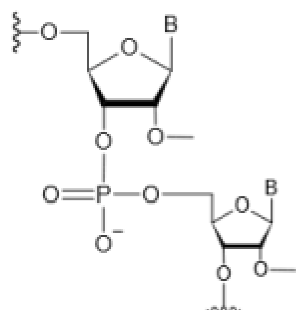
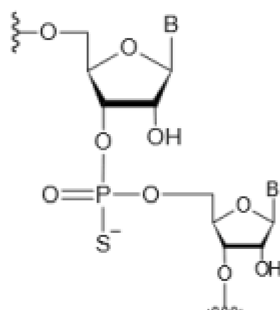
Guest Author – Helen Whitby, PhD., Technical Specialist

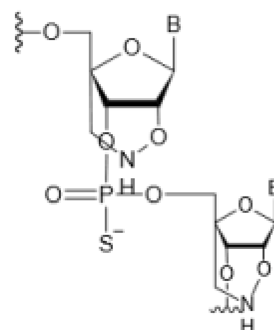
Synthetic oligonucleotides are promising therapeutic agents and their characterization is fundamental to their success. However, various impurities either product-related or arising from their synthesis must be identified and characterized post-synthesis. Their complexity in structure together with modifications conducted to improve stability together with identification of the impurities makes the analysis of these molecules a challenge.

Oligonucleotides are short DNA or RNA oligomers typically 20-60mer in length containing a phosphate backbone, which increases their polar nature and makes them extremely difficult to analyse by reversed phase HPLC. In addition to their polar functionality, the modifications to their structure to improve stability (such as OMe, MOE or 2-fluoro) increases polarity, amplifying the challenge.

Examples of Oligonucleotide Modifications used to improve stability





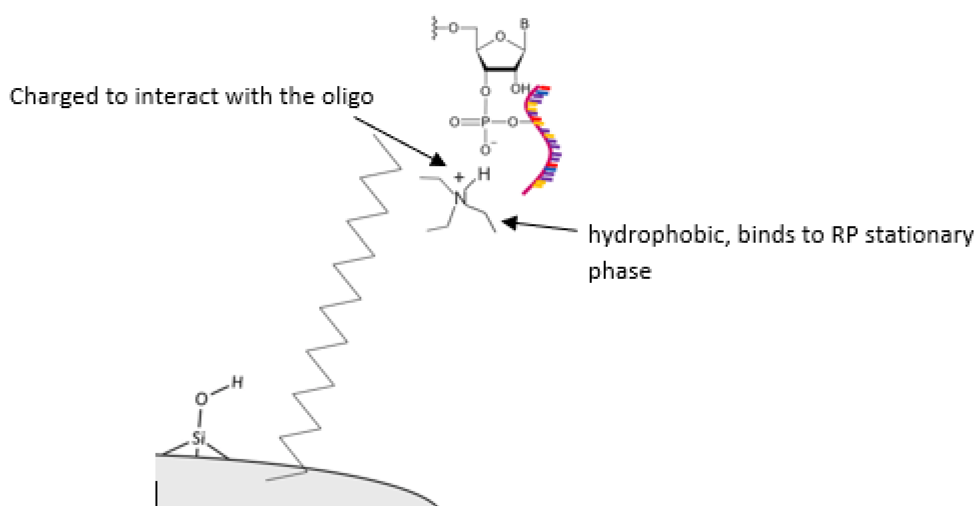


2'-O Methyl　　Phosphorothioate　　Bridged Nucleic Acid

Reversed phase HPLC involves the partitioning of an analyte between mobile phase and stationary phase with interactions typically driven through hydrophobic retention of the analyte on the stationary phase. More hydrophobic compounds will retain longer by reversed phase as their interaction with the phase is stronger. Synthetic oligonucleotides have very little hydrophobicity, so under typical reversed phase conditions do not retain on a C18 phase, which is the standard stationary phase for this type of separation. One potential solution to this retention problem would be to use a more polar stationary phase, however reversed phase chromatography with a C18 selectivity provides the highest resolution separation for HPLC. The nature of the product impurities means high methylene selectivity is imperative, for which a C18 is required to ensure separation of these n-1, n+1 sequence impurities.

