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THERAPEUTIC NUCLEIC ACIDS APPLICATION NOTEBOOK

ANALYSIS & PURIFICATION OF OLIGONUCLEOTIDES

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EDITORIAL
DEAR READER

Therapeutic nucleic acids (TNAs) are nucleic acids or closely related compounds used to treat disease by halting the expression of abnormal proteins, typically via inhibition of mRNA translation. To date, most approved therapeutic nucleic acids have been synthetic DNA or RNA oligonucleotides applied as antisense oligonucleotides (ASOs) or short interfering RNAs (siRNAs). Aptamer RNA modulating protein functions is another popular area of research.

There has been tremendous development since the first antisense approach in living cells in 1978. The growing interest in oligonucleotides is driven by several factors: their high potential to be used in the treatments of a variety of medical conditions, the growing number of FDA-approved oligonucleotide drugs, and an increased focus on personalized medicine and the development of therapies for rare diseases.

Oligonucleotides (ONs) are short synthetic single-strand or double-strand DNA or RNA molecules. In nature, oligonucleotides are usually found as small RNA molecules acting in the regulation of gene expression (e.g. microRNA). They are synthesized chemically on solid supports through repeated cycles adding the desired nucleotides to the enlarging molecule. Recently, TOYOPEARL HW-65S resin was also been successfully used for solid-phase oligonucleotide synthesis1). As a consequence of the chemical synthesis, the typical purification scheme of oligonucleotides is different and less complex than the purification of biotherapeutics produced in cell culture.

Oligonucleotide synthesis includes the repeated use of protection groups that need to be removed after the last cycle. Subsequent purification ensures the purity required for the intended use. The removal of N-1 (shortmer) and N+1 (longmer) impurities is the main purification task. These impurities emerge through the faulty addition of two similar nucleotides in one cycle or a failed coupling of the last nucleotide. Chromatography can also separate ONs carrying protection groups from deprotected oligonucleotides and it can even facilitate the cleavage and removal of protection groups. This notebook provides several applications for the purification of synthetic oligonucleotides.

Chromatographic approaches for characterization and purity control of therapeutic nucleic acids use anion exchange, reversed-phase, and size exclusion chromatography. To achieve short analysis times while retaining high resolution, non-porous particles are often used for ON analysis by HPLC or UHPLC. A more detailed characterization of the molecular structure can be achieved when hyphenating (U)HPLC with mass-sensitive detection such as multi-angle light scattering or mass spectrometry. This notebook provides examples for the characterization of several types of standard and conjugated oligonucleotides, as well as for the analysis of related targets such as plasmid vectors and virus-like particles (VLPs).

ENJOY READING

1) Philpott, M., Watson, J., Thakurta, A. et al. Nat Biotechnol (2021). https://doi.org/10.1038/s41587-021-00965-w

IMPRESSUM

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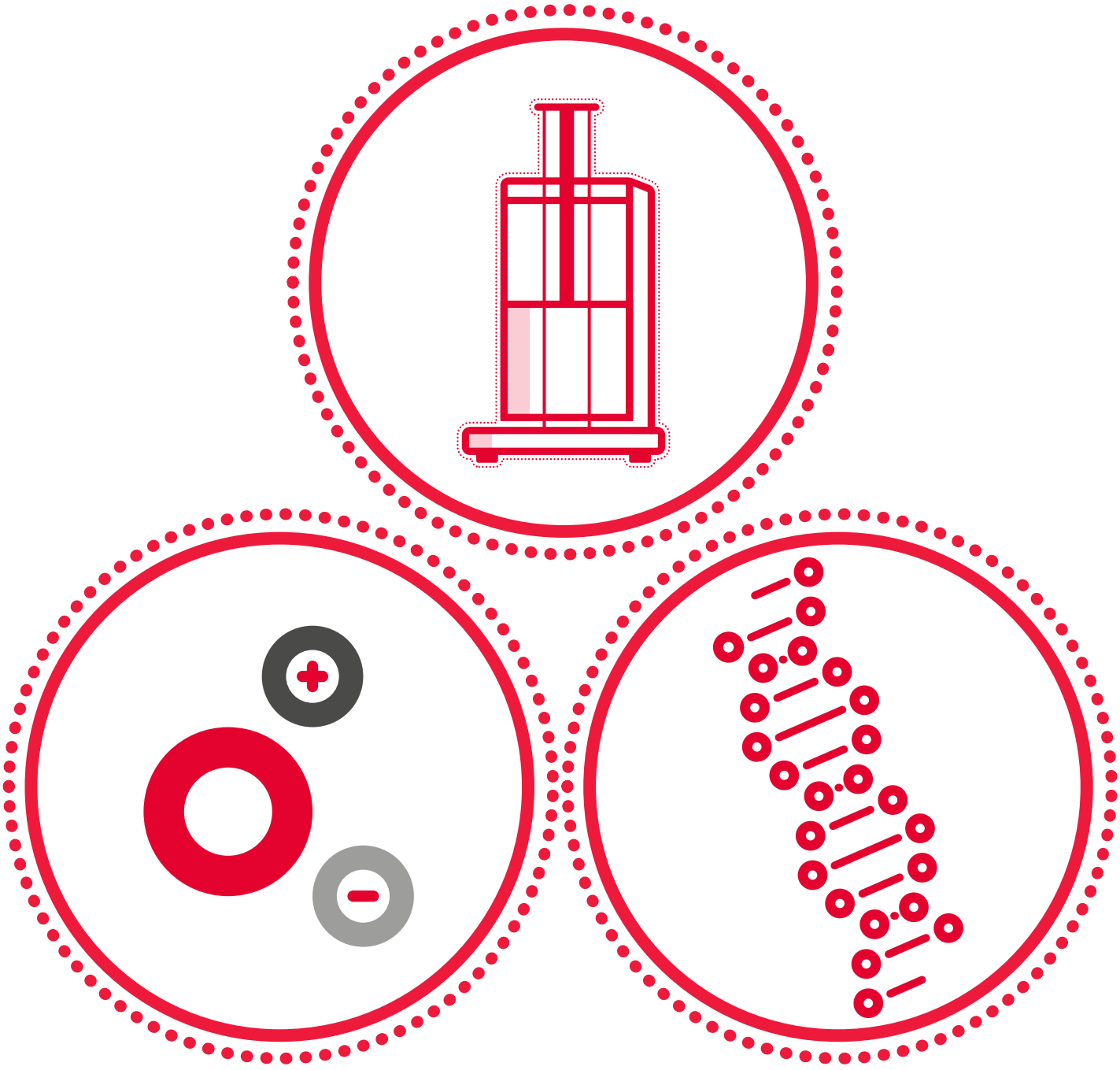
PURIFICATION



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Application Note

Development of a universal preparative AEX method to purify oligonucleotides

Introduction

Oligonucleotide-based therapeutics have been investigated over the last decades and their promise as a new drug modality is now being realized. The growing interest in oligonucleotides is driven by the high potential of oligonucleotides to be used in the treatments of a variety of medical conditions, the growing number of FDA approved oligonucleotide drugs, an increased focus on personalized medicine and on the development of therapies for rare diseases, and the wide adoption of nucleotide-based COVID-19 vaccines.

Oligonucleotides are short, linear sequences of DNA or RNA that are generally manufactured by chemical synthesis. Oligonucleotides are extremely susceptible to oxidation, enzymatic degradation, and clearance in vivo. Because of this, synthetic oligonucleotides are often chemically modified to improve their stability and make them resistant to extracellular and intracellular nuclease degradation. One of the original and still most widely used modifications is the phosphorothioate modification of the oligonucleotide backbone.

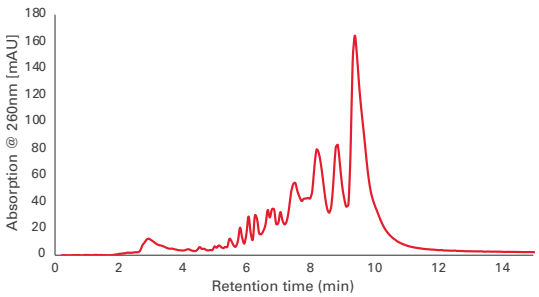
Due to errors during the oligonucleotide synthesis process, nucleosides may either be missing (N-X) or are attached in excess (N+X). Moreover, the chirality of the sulfur atoms in the backbone of ONs (due to the phosphorothioate modification) leads to diastereomers. To remove these impurities, the biopharma relies on chromatography during the purification process. The increased demand for oligonucleotides requires a cost-effective and easy scale-up from research amounts to commercial needs.

In this application note, we developed a universal method for the purification of phosphorothioate oligonucleotides using TSKgel SuperQ-5PW (20), a specially designed resin which provides high resolution and selectivity. This method can be used as a starting point in the lab to develop efficient large-scale preparative processes. Before addressing purification, we developed an anion exchange HPLC (AEX-HPLC) analytical method using a TSKgel DNA-NPR column to assess purity and recovery.

UHPLC Analysis of phosphorothioate Oligonucleotides

The crude oligonucleotides were analyzed before and after purification with analytical anion exchange HPLC (AEX-HPLC) using a linear salt gradient over 20 minutes on a TSKgel DNA-NPR column. (*Figure 1*).

Figure 1. AEX-HPLC chromatogram of the unpurified oligonucleotide sample with a purity of 42.17%



Column:	TSKgel DNA-NPR (4.6 mm ID x 7.5 cm)
UHPLC:	Thermofisher Dionex Ultimate 3000 UHPLC system
Mobile phase:	A: 10 mmol/L NaOH, pH 12.0 B: 2.0 mol/L NaCl
Gradient:	0 - 100 % B in 20 min
Flow rate:	0.5 mL/min
Detection:	UV @ 260nm
Injection vol.:	10 µm
Sample:	ssDNA full phosphorothioate (Purity 42.17%)

Purification of Oligonucleotides by AEX

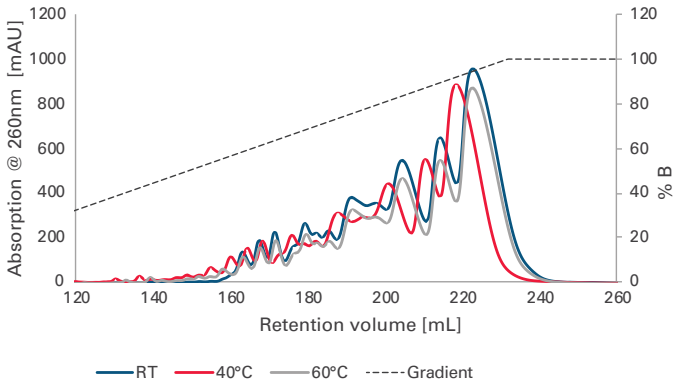
To develop a robust preparative method to purify oligonucleotides, we tested the performance of the TSKgel SuperQ-5PW resin at a 1mg/mL loading at different temperatures and buffer compositions.

Material and Methods

Column:	0.66 cm ID x 20 cm
Mobile phase:	A: 10 mmol/L NaOH, pH 12.0 B: 2.0 mol/L NaCl
Additives:	5% Acetonitrile (ACN) or 10 mmol/L NaCl
Flow rate:	1.2 mL/min
Detection:	UV@260nm
Sample:	20-mer ssDNA full phosphorothioate (Purity 42.17%)
Loading:	1 mg/mL Resin

Figure 2 shows the separation of the oligonucleotide at room temperature (blue), 40°C (red) and 60°C (grey) with a sodium hydroxide buffer as mobile phase and sodium chloride as elution phase.

Figure 2. TSKgel SuperQ-5PW (20), 1 mg load at different temperatures.



We observe a better resolution at 60°C, as confirmed by AEX-HPLC (*Table 1*). Therefore, all subsequent experiments were performed at 60°C.

The addition of chaotropic reagents or solvents reduces hydrophobic secondary interaction and can improve resolution in the purification of phosphorothioates, which are more hydrophobic than conventional phosphodiester oligonucleotides. The addition of low concentrations of salt to the binding buffer, reduces the ion exclusion effect. This leads to stronger binding of the sample and can potentially also increase resolution. To verify these assumptions, we evaluated the addition of 5 % acetonitrile and 10 mmol/L NaCl to the mobile phase.

