Preparative Anion Exchange Chromatography for the Purification of Phosphorothioate DNA Oligonucleotides

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Abstract

Oligonucleotide (ON)-based therapies are a focus of biopharma development, but economical downstream bioprocessing of these therapeutics can be challenging. Incomplete or erroneous sequences of ONs during solid-phase synthesis are key impurities that must be removed before these molecules can be used as therapeutics. Typically, anion exchange (AEX) chromatography can be utilized to remove length-based impurities due to the ON's high degree of negative charge. In this case study, preparative AEX chromatography with Nuvia HP-Q Resin from Bio-Rad Laboratories, Inc. was utilized for the purification of a fully phosphorothioated, single-stranded 20-mer DNA ON, with the specific goal of separating the desired ON from other length-based contaminants. Nuvia HP-Q Resin with a bead size of 50 µm was successfully used for crude preparation of a 20-mer, demonstrating its utility for efficient and scalable ON purification. Both small- and large-scale experiments with 5 and 25 ml columns, respectively, gave excellent yields (~82%) while maintaining high purity of the full-length ON (96–97%).

Introduction

ONs are considered an exciting new class of biopharmaceuticals for the treatment of human diseases and are regarded as the third distinct drug discovery platform after small molecule and protein therapeutics. ON-based drug platforms function through a variety of specific molecular mechanisms designed to modulate the processing of genetic information. Single-stranded antisense DNA ONs represent an important therapeutic subclass and tend to have lengths of 15–25 nucleotides. To increase their general biostability and therapeutic efficiency in terms of improved distribution and potency, ONs are commonly chemically modified at the base, sugar, or phosphate backbone. The development of suitable chemical ON modifications has proven to be very successful and is a highly innovative research field (Prakash et al. 2014, Roberts et al. 2020).

Phosphorothioation is a very common chemical modification used to improve the resistance of antisense ONs to in vivo nuclease degradation. Here, the phosphate groups in the ON backbone are replaced by phosphorothioate (PS) groups (Figure 1), where one nonbridging oxygen of the internucleotide linkage is substituted by a sulfur atom. These PS-ONs were the first class of antisense therapeutics to get marketing approval by the U.S. Food and Drug Administration (FDA) in 1998.

The manufacture of PS-ONs is a multistep process based on phosphoramidite chemistry (Verma and Eckstein 1998). Although individual cycling efficiency is high (>98%), a low percentage of the ON chain often does not grow to the desired full-length product with a chain length of n. Sutton et al. have proposed to subdivide impurities into three high-level classes: (i) shortmers: deletion sequences (n–x); (ii) longmers: addition sequences (n+x); and (iii) modified full-length product (nm) having structural modifications (Sutton et al. 2020). Shortmers are regarded as the most abundant impurities after ON synthesis. In contrast, modified full-length products typically occur only at low levels and tend to be relatively structurally uniform. An excellent overview of possible impurity profiles of phosphorothioate ON therapeutics is given in a recent review (Rentel et al. 2022). A frequently observed structural impurity is caused by incomplete sulfurization during solid-phase synthesis and leads to the formation of full-length PS-ONs containing one $[(P = O)_1]$ or more $[(P = O)_n]$ phosphodiester linkages anywhere in the sequence. Removal of $(P = O)_1$ ON impurities during a preparative chromatographic purification scheme has been reported to be challenging; therefore, much effort has been made to increase sulfurization efficiency, which is now as high as 99.9% per linkage (Krotz et al. 2004). Furthermore, chemical synthesis

of PS-ONs produces a mixture of diastereomers because the replacement of a nonbridging oxygen by sulfur makes the phosphorus center chiral. For example, a 20-mer PS-ON contains 19 linkages and is thus a mixture of 524,288 diastereomers.

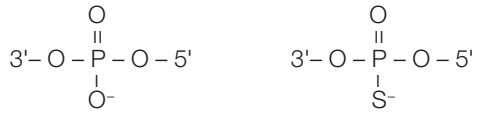


Fig. 1. Chemical structure of phosphodiester (left) and phosphorothioate (right). The replacement of one nonbridging oxygen with sulfur makes the phosphorus center chiral.

ONs are becoming more and more important as therapeutic agents, but interestingly there is still a lack of consensus among regulatory agencies on impurity reporting, identification, and qualification thresholds. According to Rupp and Cramer, regulatory agencies view ON therapeutics as "large small molecules" because they are manufactured by chemical synthesis, like small molecules, but also share properties with biologics (Rupp and Cramer 2022). The Oligonucleotide Safety Working Group (OSWG), a collaborative team from leading pharmaceutical companies, has addressed some of the analytical challenges of process-related impurities in two white papers to ensure ON quality throughout development, manufacturing, and release (Capaldi et al. 2017, Capaldi et al. 2020).