Typical strategies for oligonucleotide analysis involve an ion-pair reversed phase separation employing mobile phase additives such as alkylamine's for ion-pairing and fluorinated alcohols to improve ionization for mass spec characterization.

The ion pair (commonly such alkyl amines as diisopropylamine or triethylamine) will ion pair to the negatively charged backbone of the oligonucleotide when run at an appropriate pH to ensure its opposite charge.

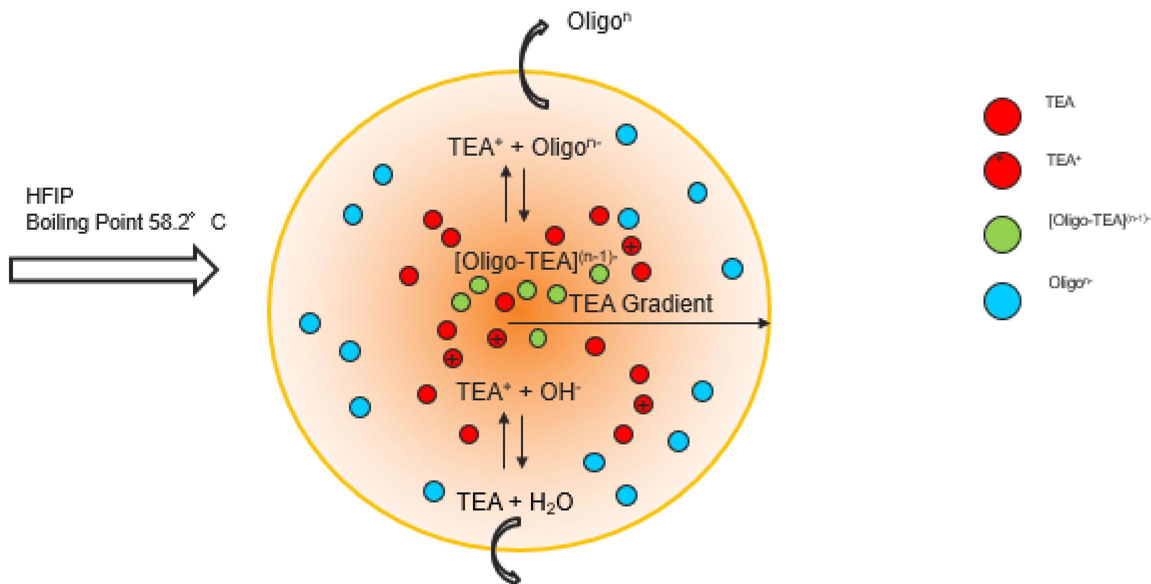


Other ion pairs also used include such longer chain alkylamines as hexylamine, however it has been documented by Bartlett et al that when used in higher quantities, these longer chain reagents have the capacity to form micelles in the mobile phase.[1]

In addition to the ion pair additive, fluorinated alcohols are required to act as acidic modifiers and facilitate ionization of the synthetic oligonucleotides leading to a better signal in the mass spectrometer. Traditional methods employ relatively high levels of these modifiers, however more recently it has been reported concentrations as low as 12.5mM are still effective for a range of different oligonucleotide types representing a 20 fold reduction

in the amount of HFIP needed compared with more traditional protocols.[2]

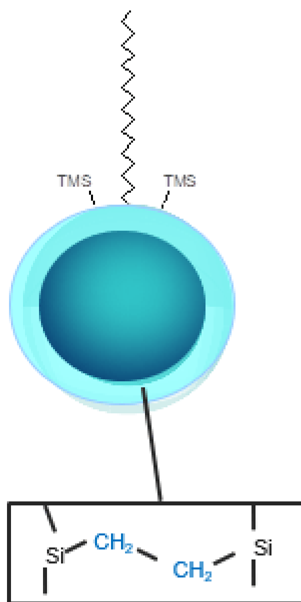
The ESI mechanism for oligonucleotides proposed by Barlett et al is represented below.[3]