Figure 3 shows the chromatograms on TSKgel SuperQ-5PW at a load of 1mg/ml with the different buffer conditions at 60°C.

Figure 3. TSKgel SuperQ-5PW (20), 1 mg load at different buffer conditions.

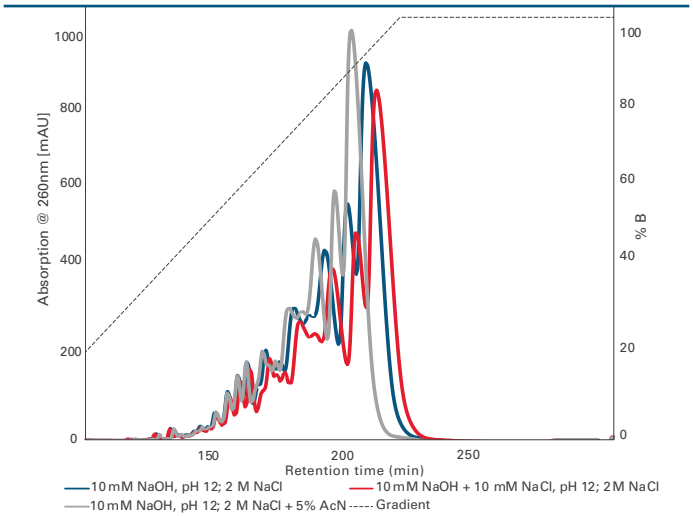


Table 1. Oligonucleotide purity and recovery from AEX purification at different temperatures and buffer conditions. Fractions with a main peak purity > 80 % were pooled for recovery and purity determination.

Condition	Recovery [%]	Purity [%]
Unpurified sample		42.17
RT	68.2	97.13
40°C	73.44	95.93
60°C	83.1	98.38
A: 10 mmol/L NaOH, pH 12.0; B: 2 mol/L NaCl	83.1	98.38
A: 10 mmol/L NaOH, pH 12.0 + 5% Acetonitrile; B: 2 mol/L NaCl + 5% Acetonitrile	75.5	97.71
A: 10 mmol/L NaOH, pH 12.0 + 10 mmol/L NaCl; B: 2 mol/L NaCl	76.7	95.2

The addition of sodium chloride suppressed ion exclusion during binding, and the sample binds stronger to the resin, resulting in a later elution time. In contrary, the addition of acetonitrile decreased the retention time as it suppressed hydrophobic interactions with the stationary phase. For our tested sample, both additives resulted in lower purity and recovery (*Table 1*).

Conclusion

We investigated the influence of temperature and buffer composition for the purification of oligonucleotides on the anion exchange resin TSKgel SuperQ-5PW (20). By increasing the temperature from room temperature to 60°C, we could separate the impurities from the target oligonucleotide with higher resolution, recovery, and purity. The mobile phase composition also has a significant influence on the purification of oligonucleotides. In our case, the addition of acetonitrile and NaCl in the equilibration buffer had a negative effect on recovery and purity.

Therefore, we recommend to purify oligonucleotides on TSKgel SuperQ-5PW (20) at elevated temperature, using a 10 mmol/L sodium hydroxide buffer at pH 12 and sodium chloride as eluent. Several of our biopharma partners already implemented similar methods for the purification of oligonucleotides on larger scale, leading to improvement of the process efficiency.

Table. Featured method development tools.

P/N	Description	Dimension
0045208	SkillPak SuperQ-5PW (20) 5 x 1 mL col.	7 mm ID x 2.5 cm
0045244	SkillPak SuperQ-5PW (20) 5 mL col.	8 mm ID x 10 cm
0043383	TSKgel SuperQ-5PW (20)	25 mL
0018249	TSKgel DNA-NPR	4.6 mm ID x 7.5 cm

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Application Note

Why is TSKgel® SuperQ-5PW (20) the industry standard for siRNA purification?

Oligonucleotide-based therapeutics represent the changing face of drug discovery and development. Designed to prevent or modulate the translation of a specific gene, this new class of biotherapeutics have the potential to tackle previously undruggable targets, and could set the tone for the future of personalized medicine.

In the application note "*Development of a universal preparative AEX method to purify oligonucleotides*", we presented a universal method for the purification of phosphorothioate oligonucleotides using Anion Exchange Chromatography (AEX). Based on this initial work, we recommend purifying oligonucleotides at elevated temperature, using a 10 mM sodium hydroxide buffer at pH 12 and sodium chloride as eluent.

Several of our biopharma partners already implemented similar methods for the purification of oligonucleotides on larger scale, leading to improvement of the process efficiency, using the anion exchange resin TSKgel SuperQ-5PW (20). To understand why TSKgel SuperQ-5PW (20) has become the industry standard for the purification of oligonucleotides, we compared the purification performances of TSKgel SuperQ-5PW (20) with another AEX resin used in the industry for the purification of oligonucleotides, Source 15Q from Cytiva. The analysis of purity and recovery was performed using an analytical TSKgel DNA-NPR column.

Purification of Oligonucleotides by AEX

In this study, we purified a ssDNA full phosphorothioate oligonucleotide with 48.47% purity after synthesis. We evaluated the performance at different loadings of 1, 5 and 10 mg oligonucleotide/mL and compared the two resins described in *Table 1*.

Table 1. Properties of TSKgel SuperQ-5PW (20) and Source 15Q.

	TSKgel SuperQ-5PW (20)	Source 15Q
Particle size	20 µm	15 µm
Pore diameter	>100 nm	
DBC oligo	46.4 mg/ml	
pH range	2-13	2-12

Material and Methods:

Column: 0.66 cm ID x 20 cm
Mobile phase: 10mmol/L NaOH, pH 12
2.0 mol/L NaCl
Flow rate: 1.2 mL/min
Detection: UV @260nm
Sample: 20-mer ssDNA full phosphorothioate (Purity 48.47%)
Load: 1, 5, 10 mg/mL resin

Figure 1, 2, and 3 show the chromatogram of TSKgel SuperQ-5PW (red) and Source 15Q (blue) at a load of 1 mg/mL, 5 mg/mL, and 10 mg/mL, respectively.

Figure 1. TSKgel SuperQ-5PW (20) and Source 15Q, 1 mg Load.

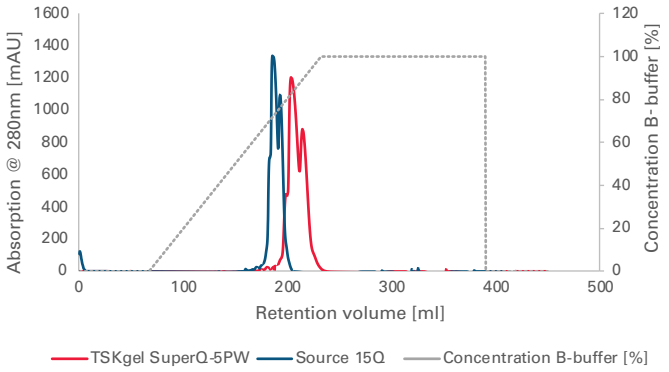


Figure 2. TSKgel SuperQ-5PW (20) and Source 15Q, 5 mg Load.

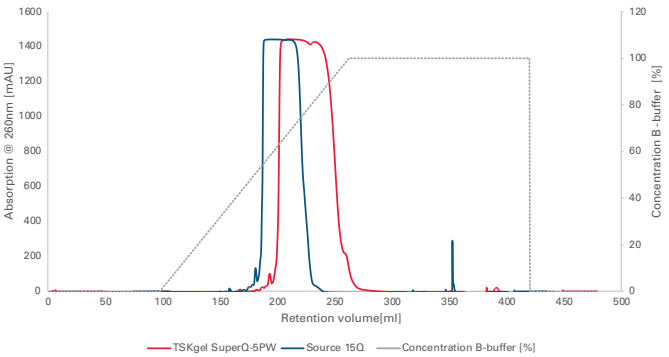
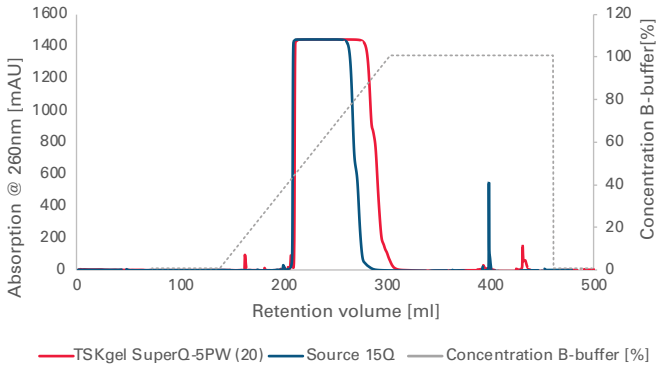


Figure 3. TSKgel SuperQ-5PW (20) and Source 15Q, 10 mg Load.



Both Source 15Q and TSKgel SuperQ-5PW (20) are suitable to purify phosphorothioate oligonucleotide at different loadings. As can be seen in the chromatograms, the impurities in the (N+1) and (N-1) region can be separated from the main peak in the center.

To compare both resins, we investigated the two critical performance parameters in the industry, purity and recovery at purity > 70 % (*Table 2*). Whereas the purity is higher on Source 15Q at the lowest loading, the purity on TSKgel SuperQ-5PW (20) is higher at the higher loadings, which are more suitable for industrial processes. Moreover, the recovery is higher with TSKgel SuperQ-5PW (20) at any loadings.

Table 2. Oligonucleotide purity and recovery from AEX purification at different loadings.

Resin	Load [mg/ml]	Recovery [%]	Purity[%]
TSKgel SuperQ-5PW	1	99,55	88,64
	5	98,26	97,45
	10	100	94,48
Source 15Q	1	80,76	96,01
	5	73,39	97,87
	10	84,12	94,99

Conclusion

TSKgel SuperQ-5PW (20) exhibits higher purification performances, especially at higher loadings than Source 15Q. Therefore, in industry processes, TSKgel SuperQ-5PW (20) will allow the development of more efficient purification methods thanks to higher purity and recovery.

Table. Featured method development tools.

P/N	Description	Dimension
0045208	SkillPak SuperQ-5PW (20) 5 x 1 mL col.	7 mm ID x 2.5 cm
0045244	SkillPak SuperQ-5PW (20) 5 mL col.	8 mm ID x 10 cm
0043383	TSKgel SuperQ-5PW (20)	25 mL
0018249	TSKgel DNA-NPR	4.6 mm ID x 7.5 cm

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Application Note

Purification of Oligonucleotides on TOYOPEARL® GigaCap® Q-650S

TOYOPEARL GigaCap Q-650S, a high capacity/ high resolution anion exchange resin for process scale applications with dynamic binding capacities approaching 190 g/L for bovine serum albumin (BSA).

TOYOPEARL GigaCap Q-650S maintains the high capacity of our popular TOYOPEARL GigaCap Q-650M and the 35 µm particle size provides high resolution for improved separation of process impurities and aggregates.

The purification of oligonucleotides using anion exchange chromatography has traditionally fallen to resins such as TSKgel® SuperQ-5PW (20) that offer high resolution and selectivity in conjunction with excellent mechanical stability at very high column pressures. TOYOPEARL GigaCap Q-650S resin offers a low pressure alternative to oligonucleotide purification while preserving the selectivity, resolution and yields of those higher pressure processes.

TOYOPEARL and TSKgel products are hydroxylated methacrylic polymer resins and are made commercially in many different pore sizes and particle diameters. TOYOPEARL resins vary from TSKgel resins by having a lower degree of crosslinking. Lower crosslinking makes available a larger number of resin sites for ligand immobilization when producing TOYOPEARL resins. This lower degree of crosslinking also makes for a less rigid bead. Therefore a functionalized TOYOPEARL resin will have a lower pressure rating than the corresponding TSKgel material.

Because similarly functionalized TSKgel and TOYOPEARL resin types have the same backbone polymer chemistry, the selectivity for proteins, oligonucleotides and their attendant impurities remains the same. TOYOPEARL resins can be used at high linear velocities and withstand operating pressures up to 0.3 MPa while TSKgel resins can withstand operating pressures of up to 2.0 MPa.

