solvents used in the synthesis of oligonucleotide products.

Compendial/Physical/Microbiology

As with any biopharmaceutical product, various other characteristics are typically required to support stability and release testing to meet expectations of the regulators – these tests may be carried out on the drug substance, product or both. Some of them are described in the compendia of both the United States and European Pharmacopeias, and may include:

- pH
- Osmolality
- Appearance
- Particulate matter
- Sterility
- Bacterial endotoxin
- Bioburden



The FDA has also been interested in subvisible particles, so newer techniques, such as micro-flow imaging, that are capable of providing qualitative as well as quantitative aspects of subvisible particles are sometimes carried out. Finally, drug developers are constantly employing novel delivery systems and formulations to increase the efficacy of their products, so additional assays may be required to provide information to cover these aspects of the final product.

Bright Future

Oligonucleotide therapies and vaccines are showing positive results in clinical trials and continue to gain traction within biopharma organisations. The techniques required to measure their critical quality attributes can be quite complex. However, advances in instrumentation, software, workflows and bioinformatics continue to provide better information to ensure well-characterised products to the patients that need them.

References

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