TOSOH

Application Note

Analysis of Oligonucleotides by SEC

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 (SEC) 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 the most ardent post synthesis clean-up, there will be some heterogeneity with regards to chain distribution. Monitoring of this distribution is a fundamental aspect of process and quality control. This assessment is typically done by capillary gel electrophoresis or anion exchange chromatography. Here we present the ability of size exclusion chromatography to discriminate oligonucleotides differing by one base in length. A TSKgel® UP-SW2000 column was used in combination with UHPLC and UHPLC-MALS systems.

Analysis of Oligonucleotides by SEC

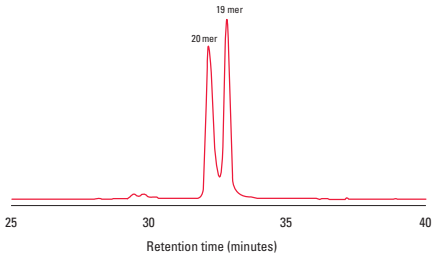
TSKgel UP-SW2000 is a newly developed silica-based 2 µm, 12.5 nm pore size SEC column designed for the separation of small proteins, peptides and oligonucleotides. The column can be used in both HPLC and UHPLC systems and is ideally suited for method transfer from conventional HPLC size exclusion columns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

Materials and Method:

Column:	TSKgel UP-SW2000, 2 µm, 4.6 mm ID × 30 cm × 2
Mobile phase:	50 mmol/L phosphate buffer, pH 6.7 300 mmol/L NaCl, 0.03% NaN_3
Flow rate:	0.2 mL/min
Detection:	UV @ 260 nm
Sample:	19-mer (5'-AATTCATCGGTTCAGAGAC-3') & 20-mer (5'-GAATTCATCGGTTCAGAGAC-3')

Figure 1 demonstrates that the TSKgel UP-SW2000 column 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 a newly developed multi-angle light scattering detector, the LenS3™ from Tosoh Bioscience.

Materials and Method:

Column:	TSKgel UP-SW2000, 2 µm, 4.6 mm ID × 30 cm
Instrument:	Thermo Fisher 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 Na_2SO_4, 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 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.

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

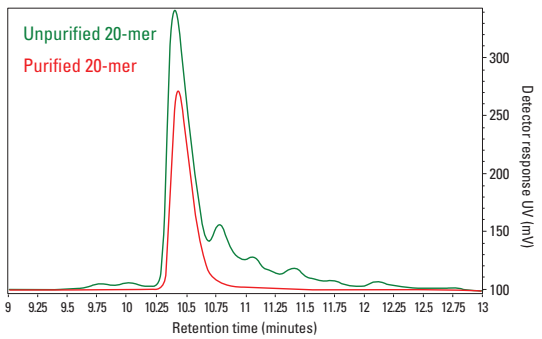
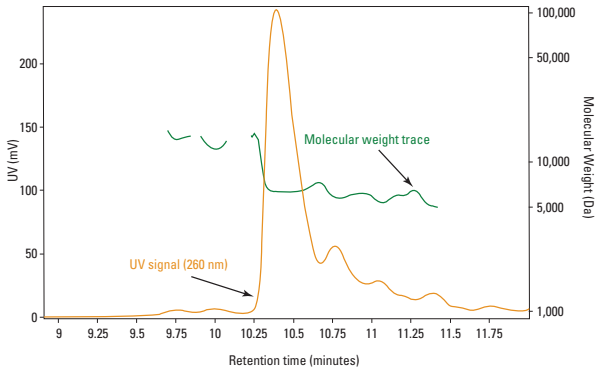


Figure 3. Molecular weight distribution (green) of the unpurified 20-mer



The peak analysis from *Figure 4*, as detailed in *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).

Figure 4. Peak analysis of the unpurified 20-mer

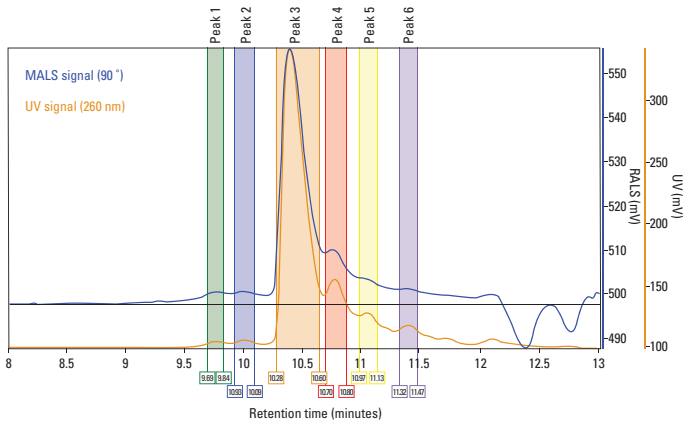


Table 1. Retention time and molecular weight of each peak (triplicate injection)

Peak	Retention time (min)	% 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

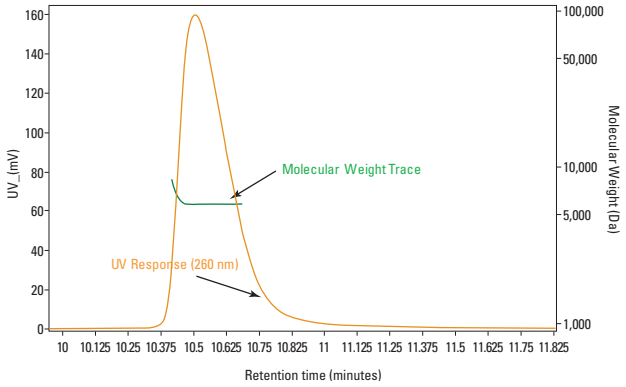


Table 2. Retention time and molecular weight of the purified 20-mer (triplicate injection)

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%***

Conclusions

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 demonstrates that this column can also be used to analyze oligonucleotides by UHPLC. Multi-angle light scattering detection delivers additional information on the molecular weight of the oligonucleotide and any impurities present in the sample.