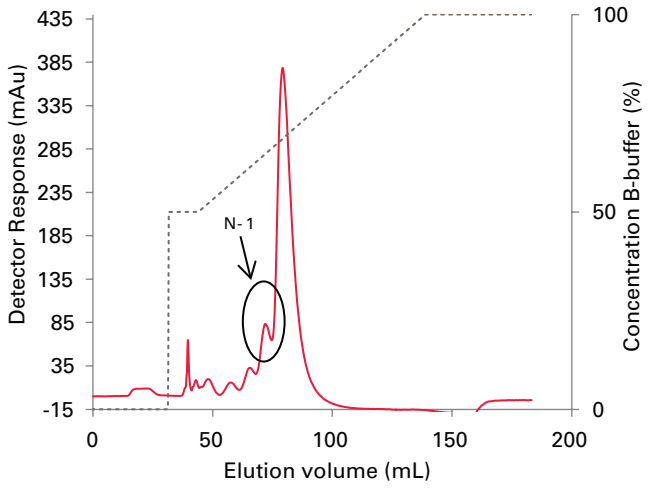
Table 1 shows the comparative properties of TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins and dynamic binding capacities for the oligonucleotide used in these experiments. The following experiments detail the purification of an oligonucleotide using TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins.

Oligonucleotides are short, linear sequences of deoxyribonucleic acid or ribonucleic acid that are generally manufactured by chemical synthesis. Because of the unique structure of these molecules and the way they are synthesized, oligonucleotides require special consideration during chromatographic purification.

Table 1. Properties of TSKgel SuperQ-5PW and TOYOPEARL GigaCap Q-650S.

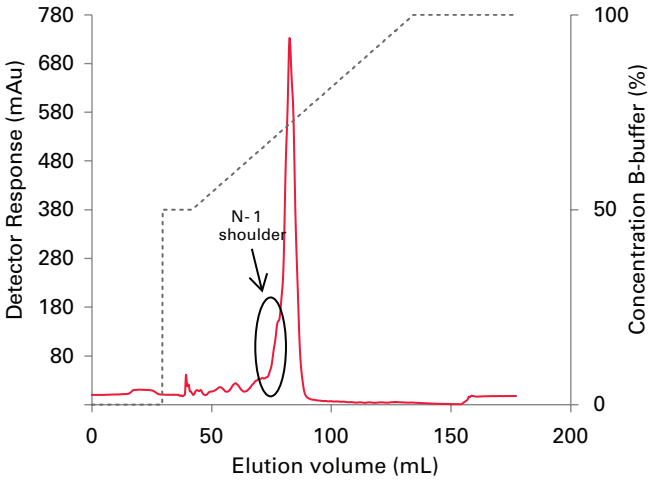
	TSKgel SuperQ-5PW (20)	TOYOPEARL GigaCap Q-650S
Particle size (µm)	20	35
Pore diameter (nm)	100	100
Ion exchange capacity (eq/L resin)	0.14	0.17
DBC oligo (g/L resin)	46.4	36.8
Max pressure	2.0 MPa	0.7 MPa

Figure 1. TSKgel SuperQ-5PW (20), 1.0 mg load.



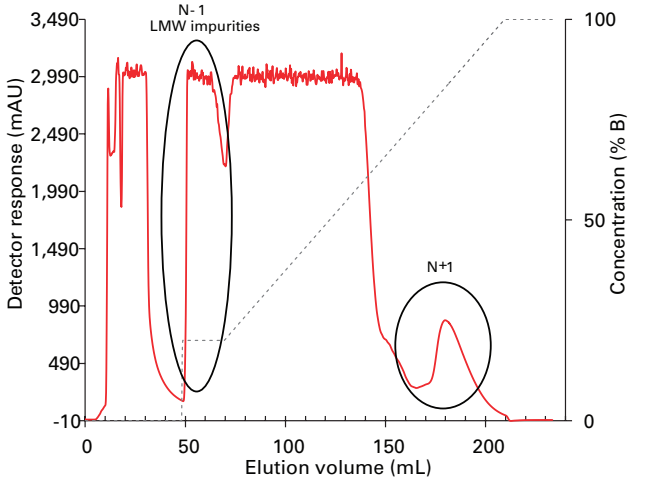
Resin: TSKgel SuperQ-5PW (20)
Column size: 6.6 mm ID × 18.5 cm (6.3 mL)
Mobile phase: A: 20 mmol/L NaOH;
B: 20 mmol/L NaOH, 3.0 mol/L NaCl;
Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV);
Flow rate: 200 cm/hr(1.14 mL/min)
Detection: UV @ 254 nm
Sample load: 1.0 mg
Sample: crude phosphorothioate deoxyoligonucleotide

Figure 2. Toyopearl GigaCap-Q-650S, 1.0 mg load.



Resin: TOYOPEARL GigaCap Q-650S
Column size: 6.6 mm ID × 18.5 cm (6.3 mL)
Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl;
Gradient: 50% B (2 CV); 50-100% B (15 CV); 100% B (2 CV);
Flow rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Sample load: 1.0 mg
Sample: crude phosphorothioate deoxyoligonucleotide

Figure 3. Purification of oligonucleotide at 80% DBC on TSKgel SuperQ-5PW (20) resin.



Resin: TSKgel SuperQ-5PW (20)
Column size: 6.6 mm ID × 18.5 cm (6.3 mL)
Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl
Gradient: 20% B (2 CV), 20-100% B (20 CV); 100% B (2 CV);
Flow rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Sample load: 235 mg
Sample: crude phosphorothioate deoxyoligonucleotide

During the synthesis of the oligonucleotide, there are a small percentage of sequences where a segment may either be deleted or have more than one segment attached (N-1 and N+1 respectively are the common nomenclature). Taken collectively, these synthesis errors may produce measurable amounts of impurities. The similarity in the impurities to the target molecule requires a high resolution technique to adequately isolate the target molecule.

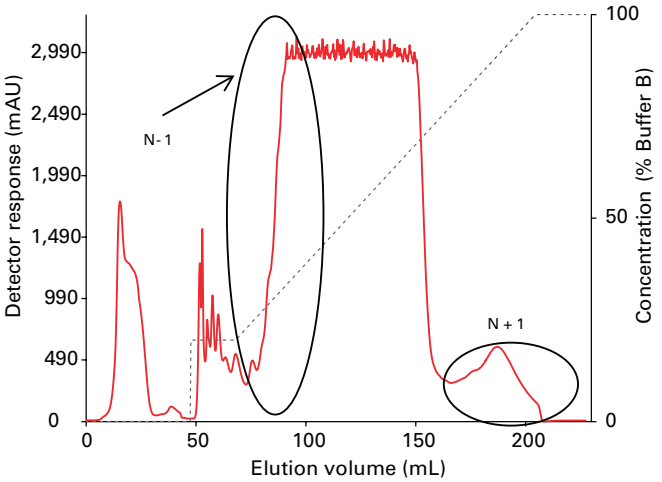
Experimental Conditions / Results

The data presented here demonstrate the similar capabilities of TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins to purify a phosphorothioate deoxyribonucleotide (24-mer).

Experiments were carried out on 6.6 mm ID × 18.0 ± 0.5 cm columns packed with TOYOPEARL GigaCap Q-650S and TSKgel SuperQ-5PW (20) resins. The columns were first under-loaded with a 1.0 mg sample of crude oligonucleotide to better visualize resin performance, Figures 1-2. As can be seen from these chromatograms, the N-1 peak was slightly better resolved with the TSKgel SuperQ-5PW (20) than with the TOYOPEARL GigaCap Q-650S, perhaps due to the smaller particle size of the TSKgel resin. HPLC analysis of fractions taken across the peaks (data not shown) revealed that both resins were able to adequately resolve the full length oligonucleotide.

After optimizing the elution gradient, the performance of the resins was then compared at 80% of each resin's respective dynamic binding capacity for this

Figure 4. Purification of oligonucleotide at 80% DBC on TOYOPEARL GigaCap Q-650S resin.



Resin: TOYOPEARL GigaCap Q-650S
Column size: 6.6 mm ID × 18 cm (6.16 mL)
Mobile phase: A: 20 mmol/L NaOH;
B: mobile phase A + 3.0 mol/L NaCl
Gradient: step to 20% B (2 CV)
20% - 100% B (20 CV); 100% B (2 CV)
Flow rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Injection vol.: 181.4 mg
Sample: crude phosphorothioate deoxyribonucleotide

oligonucleotide, *Figures 3-4*. As can be seen in the chromatograms, there was a visible N+1 peak that was resolved from the largest oligonucleotide peak in addition to the N-1 peak. Many of the low molecular weight impurities are visually resolved as well.

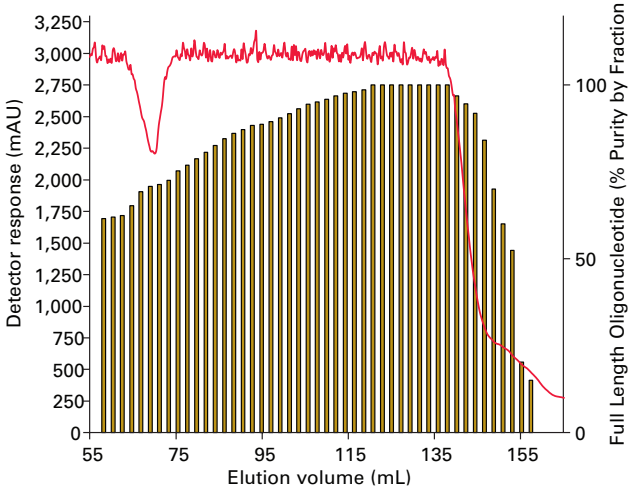
Though the chromatograms in *Figures 3 and 4* went off scale for UV, the general shape of the chromatograms is unchanged from that of the corresponding chromatogram when only 1.0 mg was loaded. HPLC analysis of fraction purity (data not shown) indicates that selectivity and resolution are maintained even at 80% DBC loading conditions.

An enlarged image of the main oligonucleotide peak, overlaid with a histogram showing HPLC results for fraction purity, highlights the chromatographic separation of the full length oligonucleotide, *Figures 5-6*. At 80% DBC, the TSKgel SuperQ-5PW (20) resin had some breakthrough of the full length product from the main peak into the N-1 peak while the TOYOPEARL GigaCap Q-650S did not. This indicates that the TOYOPEARL GigaCap Q-650S was better able to maintain resolution at 80% DBC loading conditions.

After pooling fractions of purified oligonucleotide, the yield and purity of the final product was determined for each resin, *Table 2*. The TSKgel SuperQ-5PW (20) and TOYOPEARL GigaCap Q-650S generated very high purity full length oligonucleotide (96.4% and 96.9% respectively) from crude synthesis material. The yield of full length oligonucleotide was almost 9% greater on the TOYOPEARL GigaCap Q-650S than the yield from the TSKgel SuperQ-5PW (20).

Product yield is affected by the amount of crude material loaded onto the column. In general, as column loading approaches saturating conditions, yield will decrease.

Figure 5. TSKgel SuperQ-5PW (20) resin: 80% DBC elution peak with fraction purity histogram.



Resin: TSKgel SuperQ-5PW (20)
Column size: 6.6 mm ID × 18.5 cm (6.3 mL);
Mobile phase: A: 20 mmol/L NaOH; B: 20 mmol/L NaOH, 3.0 mol/L NaCl; Gradient: 20% B (2 CV), 20-100% B (20 CV); 100% B (2 CV);
Flow rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Sample load: 235 mg
Sample: crude phosphorothioate deoxyoligonucleotide

Table 2. Oligonucleotide purity and yield from 80% DBC purifications.