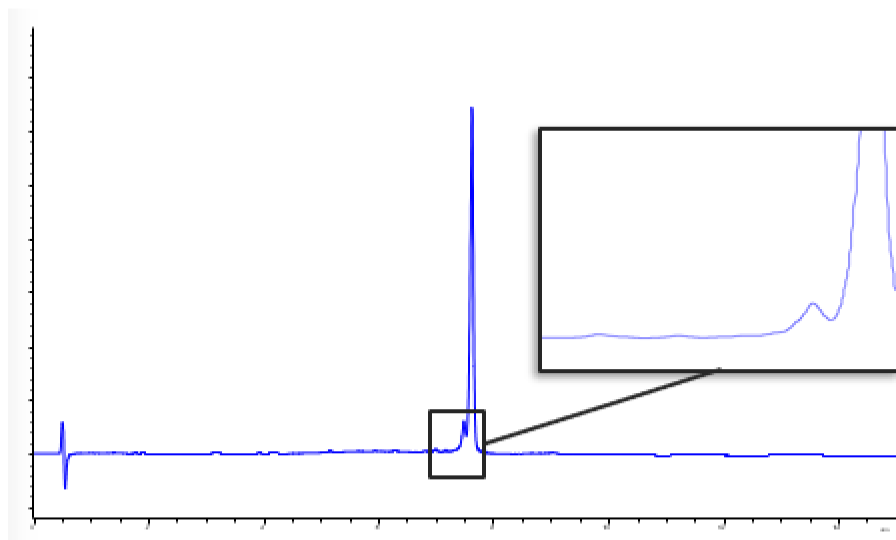
HFIP, the acidic modifier, has a low boiling point; this coupled with a high pH solvent facilitates ionization of the oligonucleotide. As TEA donates a proton to water, it will evaporate off, leaving the negatively charged oligo in gas phase to enter the MS.

As oligonucleotide analysis is an efficiency driven separation and requires the use of elevated pH (due to the alkylamine ion pair reagents), the use of a highly efficient pH stable columns is imperative. Unmodified silica is susceptible to dissolution at elevated pH so

commonly organo-silica hybrid particles incorporating ethane linkages are used for these methods. When pH stability is coupled with a core shell particle technology, it offers a unique high efficiency pH solution to achieve resolution between these n-1 / n+1 impurities together with increased sensitivity and stability from the advantages a core shell particle offers.



Standard analytical conditions for the analysis of oligonucleotides employ shallow gradients using acetonitrile as the strong solvent to modulate elution. Below we show the separation of an n-1 impurity using HFIP and diisopropylamine under gradient conditions of a hydrophobic bridged nucleic acid.



A: 50mM HFIP & 5mM DIEA in water; B: 50mM HFIP & 5mM DIEA in ACN; 5-20% B; 60°C

Although not an easy class of compounds to separate, the advances in column technologies and greater depth of research into mobile phase additives has offered a significant improvement in the characterization of oligonucleotides in recent years. In our final piece in this series we will review some of these developments together with recent news about new classes of therapeutic oligonucleotides making the news.

And don’t forget to visit the first two parts to this blog series focusing on oligonucleotides.

Part 1 – Oligonucleotides – What are They and Why are They of Interest

> Oligonucleotides – What Are They and Why Are They of Interest

Part 2 – Antisense Oligonucleotides – The Significance of the DNA-like Molecules

> Antisense Oligonucleotides – The Significance of the DNA-like Molecules

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Have any questions regarding the above information on synthetic oligonucleotides or any other technical inquires? Reach out to our technical specialists today through Chat Now – our free online service that can help with method optimization, chromatographic tips, product recommendations, and provide quotes for an easier buying process.

Start chatting today at www.phenomenex.com/chat.

1. Li, N.; El Zahar, N. M.; Saad, J. G.; van der Hage, E. R. E.; Bartlett, M. G. *J. Chrom. A* **2018**, *1580*, 110-119
2. *Need to reference new Technote for bioZen Oligo*
3. Bartlett et al. J Am Soc Mass Spectrum. 2013 Feb;24(2):257-64.

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