TOSOH

Application Note

Analysis of Oligonucleotides by SEC-MALS

The oligonucleotide therapeutics field has seen remarkable progress over the last few years and oligonucleotides are increasingly recognized as potential therapeutic agents for a variety of diseases. Here we describe the ability of ultra-high performance size exclusion chromatography to distinguish N and N-1 oligonucleotide species.

Introduction

Oligonucleotide-based therapeutics have made rapid progress in the clinic for treatment of a variety of disease indications. In recent years, several oligonucleotide drugs for gene silencing, such as short interfering RNA (siRNA) and antisense oligonucleotides (ASOs) have been approved and microRNA (miRNA) and aptamers are being developed as therapeutic platforms. The promising CRISPR-Cas system also requires a specific RNA moiety - guiding RNA - to recruit and direct the Cas nuclease activity.

Therapeutic oligonucleotides are produced through a synthetic solid-phase chemical synthesis. Despite improvements in oligonucleotide synthesis, and despite the most ardent post synthesis clean-up, there will be some heterogeneity with regards to chain distribution. Monitoring of this distribution is fundamental aspect of process and quality control. This is a fundamental assessment is typically done by capillary gel electrophoresis (CGE) or anion exchange chromatography. Here we present the ability of size exclusion chromatography to discriminate oligonucleotides differing by one base in length. The 2 µm silica-based stationary phase TSKgel® UP-SW2000 with a pore size of 12.5 nm was used in combination with UHPLC and UHPLC-MALS systems.

Analysis of Oligonucleotides by SEC

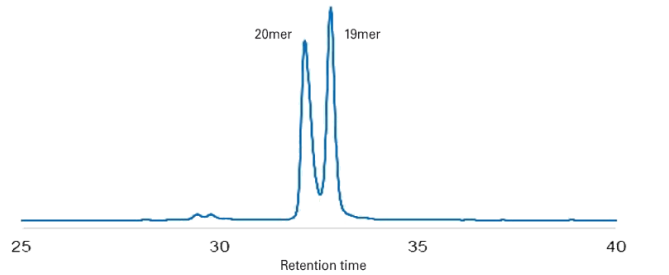
TSKgel UP-SW2000 is a newly developed silica-based 2 µm, 12.5 nm pore size SEC column designed for the separation of small proteins, peptides, and oligonucleotides. The column can be used in both, HPLC and UHPLC systems and is ideally suited for method transfer from conventional silica-based size exclusion columns to UHPLC technology. Two 30 cm TSKgel UP-SW2000 columns in a row were used to analyze a mixture of two oligonucleotides differing by only one base.

Material and Method

Column:	TSKgel UP-SW2000 2 x (2 µm 4.6 x 30 cm L, P/N 0023514)
Mobile phase:	50 mmol/L phosphate buffer, pH 6.7 300 mmol/L NaCl, 0.03% NaN3
Flow rate:	0.2 mL/min
Detection:	UV @ 260 nm
Sample:	19-mer (5'-AATTCATCGGTTCAGAGAC-3') & 20-mer(5'-GAATTCATCGTTCAGAGAC-3')

Figure 1 demonstrates that UP-SW2000 can be used to separate a 20-mer and its N-1 19-mer.

Figure 1. Separation of N and N-1 Oligonucleotides.



SEC-MALS Analysis of Oligonucleotides

Crude and purified oligonucleotide samples were analyzed by SEC-MALS using the new LenS3 multi-angle light scattering detector.

Material and Method

Columns:	TSKgel UP-SW2000 (4.6 x 30 cm L)
UHPLC:	Thermo Fisher Dionex Ultimate 3000 UHPLC system with LenS3 MALS
Mobile phase:	0.5 mol/L NaCl, 0.1 mol/L EDTA, pH 7.5 0.1 mol/L Na2SO4, 0.03% NaN_3 in 0.1 mol/L phosphate buffer
Flow rate:	0.3 mL/min
Detection:	UV @ 260 nm
Injection vol.:	10 µl
Sample:	20 bases custom oligonucleotide with MW= 6141 Da (purified sample 0.3 mg/mL; crude sample 1 mg/mL)

Figure 2 shows the comparison of chromatograms of the crude and purified oligonucleotide samples.

Figure 2. Overlay of unpurified and purified 20-mer UV chromatograms.

