TOSOH

Application Note

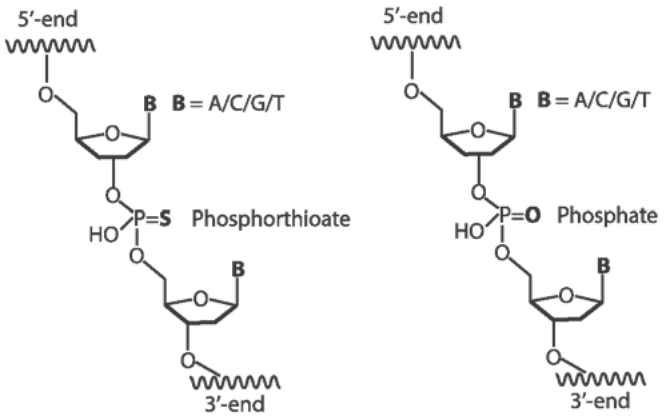
Anion Exchange Analysis of Unmodified and Phosphorothioate Oligonucleotides

Oligonucleotide therapeutics employ phosphate backbone modifications to increase resistance towards nucleases. The most common modification is the replacement of the phosphodiester (PO) bond between single nucleotides by a phosphorothioate (PS) linkage. This modification exchanges a non-bridging oxygen with a sulfur atom (*Figure 1*). Oligonucleotides exhibit this modification either at some or all nucleotides. Because of this modification, phosphorothioate modified oligonucleotides potentially exhibit additional impurities: insufficient or excessive modification, or wrong positioning of the modification. These impurities come on top of chain length variations resulting from the synthesis process. The phosphorothioate modification impacts on the physical properties of the molecule and thus separation by anion exchange chromatography (AEX) is required. AEX is typically employed to identify impurities of oligonucleotides having additional or missing nucleotides. We show the differences of AEX analysis of an unmodified and a thioated oligonucleotide and how to adjust the method for modified oligonucleotides.

Experimental Conditions

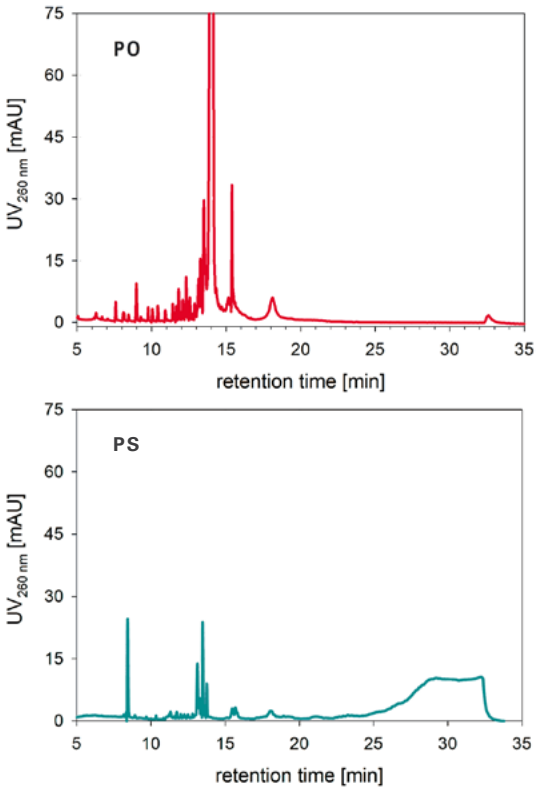
For this study, we used the TSKgel® DNA-NPR column as it offers features dedicated for nucleic acid analyses. These features include a polynucleotide-based quality control test as well as non-porous 2.5 µm particles to guarantee fast but high resolution separation.

Figure 1. Structure of phosphorothioate (PS, left) and phosphodiester (PO, right) linkage in oligonucleotides.



Column:	TSKgel® DNA-NPR, 4.6 mm ID x 7.5 cm, 2.5 µm (*P/N 818249*)
Sample:	Crude 17mer ssDNA Thioate Crude 20mer ssDNA unmodified
Mobile phase: 'standard'	A: 50 mmol/L Tris-HCl; pH 9.0 at the respective temperature; B: A + 1 mol/L Sodium chloride
Mobile phase: 'thioate'	A: 10 mmol/L Sodium hydroxide in Milli-Q® water; pH 12.0; B: A+ 2 mol/L Sodium chloride
Flow rate:	0.5 mL/min
Linear gradient:	0% A – 100% B in 10 column volumes (0-25 min)
CIP step:	3 mol/L Sodium chloride; 50 µL injection
Detection:	UV@ 260 nm, 2 µL flow cell
Injection vol.:	3 µL
Temperature	20 °C, 60 °C

Figure 2. Anion exchange (AEX) analysis of PO and PS oligonucleotides.



