TOSOH

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.

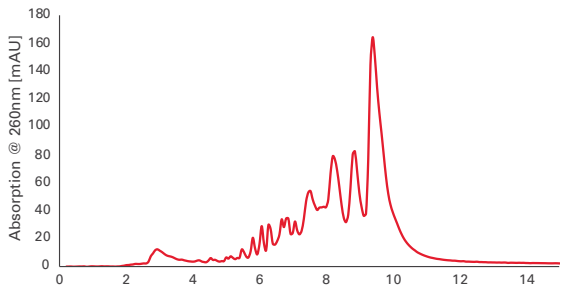
Due to errors during the oligonucleotide synthesis process, nucleosides may either be missing (N-X) or are attached in excess (N+X). Moreover, the chirality of the sulfur atoms in the backbone of ONs (due to the phosphorothioate modification) leads to diastereomers. To remove these impurities, the biopharma 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 L) |
| UHPLC: | Thermofisher Dionex Ultimate 3000 UHPLC system |
| Mobile phase: | A: 10 mmol/L NaOH, pH 12.0<br>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

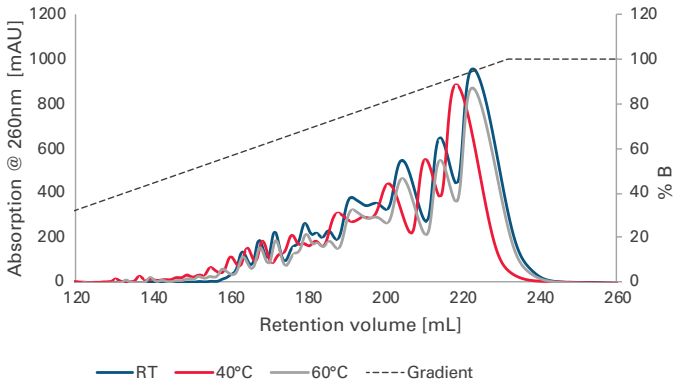
To develop a robust preparative method to purify oligonucleotides, we tested the performance of the TSKgel SuperQ-5PW resin at a 1mg/mL loading at different temperatures and buffer compositions.

## Material and Methods

| | |
|---|---|
| Column: | 0.66 cm ID x 20 cm L |
| Mobile phase: | A: 10 mmol/L NaOH, pH 12.0<br>B: 2.0 mol/L NaCl |
| Additives: | 5% Acetonitrile (ACN) or 10 mM 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.

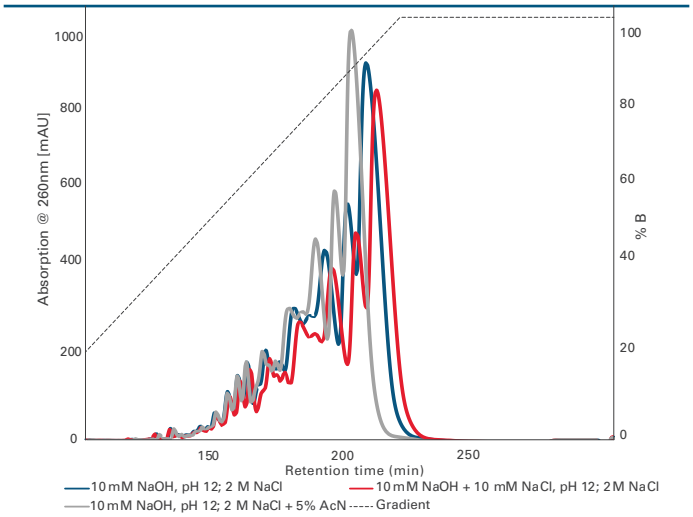



Table 1. Oligonucleotide purity and recovery from AEX purification at different temperatures and buffer conditions. Fractions with a main peak purity > 80 % were pooled for recovery and purity determination.

| 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 mM sodium hydroxide buffer at pH12 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 2. 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 L |
| 0045244 | SkillPak SuperQ-5PW (20) 5 mL col. | 8 mm ID x 10 cm L |
| 0043383 | TSKgel SuperQ-5PW (20) | 25 mL |
| 0018249 | TSKgel DNA-NPR | 4.6 mm ID x 7.5 cm L |