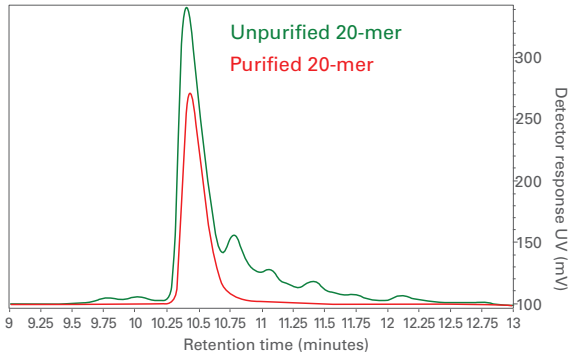


Figure 3. Molecular weight distribution (green) of the unpurified 20-mer.

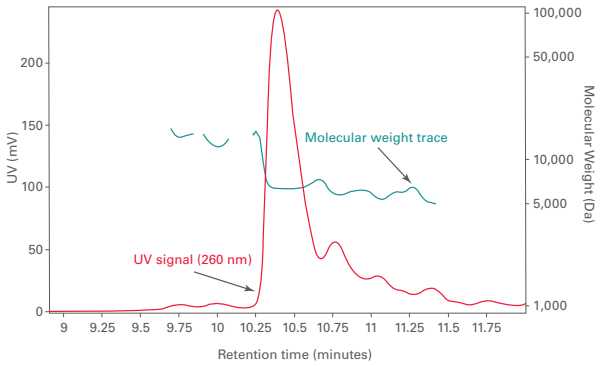


Figure 4. Peak analysis of the unpurified 20-mer.

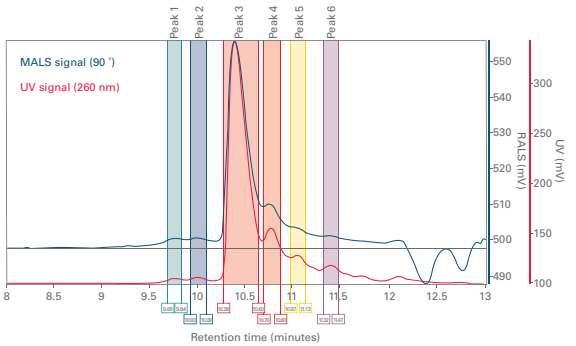


Figure 3 shows the molecular weight distribution of the unpurified 20-mer. The molecular weight trace clearly indicates the presence of higher and lower molecular weight impurities.

The peak analysis (*Figure 4* and *Table 1)* allows a molecular weight profiling of the product and the impurities. The MALS analysis of the purified sample (*Figure 5*) proves the high purity of the 20-mer oligonucleotide. The good reproducibility of retention time and calculated molecular weight of the purified 20-mer is shown in *Table 2* (triplicate injection).

Table 1. Molecular weight profiling.

Peak	Retention time	% RSD	MW (Da)	% RSD
1	9.774	0.1%	13,599	2.1%
2	10.012	0.0%	11,550	1.9%
3	10.398	0.1%	6,398	0.7%
4	10.776	0.1%	5,751	1.5%
5	11.053	0.1%	5,177	2.3%
6	11.422	0.2%	4,446	5.5%

Figure 5. Molecular weight distribution (green) of the purified 20-mer.

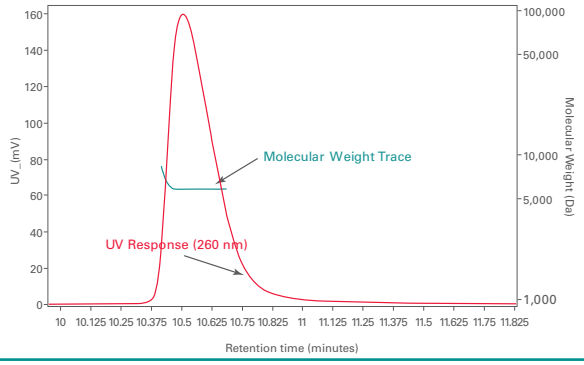


Table 2. Reproducibility of retention time.

Injection	Retention time (min)	MW (Da)
1	10.431	6,066
2	10.443	6,023
3	10.445	6,038
Average	10.440	6,042
% RSD	0.1%	0.3%

Conclusion

TSKgel UP-SW2000 is a size exclusion column designed for UHPLC analysis of biomolecules of a molecular weight of 1 to 150 kDa. The separation range is ideally suited to analyze small proteins or peptides and their aggregates.

This application note shows that this column can also be used to analyze oligonucleotides by (U)HPLC. Multi-angle light scattering detection delivers additional information on the molecular weight of the oligonucleotide and any impurities present in the sample.