Resin	Crude Oligo Purity	Final Oligo Purity	% Yield
TSKgel SuperQ-5PW (20)	66.5%	96.4%	72.5%
TOYOPEARL GigaCap Q-650S	66.5%	96.9%	81.3%

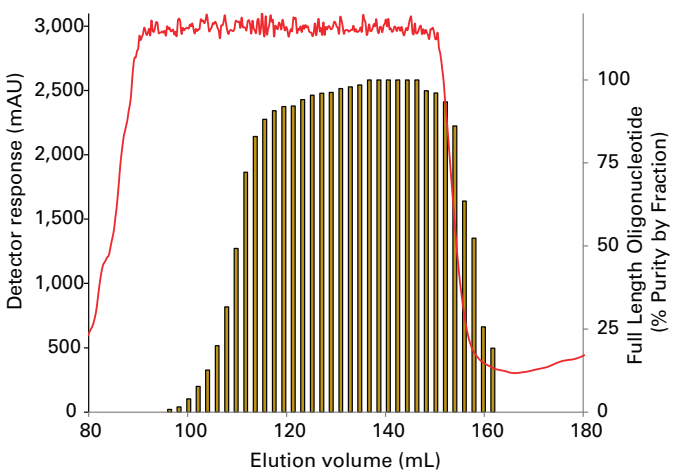
This phenomenon appears to be more pronounced with the TSKgel SuperQ-5PW (20) resin than with the TOYOPEARL GigaCap Q-650S resin.

Recovery was determined by comparing the amount of full length oligonucleotide present in the crude sample loaded onto the column with the amount of full length oligonucleotide present in the fraction pool.

Conclusion

TOYOPEARL GigaCap Q-650S is capable of delivering oligonucleotides of comparable purity to that seen with the TSKgel SuperQ-5PW (20) resin and at slightly higher process yields under the same loading conditions but at lower pressures. This capability allows chromatographers to purify oligonucleotides without the added expense of purchasing high pressure manufacturing equipment.

Figure 6. Toyopearl GigaCap Q-650S resin: 80% DBC elution peak with fraction purity histogram.



Resin: TOYOPEARL GigaCap Q-650S
Column size: 6.6 mm ID × 18 cm (6.16 mL);
Mobile phase: A: 20 mmol/L NaOH; B: mobile phase A + 3.0 mol/L NaCl; Gradient: step to 20% B (2 CV); 20% - 100% B (20 CV); 100% B (2 CV)
Flow rate: 200 cm/hr (1.14 mL/min)
Detection: UV @ 254 nm
Injection vol.: 181.4 mg;
Sample: crude phosphorothioate deoxyribonucleotide

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Application Note

Simple and Effective Method for Purification of DMT-on Oligonucleotides using HIC Resins

Introduction

The use of synthetic oligonucleotide therapeutics continues to grow because of their effectiveness in the treatment of devastating diseases. Within the bio-pharmaceutical industry, oligonucleotide drug pipelines have increased significantly, along with the need for purification techniques of these highly valuable materials.

Dimethoxytrityl (DMT), a 5′ protecting group, is used in the synthesis of oligonucleotides to temporarily mask the characteristic chemistry of a 5′-hydroxy functional group. In many preparations of delicate oligonucleotides, DMT may be left on an oligonucleotide following synthesis to give stability to the molecule during subsequent processing.

In this note, a novel, effective and high recovery method for purification of a DMT-on oligonucleotide and the effective removal of a DMT-group from an oligonucleotide in a single purification step are described. This purification can be achieved by using hydrophobic interaction chromatography (HIC) since the DMT-on group is strongly hydrophobic.

Materials and Methods

Oligonucleotide: 5′-GAA TTC ATC GGT TCA GAG AC-3′, a single stranded DNA oligonucleotide, 20-mer in length with a molecular weight of 6.141 kDa. It was supplied (AEX-HPLC) at ~55% purity from Trilink.

Salts: 3 different salts purchased from Millipore

Sigma were used in the study: sodium chloride (NaCl), sodium sulfate (Na_2SO_4) and ammonium sulfate $(NH_4)_2SO_4$.

HIC resins: Four TOYOPEARL® HIC resins were selected for the study: PPG-600M, Phenyl-650M, Butyl-650M, and Hexyl-650C.

Conditions: See chromatograms

Results and Discussions

Resin selection

Hydrophobic interaction chromatography is a powerful tool for the process purification of biomolecules. The technique utilizes the accessible hydrophobic regions located on the surfaces of the molecules and their interactions with a hydrophobic stationary phase.

Proteins and other molecules with hydrophobic surfaces are attracted to the hydrophobic ligands of HIC resins by employing an aqueous high salt mobile phase. The salt conditions contribute to a lyotropic effect, which allows the proteins to bind to a hydrophobic ligand. Bound molecules are eluted by decreasing the salt concentration. Most therapeutic targets are eluted in a low salt or a no salt buffer. Since HIC separations are done under mild eluting conditions, biological activity is typically retained.

In order to determine the ligand able to provide the best purity, recovery and yield for the purification of an oligonucleotide, four TOYOPEARL HIC resins were selected: PPG-600M, Phenyl-650M, Butyl-650M, and Hexyl-650C. These stationary phases are ranked from the least to the most hydrophobic, as shown in *Figure 1*. These HIC resins are polymethacrylic polymer beads featuring ligands with different degrees of hydrophobicity and selectivity.

Figure 1. Degrees of Hydrophobicity of TOYOPEARL HIC resins.

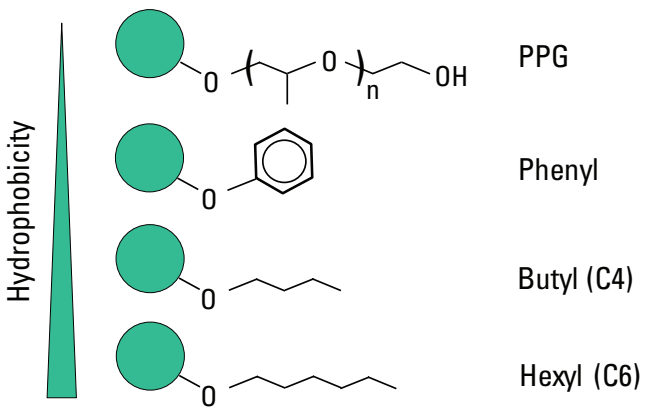
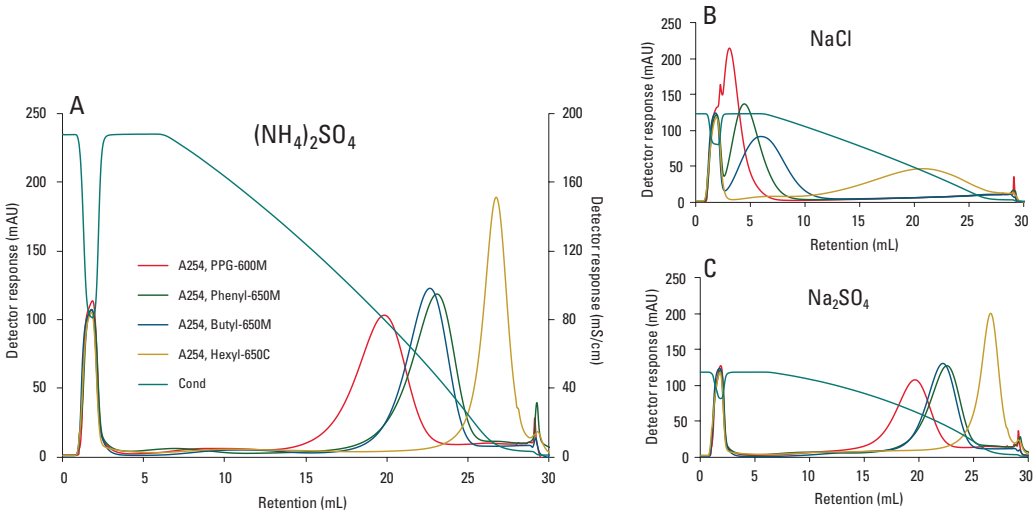


Figure 2. Impact of varying salt conditions on HIC oligonucleotide purification.

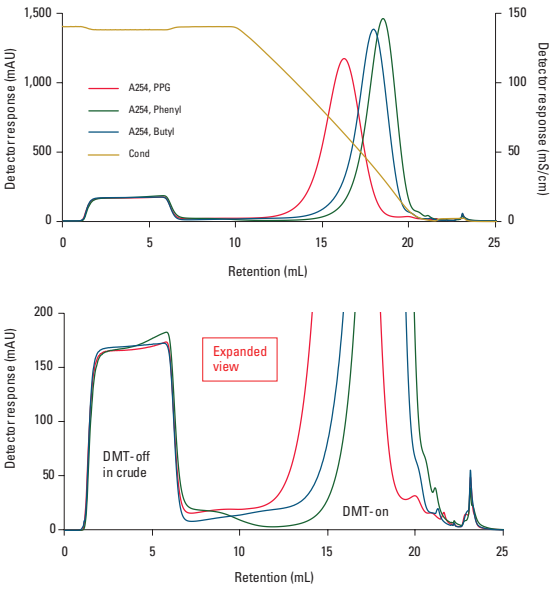


Media:	TOYOPEARL PPG-600M, Phenyl-650M, Butyl-650M, or Hexyl-650C
Column:	5 mm ID × 5 cm (1 mL)
Mobile phase:	A: 20 mmol/L Tris, 1 mmol/L EDTA, 1.5 mol/L salt as indicated, pH 7.5, B: 20 mmol/L Tris, 1 mmol/L EDTA, pH 7.5
Gradient:	0 - 100% B, 20 mL, 100% B, 4 mL, columns were washed with 5 CV of 30% isopropyl alcohol between runs
Flow rate:	1.0 mL/min (1 min residence time)
Detection:	UV @ 254 nm (mAU), conductivity (mS/cm)
Temperature:	ambient
Injection vol.:	1 mL (0.1 mg/mL load ratio)
Sample:	unpurified DMT-oligo, 0.1 mg/mL

Impact of varying salt conditions on purification

Prior to the resin screening process, different salts were used to determine the salt tolerance limit for the oligonucleotide that will be purified. Different concentrations of salts added to the sample were tested in order to verify the concentration at which precipitation occurs: 0.5 mol/L, 1.0 mol/L and 1.5 mol/L. Experimental results (data not shown) indicated that at 1.5 mol/L salt concentration, the oligonucleotide was bound strongly to all of the HIC resins without sample precipitation. Therefore, 1.5 mol/L salt concentration was selected for use in subsequent studies.

Figure 3. Comparison of different TOYOPEARL HIC resins.



In this study, 3 different salts were screened: sodium chloride (NaCl), sodium sulfate (Na_2SO_4) and ammonium sulfate ($(NH_4)_2SO_4$). *Figure 2*, panels A and C, show that $(NH_4)_2SO_4$ and Na_2SO_4 generated very similar oligonucleotide peaks. In fact, all HIC resins gave similar elution profiles and all selected resins effectively separated a DMT-off (flow-through peak) from a DMT-on oligonucleotide. In addition, the data also showed that TOYOPEARL Phenyl-650M and Butyl 650M bound the oligonucleotide stronger compared to the TOYOPEARL PPG-600M resin. TOYOPEARL Hexyl-650C showed the strongest binding, therefore, the oligonucleotide peak was eluted at the latest retention time. It was also noticed that NaCl (*Figure 2*, panel B) did not provide strong binding for the oligonucleotide on the resin, thus the reason why the oligonucleotide was eluted very early at the beginning of the decrease of the salt gradient.

It is known that sulfates of sodium and ammonium are most effective at promoting ligand-oligonucleotide interactions and are known to have little destructive effect on sample structure. Ammonium sulfate demonstrated a more linear conductivity response when used in a gradient (compare *Figure 2*, panel A to panel C) and thus was chosen for these studies.

Effect of HIC stationary phase on purification

After the initial resin screening, TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M were selected for the loading study consisting of both a DMT-off and DMT-on oligonucleotide. These resins were selected because they generated similar peak elution profiles under similar gradient conditions (*Figure 3*). TOYOPEARL PPG-600M eluted the DMT-on oligonucleotide earlier than the TOYOPEARL Phenyl-650M and Butyl-650M resins.