A 20mer ssDNA (PO) and a phosphorothioate modified ssDNA oligonucleotide (17mer) (PS) were separated on a TSKgel® DNA-NPR column with the following conditions: 20 °C, mobile phase A: 50 mmol/L Tris-HCl; pH 9.0, mobile phase B: (A + 1 mol/L Sodium chloride), linear gradient over 10 column volumes (25 min).

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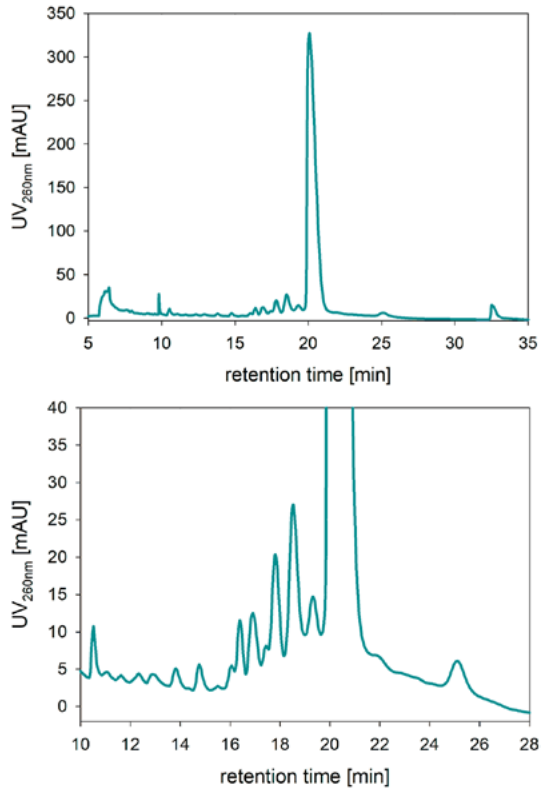
Results and Discussion

Initially, a single stranded DNA 20mer and a fully thioated single stranded DNA 17mer were analyzed using standard anion-exchange conditions: mobile phase A: 50 mmol/L Tris-HCl; pH 9.0, 20 °C; mobile phase B: A + 1 mol/L Sodium chloride. The non-modified sample eluted completely and showed a good separation of the main product eluting at 14 min and shorter (earlier eluting) and longer impurities (later eluting) (*Figure 2a*). In contrast, the fully PS-modified oligonucleotide was more strongly retained by the column, despite being shorter. This result leads to late retention times and the bulk eluting without resolution of different species at 25–33 min (*Figure 2b*).

Figure 3. Adapted method for AEX analysis of PS oligonucleotides.



A PS modified oligonucleotide (17mer) was separated on a TSKgel® DNA-NPR column with the following conditions: mobile phase A: 10 mmol/L Sodium hydroxide pH 12* 10% Acetonitrile, 60 °C*, mobile phase B: (A + 2 mol/L Sodium chloride) linear gradient: 10 CV.
**pH and temperature were increased to resolve possible secondary structures*

PS oligonucleotides were reported to have a lower pKa and being more acidic as compared to PO oligonucleotides[1] which is one possible explanation for the increased relative retention of the PS 17mer. In order to increase elution from the column, a higher salt concentration (2 mol/L Sodium chloride) was chosen for the PS oligonucleotide. Additionally, it was shown that sulfur modified oligonucleotides are more hydrophobic[2], a second factor explaining the retention differences between PO and PS oligonucleotides as secondary hydrophobic interaction with the stationary phase adds onto the anion exchange retention mechanism. Therefore, acetonitrile (ACN) was added to the mobile phase in order to suppress hydrophobic interactions and to achieve separation of the impurities of the PS-modified oligonucleotide. *Figure 3* shows the adapted method allows for complete elution of the PS-modified 17mer and separation of the main product (25 min) and impurities such as longmers, shortmers and potentially incompletely thioated moieties.

The relatively broad peaks may stem from numerous diastereomers (for the analyzed 17mer full thioate up to 2^{16} possible diastereomers) that elute differently and overlap with the according shortmers and longmers.

Conclusion

The insertion of phosphorothioate instead of phosphate linkages in oligonucleotides not only increases stability against nucleases, but influences the physicochemical properties of the molecule leading to differences in anion-exchange analysis. On the one hand, the higher hydrophobicity of PS oligonucleotides requires the use of an organic modifier to reduce secondary interactions and strengthen the anion-exchange separation mechanism. On the other hand, the introduction of stereocenters leads to a drastic number of diastereomers with slightly different retention mechanisms that overlap with the separation of longmers and shortmers. However, a number of impurities can be identified in addition to the main product using the DNA-NPR weak anion exchange column.

References

1. M. Gilar *et al.* Kinetics of phosphorothioate oligonucleotide metabolism in biological fluids. Nucleic Acids Research, 1997, Vol. 25, No. 18 3615–3620

2. Martin Enmark *et al.* (2019) Investigation of factors influencing the separation of diastereomers of phosphorothioated oligonucleotides. Analytical and Bioanalytical Chemistry volume 411, pages3383–3394 (2019)

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