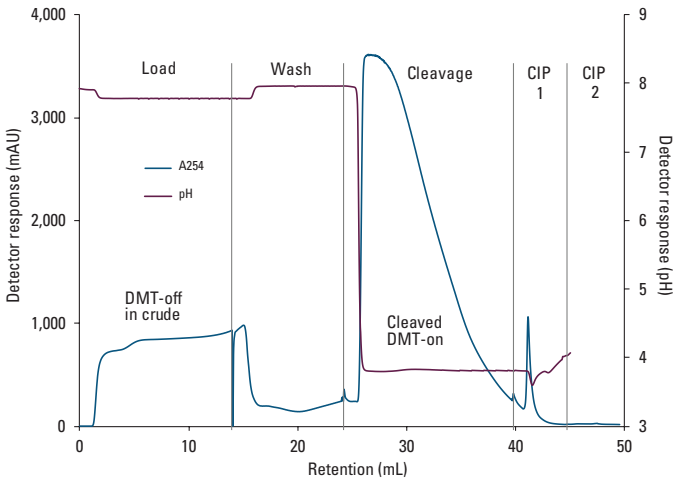
The expanded view chromatogram indicated that the DMT-off oligonucleotide was not bound to any of the three resins and was eluted in the void peak, whereas, the DMT-on oligonucleotide was bound to all three resins and eluted when the salt concentration was decreased. TOYOPEARL PPG-600M did not bind the DMT-on oligonucleotide as strongly as the other two resins. High resolution and baseline separation between DMT-off and DMT-on oligonucleotide peaks were provided by TOYOPEARL Phenyl-650M and Butyl-650M.

Table 1 demonstrates that TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M gave similar performance in the purity (99%) and recovery (89%) of a DMT-on oligonucleotide as determined by reversed phase HPLC.

Table 1. Summary of purity and recovery.

Fraction	Fraction Volume (mL)	Avg. conductivity (mS/cm)	Purity (% DMT-on)	Recovery (% DMT-on)
Load			77.9	
PPG elution	4.9	68.0	98.7	89.1
Butyl elution	4.5	45.9	99.0	89.0
Phenyl elution	4.2	36.8	99.0	88.9

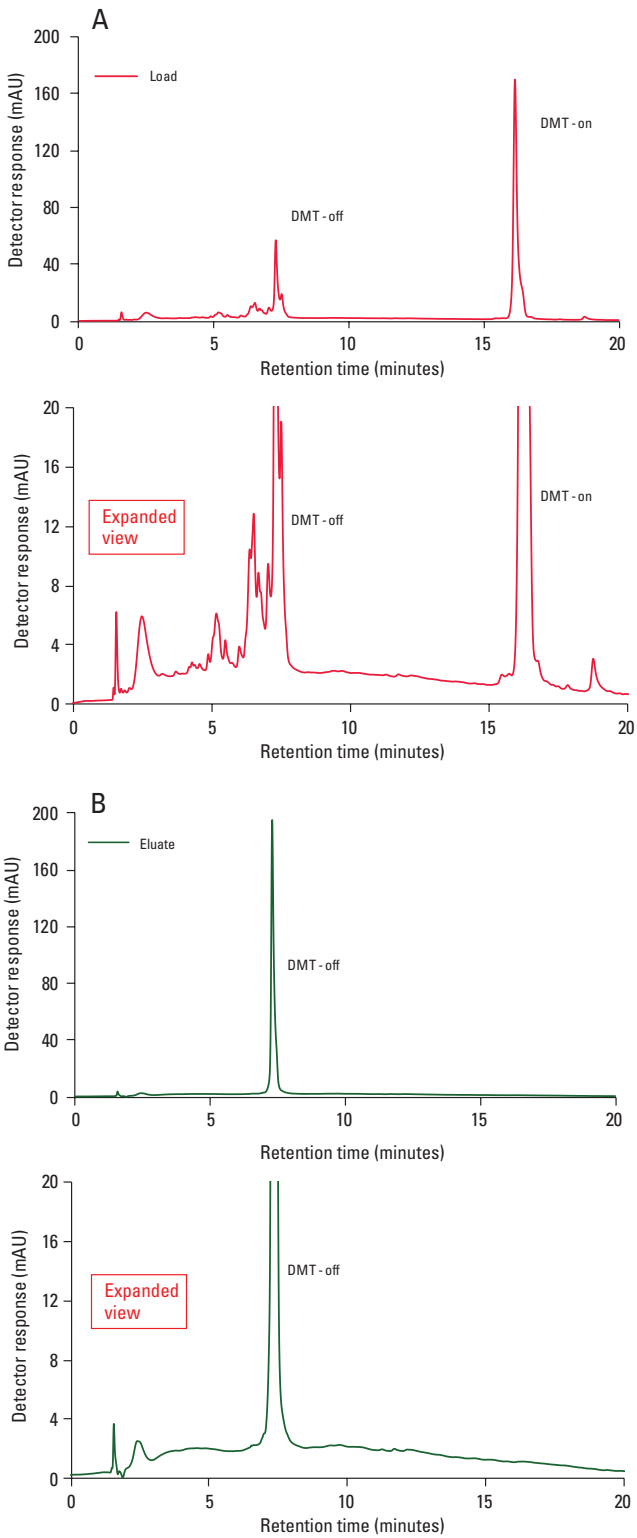
Figure 4. Cleavage of DMT-group directly on column.



Media: TOYOPEARL Phenyl-650M
Column: 6.6 mm ID × 3.0 cm (1 mL)
Flow rate: 0.25 mL/min (4 min residence time)
Detection: UV @ 254 nm (mAU), pH
Temperature: ambient

Phase	Volume (mL)	Buffer
Equilibrium	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Load	14	DMT-oligo, 0.5 mg/mL (7 mg)
Wash	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Cleavage	15	50 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$
CIP 1	5	water
CIP 2	5	30% (v/v) 2-propanol

Figure 5. Cleavage of DMT-group directly on column.



Column: TSKgel OligoDNA-RP, 4.6 mm ID × 15 cm
Mobile phase: A: 100 mmol/L TEAA, pH 7.0
B: acetonitrile
Gradient: 5 - 35% B, 20 min
Temperature: 45 ˚C
Flow rate: 1.25 mL/min
Detection: UV @ 254 nm (mAU)
Injection Vol.: 10 µL

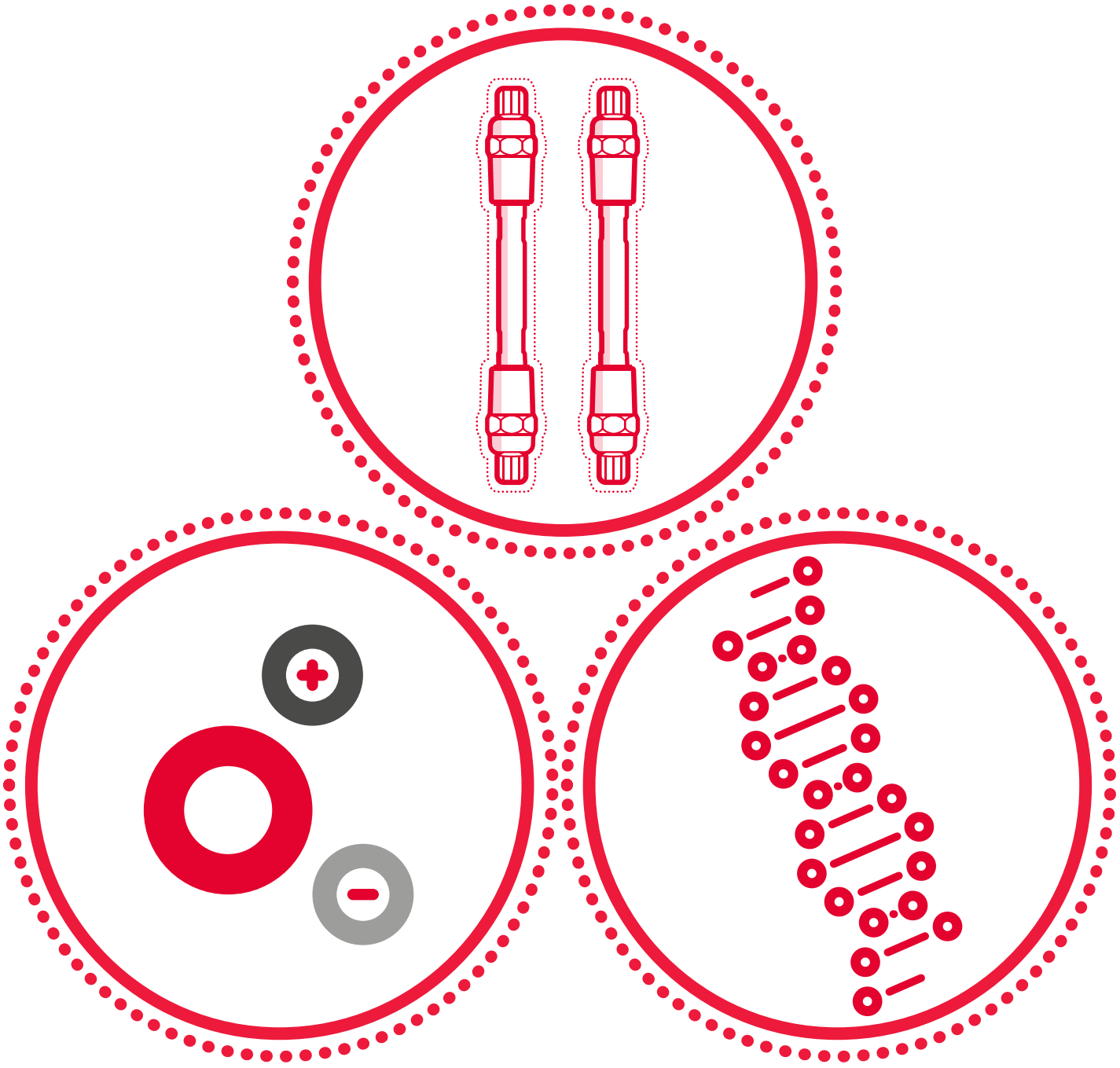
One-step removal of DMT protection group
For the on-column removal of the DMT-group from the oligonucleotide, TOYOPEARL Phenyl-650M was chosen for further study as a representative of the screened TOYOPEARL HIC resins. *Figure 4* shows that removal (cleavage) of the DMT-group from the oligonucleotide was successfully accomplished directly on the column. DMT was cleaved by acidification at approximately pH 4 and the DMT-off oligonucleotide eluted.
The DMT-group was removed from the stationary phase during cleaning-in-place (CIP) of the column.

Figure 5 shows the analysis of a crude oligonucleotide sample (panel A) and the on-column cleaved former DMT-on oligonucleotide fraction from *Figure 4* (panel B) using a TSKgel® OligoDNA-RP HPLC column. Data confirms that the DMT-group was effectively removed from the oligonucleotide and that on-column DMT cleavage resulted in a > 99% pure DMT-off oligo-nucleotide at 99% recovery.

Conclusion

TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M hydrophobic interaction chromatography resins are effective in separating DMT-on and DMT-off oligonucleotides in a crude preparation. On-column cleavage at low pH was used effectively to remove a DMT-group and elute a DMT-off oligonucleotide. High purity and recovery were achieved for an on-column cleavage procedure.

CHARACTERIZATION



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Application Note

Optimization parameters for anion exchange analysis of oligonucleotides

The development of oligonucleotide therapeutics is on the rise since the first approval in 1998. The 15-25 nucleotide long single or double-strands are designed to interact with a specific RNA sequence and thus modulate mRNA splicing or stability[1]. In order to do so, the sequence and length of oligonucleotide therapeutics are essential. However, due to errors in the oligonucleotide synthesis process, nucleotides may either be missing (N-x) or are attached in excess (N+x). The addition or lack of a nucleotide goes along with an additional or missing phosphate/phosphorothioate changing the overall negative charge of the molecule, which makes anion-exchange a method to analyze N+/-x impurities.

This application note demonstrates how to optimize a (U)HPLC method for oligonucleotide analysis by modifying the buffer composition and temperature. This helps adjusting the method to the need of individual oligonucleotides and results in a universal method serving as a starting point for in-depth method optimization. A single-stranded oligonucleotide was employed as an exemplary sample and optimization was performed on a TSKgel® DNA-NPR anion exchange column. With its small, non-porous particles with a weak anion exchange ligand, it is well suited for fast nucleic acid analysis.

Material and Methods

Column:	TSKgel DNA-NPR (4.6 mm ID x 7.5 cm)
UHPLC:	Thermofisher Dionex Ultimate 3000 UHPLC system
Mobile phase:	A: 10 mM NaOH pH 12 or 20 mM Tris-HCl pH 8 as indicated B: 10 mM NaOH pH 12, 2 M NaCl or 0.8 M NaBr as indicated
Gradient:	0 min (0% B) 3 min (0% B) 16 min (100% B)
Flow rate:	0.5 mL/min
Detection:	UV @ 260nm
Injection vol.:	5 µL
Temperature:	25 ° C if not stated differently
Sample:	ssDNA full phosphorothioate

Results

The separation of an oligonucleotide sample was tested at pH 8 and 12 (*Figure 1*). At the more basic pH of 12, the major product as well as shorter impurities (eluting earlier) and longer ones (eluting later) were separated. In contrast, these sample components were hardly separated at lower pH. This is explained by reduced ionization of the oligonucleotide at the given pH of 8.

Further experiments were, hence, conducted at pH 12.

Figure 1. Influence of pH on the analysis of oligonucleotides by AEX.

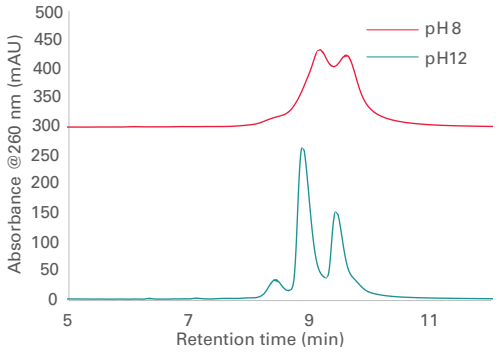


Figure 2. Influence of the temperature on the characterization of oligonucleotides by AEX.

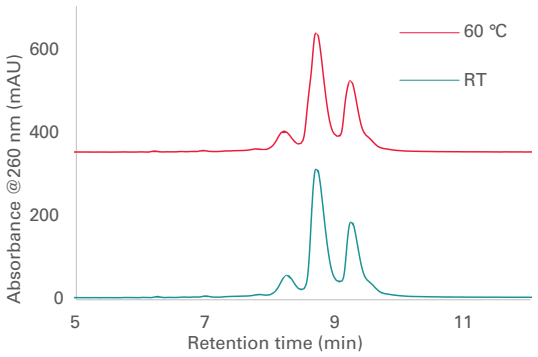


Figure 3. Influence of the type of salt on analysis of oligonucleotides by AEX.

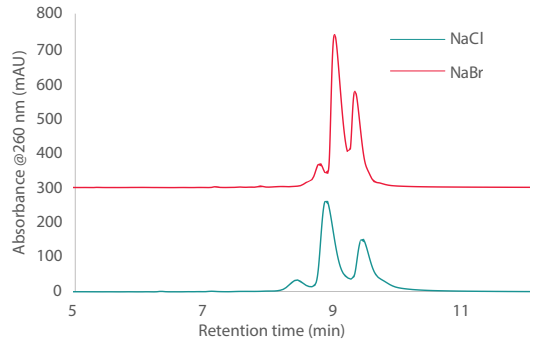


Figure 2 shows the analysis of an oligonucleotide at room temperature (25 °C) and 60 °C. For both conditions, the major product, shortmers, and longmers were separated. No difference in retention was observed for the analyzed oligonucleotide.

As higher temperature resolves secondary structures when present or -as in our example- have no negative effect, a universal method would still be performed at 60 °C.

The effect of sodium chloride and the less ionic and more soluble sodium bromide on oligonucleotide separation was tested. Due to a better elution, a lower concentration for NaBr (0.8 M) was employed, allowing for evaluation of selectivity differences. Both salt types separated the major product from smaller and longer impurities. While sodium chloride resulted in broader peaks, sodium bromide led to sharper peaks but reduced resolution (*Figure 3*). Further gradient optimization could potentially lead to a better separation with both salts.

The addition of 5 % acetonitrile decreased the retention time and led to peak-sharpening to both mobile phases (NaCl) (*Figure 4* when eluting with NaCl). This is due to decreased hydrophobic interactions between phosphorothioates and the stationary phase in the presence of organic modifiers.

Figure 4. Influence of 5 % acetonitrile (ACN) on analysis of oligonucleotides by AEX.

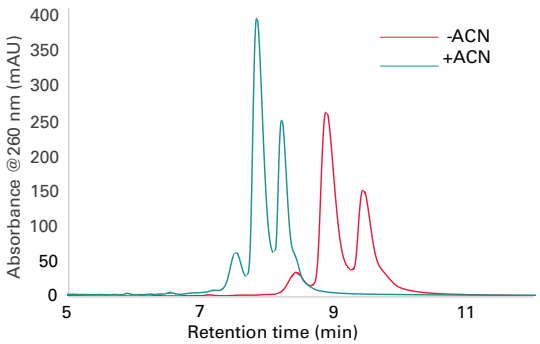
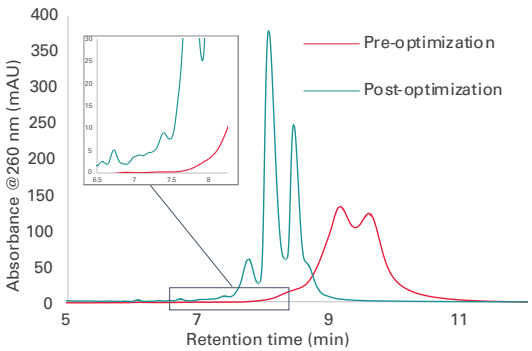


Figure 5. Result of optimization process.



Conclusion

We investigated how various conditions influence oligonucleotide analysis by anion-exchange chromatography. By modifying the buffer conditions (i.e., salt type, organic modifier addition, and temperature), we developed a universal method, which is a good starting point for oligonucleotide method development (A: 10 mM NaOH, 5% ACN, pH12, B: 2M NaCl, 5% ACN, *Figure 5*).

The TSKgel® DNA-NPR anion exchange column has proven ideal for this application as it is stable at high pH and high temperatures, which is required for oligonucleotide analyses to prevent formation of secondary structures. In addition, the non-porous base beads offer fast mass transfer and consequently an analysis in less than 15 min.

Reference

1) Dhuri K *et al.* (2020) Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development. J Clin Med. 2020 Jun; 9(6): 2004. doi: 10.3390/jcm9062004

Table 1. Featured product.

P/N	Description	Dimension
0018249	TSKgel DNA-NPR	4.6 mm ID x 7.5 cm L

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Application Note

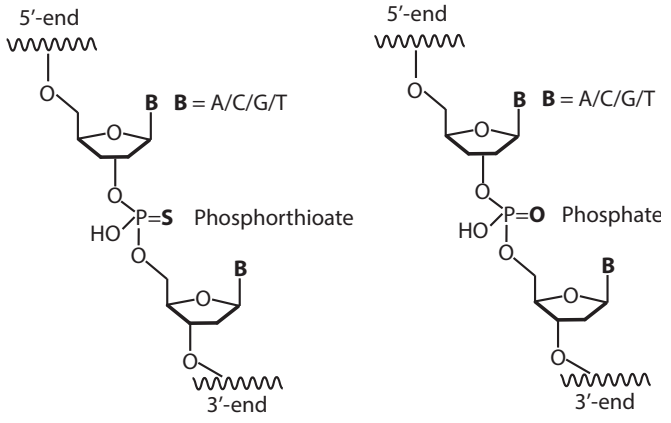
Anion exchange analysis of unmodified and phosphorothioate oligonucleotides

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 exchanges a non-bridging oxygen with a sulfur (*Figure 1*). Oligonucleotides exhibit this modification either at some or all nucleotides. According to this, phosphorothioate modified oligonucleotides potentially exhibit additional impurities: insufficient or excessive modification, or wrong positioning of the modification. These comes 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). 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

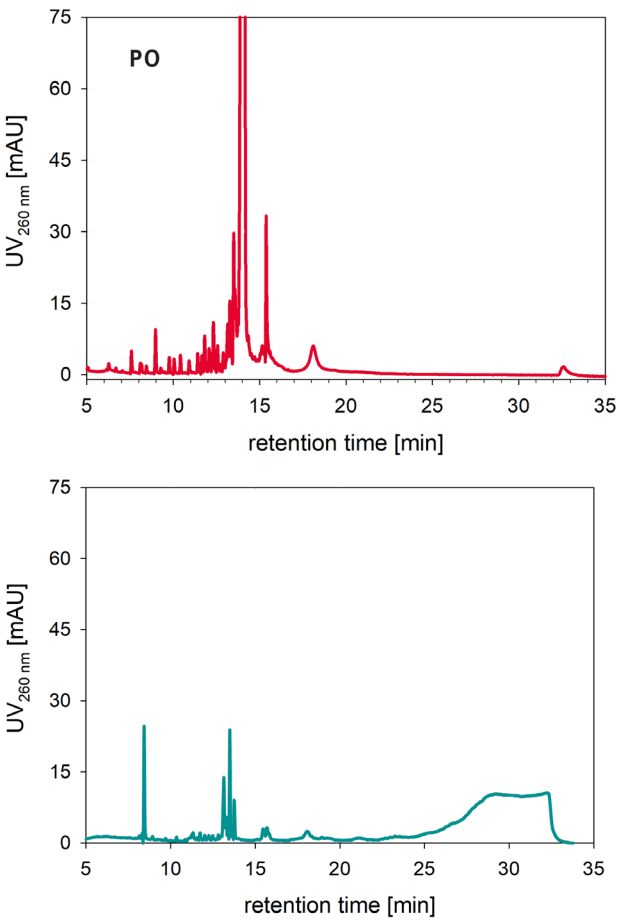
For our experiment we used the TSKgel DNA-NPR column as it offers features dedicated for nucleic acid analyses. This comprises a polynucleotide-based quality control as well as non-porous 2.5 µm particles to guarantee fast but high resolution separation.

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
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 NaCl
Mobile phase: 'thioate'	A: 10 mmol/L NaOH in MilliQ water; pH 12.0; B: A+ 2 mol/L NaCl
Flow rate	0.5 mL/min
Linear gradient:	0% A – 100% B in 10 Column volumes V (0-25 min)
CIP step:	3 mol/L NaCl; 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.



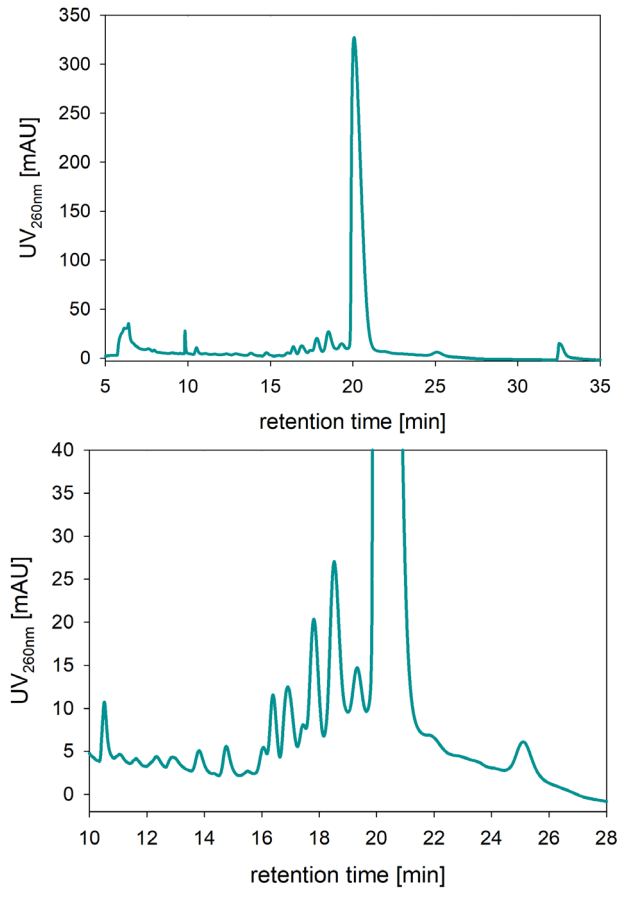
A 20mer ssDNA (a) and a phosphorothioate modified ssDNA oligonucleotide (17mer) (b) were separated on a TSKgel DNA-NPR column with the following conditions: 20 °C, mobile phase A: 50 mmol/L TRIS-HCl; pH 9.0, mobile phase B: (A + 1 mol/L NaCl), linear gradient over 10 column volumes (25 min).

Results & 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 NaCl. 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 of being shorter. This leads to late retention times and the bulk eluting without resolution of different species at 25-33 min (*Figure 2b*).

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 NaCl) 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.

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 NaOH pH 12* 10% ACN, 60°C*, mobile phase B: (A + 2 mol/L NaCl) linear gradient: 10 CV.
**pH and temperature were increased to resolve possible secondary structures*

The relatively broad peaks may stem from numerous diastereomers (for the analyzed 17mer full thioate up to 216 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. One the one hand, the higher hydrophobicity of PS ONs 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)

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

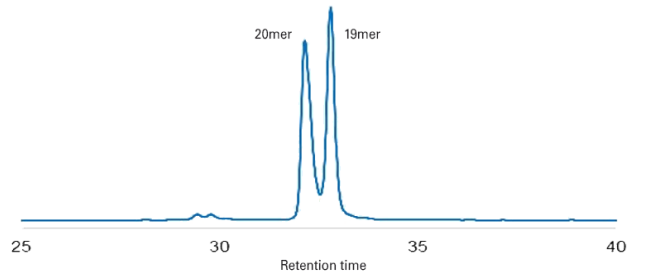
TSKgel UP-SW2000 is a newly developed silica-based 2 µm, 12.5 nm pore size SEC column designed for the separation of small proteins, peptides, and oligonucleotides. The column can be used in both, HPLC and UHPLC systems and is ideally suited for method transfer from conventional silica-based size exclusion 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, 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.

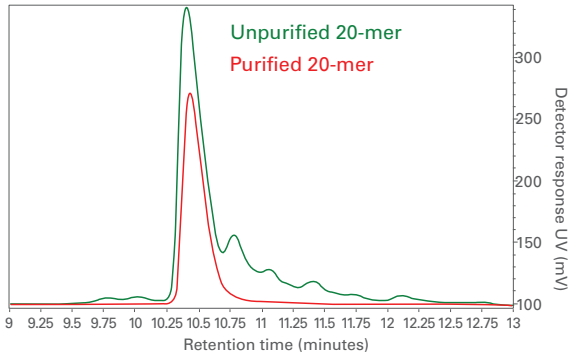


Figure 3. Molecular weight distribution (green) of the unpurified 20-mer.

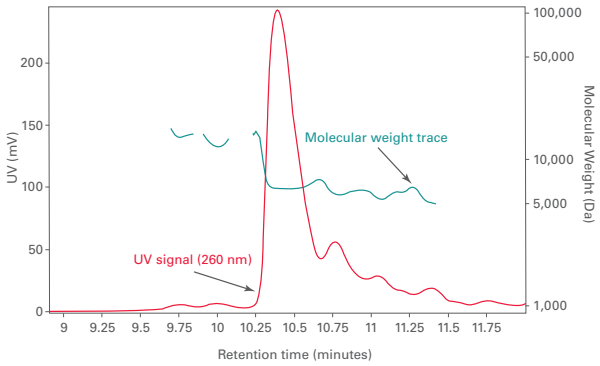


Figure 4. Peak analysis of the unpurified 20-mer.

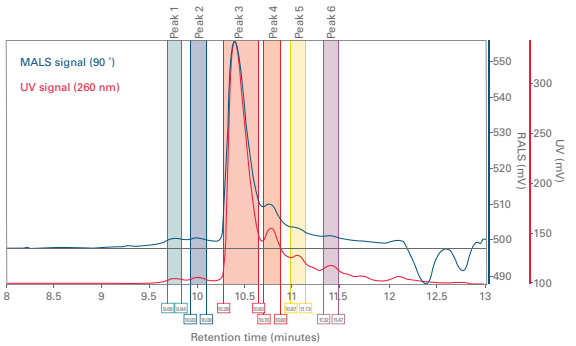


Figure 3 shows the molecular weight distribution of the unpurified 20-mer. The molecular weight trace clearly indicates the presence of higher and lower molecular weight impurities.

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.

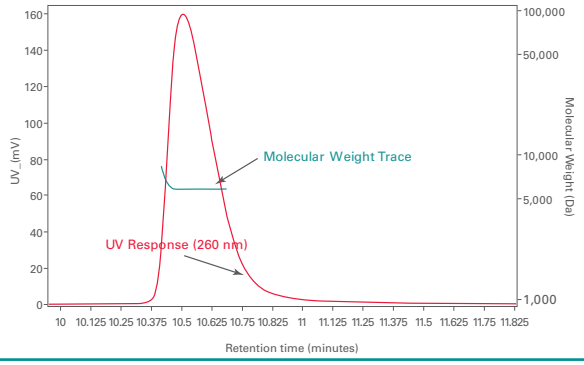


Table 2. Reproducibility of retention time.

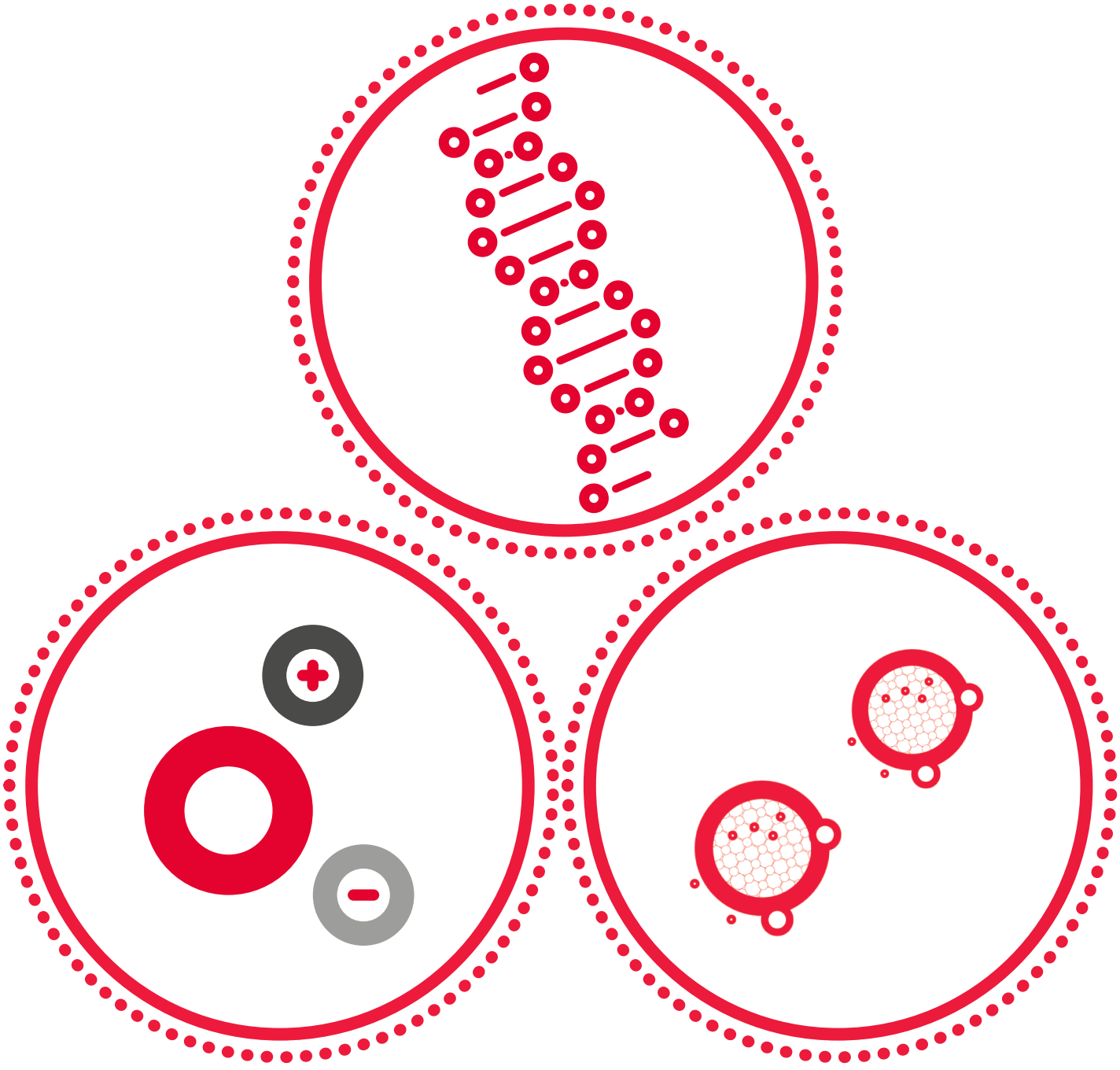
Injection	Retention time (min)	MW (Da)
1	10.431	6,066
2	10.443	6,023
3	10.445	6,038
Average	10.440	6,042
% RSD	0.1%	0.3%

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.

RELATED APPLICATIONS



TOSOH

Application Note

Rapid Analysis of Plasmid Topoisomers by Anion Exchange Chromatography

Recombinant plasmid DNA (pDNA) is increasingly used as a raw material in gene therapy (e.g. in lentiviral and AAV vector production) and as an active ingredient for DNA vaccination. Pharmaceutical grade plasmid DNA must meet specifications concerning both host-related-impurities as well as homogeneity (i. e. the content of pDNA topoisomers (ccc, oc, lin) and di- or multimeric variants). During large-scale plasmid fermentation, plasmids are maintained predominantly in a supercoiled, covalently closed circular form, during downstream process some of the plasmids might become nicked and they will be transformed in open-circular and linear forms.

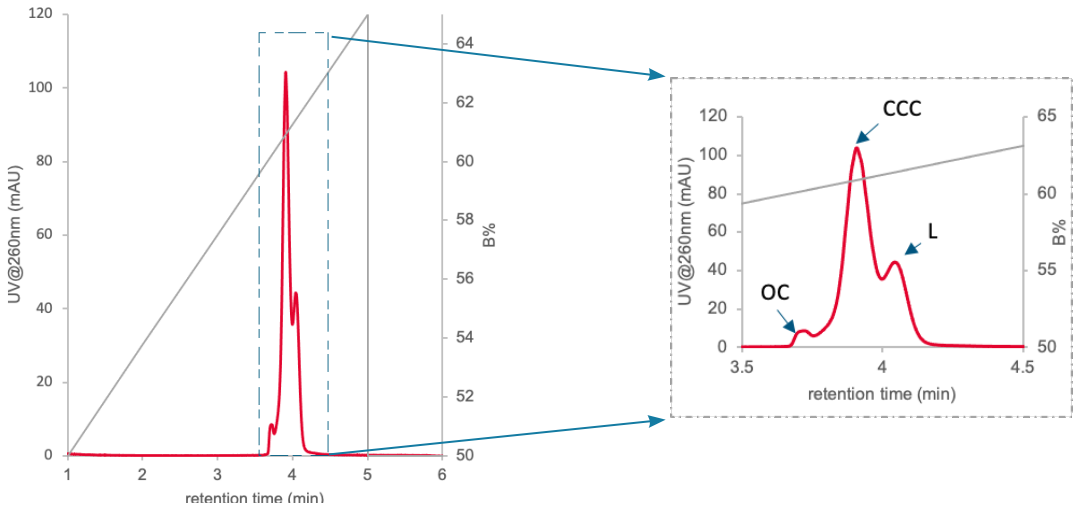
For a fast and reliable characterization of pDNA samples Schuchnigg et al. developed an HPLC method with a high resolving power based on the TSKgel DNA-NPR anion exchange HPLC column[1]. TSKgel DNA-NPR is packed with 2.5 µm hydrophilic non-porous polymer beads modified with a weak anion exchange group. The non-porous particle offers fast mass transfer, a key to achieve high resolution. The small particle size and the fast mass transfer of non-porous beads can be exploited to speed up the analysis. By using pBR322, one of the first widely used *E. coli* cloning vectors, we demonstrate that the method can be transferred from HPLC to UHPLC systems.

Material and Methods

Column:	TSKgel DNA-NPR, 2.5 µm, 4.6 mm ID x 7.5 cm (*P/N 0018249*)
Mobile Phase	A: 20 mmol/L Tris/EDTA pH 9.0 B : A + 1.0 mol/L sodium chloride
Gradient:	50 % B to 65% B in 5 min
Flow rate:	1.0 mL/min
UHPLC system:	Ultimate®3000RS
Detection:	UV @ 260 nm
Temperature:	25 °C
Inj. vol:	5 µL
Sample:	pBR322 (NEBiolabs)

The plasmid pBR322 has a length of 4,361 base pairs, which results in a molecular weight of 2.83 x 10^6 Da. In order to classify the topoisomers properly, the linear form was prepared by incubation with the single-cutting restriction Enzyme EcoRI (NEB). pBR322 has a single EcoR1 restriction site at position 4359. For the digest, one unit of restriction enzyme per µg DNA was used. The sample was incubated at 37°C for 60 min. The reaction was stopped by heating to 65°C for 20 min.

Figure 1. AEX analysis of pBR322 topoisomers on TSKgel DNA-NPR.



Results

The chromatographic analysis of pBR322 on TSKgel DNA-NPR is shown in *Figure 1*. The plasmid can be analyzed within a 5 min linear gradient from 50% to 65% mobile phase B at a flow rate of 1.0 mL/min. The plasmid elutes in three peaks representing the different species, supercoiled, open circular, and linear. The highest peak corresponds to the supercoiled plasmid.

Figure 2. AEX analysis of linear EcoR1 digested pBR322 on TSKgel DNA-NPR

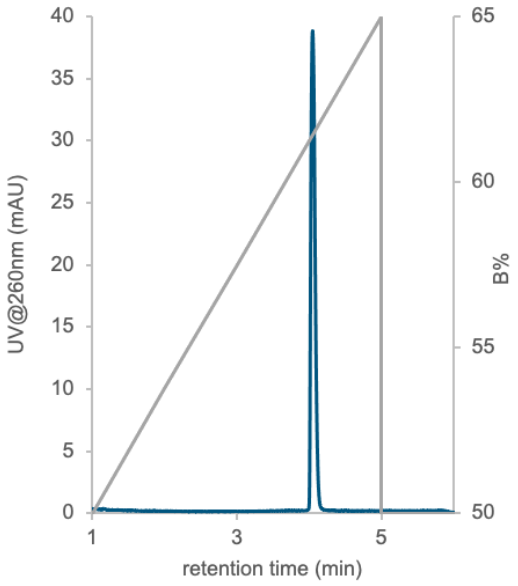
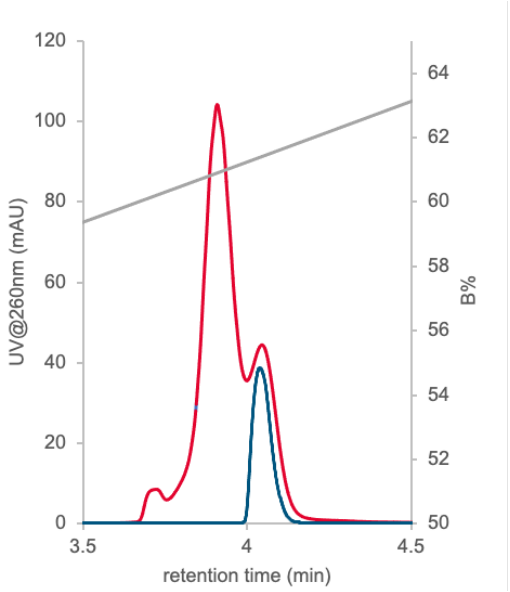


Figure 3. Overlay of pBR322 (oc, ccc, linear) and linear pBR322 analyzed by anion exchange chromatography.



The linear form of the plasmid was analyzed at the same conditions as the plasmid. *Figure 2* shows the corresponding chromatogram with a single peak, representing the EcoR1 digested linear form. *Figure 3* shows an overlay of the two chromatograms that confirms that peak three corresponds to the linear form.

Conclusion

Analytical ion exchange chromatography on a TSKgel DNA-NPR column offers a simple and rapid method for discriminating between covalently closed circular (CCC), open circular (OC), and linear (L) forms of plasmid DNA. Schuchnigg et al. showed that elution pattern changes with plasmid size. Therefore, it is recommended to confirm the position of the peak representing the linear form. The method is ideally suited to be used at various stages of pDNA R&D and manufacturing for pharmaceutical purposes and can be used in both, HPLC and UHPLC systems.

References

1.) Characterization of Plasmid DNA Samples by Chromatogrpraphic Methods; Hermann Schuchnigg, Patricia Cantarell, Christoph Pollak, Jochen Urthaler and Wolfgang Buchinger; Boehringer Ingelheim Austria GmbH, Poster HPLC 2008, Baltimore, MD, USA

TOSOH

Application Note

Molecular Weight Determination of VLPs Using LenS3 Multi-Angle Light Scattering Detector

Viruses and virus like particles (VLPs) are multimeric protein structures that mimic native viruses but are non-infectious. VLPs are subjects of interest, as their potential continues to grow as candidates in new vaccines and gene therapy products. For example, commercially available VLP-based vaccines are available for Hepatitis B and human papillomavirus. Robust analytical techniques are needed to not only ensure quality of final products but provide data for informed decision-making during the development process.

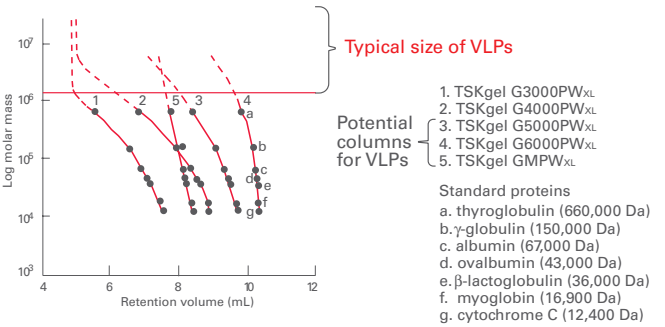
Size exclusion chromatography (SEC) is an analytical technique that provides results on the size and purity of macromolecules. When coupled with multi-angle light scattering (MALS), it offers both molecular weight (MW) and radius of gyration (Rg or size). Importantly, AU280 detection is only concentration dependent, whereas MALS corresponds to both concentration and molecular weight. Thus, the large molecular weight characteristic of VLPs inherently provides MALS with a strong scattered light response and enables VLP detection even in a dilute solution that is well below AU280 detection limit.

The primary challenge in the analysis of very large macromolecules by SEC is the selection of the appropriate analytical column. Here we explore TSKgel® PWXL series of SEC columns, which include a wide range of different pore sizes on a polymethacrylate stationary phase, for their utility in the analysis of large macromolecules such as VLPs. The protein calibration curves (*Figure 1*) show the separation range of TSKgel PWXL columns. The majority of VLPs have a molecular weight of 1 mega Daltons, which make the TSKgel G5000PWXL (100 nm pore size), TSKgel G6000PWXL (>100 nm pore size) and TSKgel GMPWXL (mixed bed) ideal columns of choice for analysis of VLPs.

Material and Methods

Columns:	TSKgel GMPWXL, 13 µm, 7.8 mm ID × 30 cm (P/N 0008023) TSKgel G5000PWXL, 10 µm, 7.8 mm ID × 30 cm (P/N 0008025)
Instrument:	Thermo Scientific UltiMate® 3000
Mobile phase:	0.145 mol/L NaCl, 0.01 mol/L HEPES, 0.05% sodium azide, pH 7.4 (refractive index, 1.333)
Flow rate:	0.3 mL/min or as indicated
Detection:	UV: UltiMate 3000 multiple wavelength detector RI: Shodex RI-504 semi-micro RI detector MALS: LenS3 MALS detector
Sample:	Parvovirus VLP (MVM-MVP) (Cygnus Technologies), stock 1 × 10^{12} particles/mL (10-15 µL injection), (dn/dc = 0.19, dA/dc = N/A)
MALS calibrant:	BSA, 5 mg/mL (dn/dc = 0.185, dA/dc = 0.66)

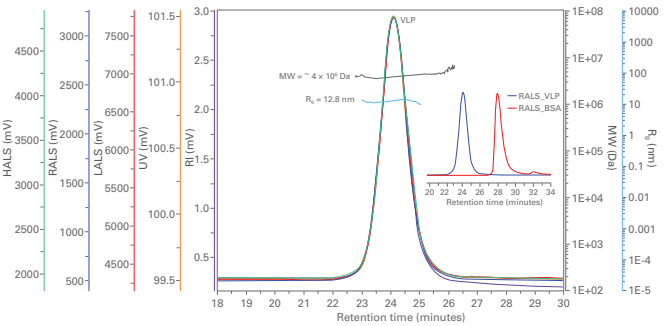
Figure 1. Protein calibration curves on TSKgel PWXL.



In this application, parvovirus VLP was separately analyzed on both a TSKgel GMPWXL and TSKgel G5000PWXL SEC column coupled with the LenS3 MALS detector. Either RI or UV can function as the concentration detector. RI was used with the right angle light scattering signal (RALS) to measure MW. Extreme low angle (LALS), right angle, and extreme high angle (HALS) signals were used to plot angular dissymmetry and to determine R_g. The MALS detector was calibrated with BSA prior to sample analysis and all data were processed and analyzed using SECview® software.

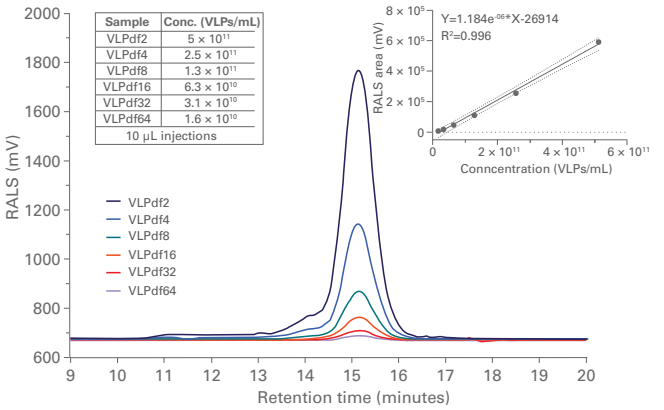
Analysis of parvovirus VLP by SEC-MALS using the TSKgel GMPWXL column revealed a MW of ~4 mega Daltons and Rg of 12.8 nm (*Figure 2*). These results closely align with reported values for this VLP (Biotech. Prog. 34, 1213-1220, 2018).

Figure 2. Analysis of parvovirus VLP and BSA on TSKgel GMPWXL mixed bed pore size SEC column.



As seen in *Figure 3*, parvovirus VLP was diluted up to 64-fold and injected at 10 µL onto a TSKgel G5000PW$_{XL}$ column. Approximately 3 × 10^{10} particles per mL can still be detected using the RALS signal from the LenS$_3$ MALS detector, which allows for analysis of materials with low concentration or when working with limited sample.

Figure 3. Limit of detection by RALS using TSKgel G5000PW$_{XL}$ at 0.5 mL/min.



Conclusion

Mass spectrometry is the most common method previously used for VLP size determination, but this technique is costly and impractical for frequent analysis. Inclusion of SEC-MALS as an analytical technique to determine the MW and Rg is a preferred alternative and allows for both routine analysis and process monitoring. The wide range in pore sizes and separation ranges of TSKgel PW$_{XL}$ SEC columns overcome challenges in analytical SEC where separations of large macromolecules require a larger pore sized stationary phase. When these SEC columns are then combined with the greatly enhanced sensitivity of Tosoh Bioscience's LenS$_3$ MALS detector, fast and easy analysis of MW and R_g with an improved level of detection (LOD) is provided.

TOSOH BIOSCIENCE

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