The cost-effective manufacture of therapeutic antisense PS-ONs depends on both a well-controlled chemical synthesis process and a highly efficient downstream processing strategy for impurity removal. A very successful purification approach for the large-scale production of antisense oligonucleotides (ASOs) is based on the combination of hydrophobic interaction chromatography (HIC) and AEX. HIC is usually applied for the initial purification of DMT (dimethoxytrityl)-on material, whereas AEX is performed on the HIC eluate after detritylation as a second polishing step (Andrews et al. 2021). This powerful two-step purification process was pioneered by Puma and colleagues in 1995 and provides a generalized scheme for economically purifying PS-ONs at process scale (Puma et al. 1995). AEX chromatography holds a key position in this workflow and easily separates standard ONs by their chain length, resulting in the removal of key impurities, such as deletion and addition sequences. This was recently demonstrated with Nuvia HP-Q Resin from Bio-Rad at a preparative scale (Posch et al. 2021). We now aim to extend these studies to the purification of fully thioated ONs, which are generally accepted to present an especially difficult challenge to any AEX resin at large-scale (Gerstner et al. 1995). In this report, we provide a careful evaluation of Nuvia HP-Q Resin for its applicability in a preparative PS-ON purification process.

Materials and Methods

Oligonucleotides

We used three custom-made, fully thioated, desalted, lyophilized single-stranded DNA ONs, as well as a phosphodiester analog of one of the ONs (Sigma-Aldrich) (Table 1). In this manuscript, we will refer to the thioated ONs as PS-ONs 1–3, and the phosphodiester analog as ON 2. PS-ONs 1–3 were used during method development, and PS-ON 2 was used for large scale purification experiments. For chromatography, the dried ON and PS-ONs were dissolved in buffers made with nuclease-free water.

Table 1. DNA oligonucleotides used in this study.

Name*	Length, nucleotides	Sequence (5'–3')	Molecular Weight, Da
PS-ON 1	19	ATA CCG ATT AAG CGA AGT T	6,125
PS-ON 2	20	ATA CCG ATT AAG CGA AGT TT	6,445
ON 2	20	ATA CCG ATT AAG CGA AGT TT	6,140
PS-ON 3	21	ATA CCG ATT AAG CGA AGT TTT	6,766

* PS-ONs 1–3 are fully thioated. ON 2 has a phosphodiester backbone.
PS-ON, phosphorothioate oligonucleotide.

Chromatography

Equipment and resins used for chromatography experiments are listed in Table 2. Chromatography was monitored at 260 and 280 nm and was performed at room temperature using an NGC Quest 10 Plus Chromatography System (Bio-Rad, catalog #7880003). The chromatography buffers were thoroughly degassed and filtered through 0.45 µm membranes (Sartolab RF 500, PES Vacuum Filtration Units for 500 ml Samples; Sartorius AG, #180F04). Chromatography operating conditions are presented in Figure 2. Samples from analytical and preparative runs were stored at 4°C and pH was not adjusted prior to analysis by electrophoresis.

Table 2. Overview of chromatography columns used for DNA PS-ON purification and fraction analysis, including operating and buffer conditions.

Column Type	Resin Type and Bead Size	Column Dimensions and Pack Type	Flow Rates, cm/hr	Application	Buffer A Composition	Buffer B Composition
Foresight Nuvia HP-Q Column, 5 ml (Bio-Rad #12007021)	Strong AEX 50 µm	8 x 100 mm prepacked	120–240	Method development	25 mM arginine-NaOH, pH 11.7	25 mM arginine-NaOH, 2 M NaCl, pH 11.7
Nuvia HP-Q Resin, 25 ml (Bio-Rad #12006693)	Strong AEX 50 µm	25 x 100 mm (packed dimensions: 25 x 50 mm) Self-packed in an Econo Alpha Column (Bio-Rad #12009461)	100–120	Large scale purification		
ENrich Q 5 x 50 Column, 1 ml (Bio-Rad #7800001)	Strong AEX 10 µm	5 x 50 mm prepacked	300	Fraction purity analysis		
ENrich Q 10 x 100 Column, 8 ml (Bio-Rad #7800003)	Strong AEX 10 µm	10 x 100 mm prepacked	300	Fraction purity analysis		

AEX, anion exchange; PS-ON, phosphorothioate oligonucleotide.

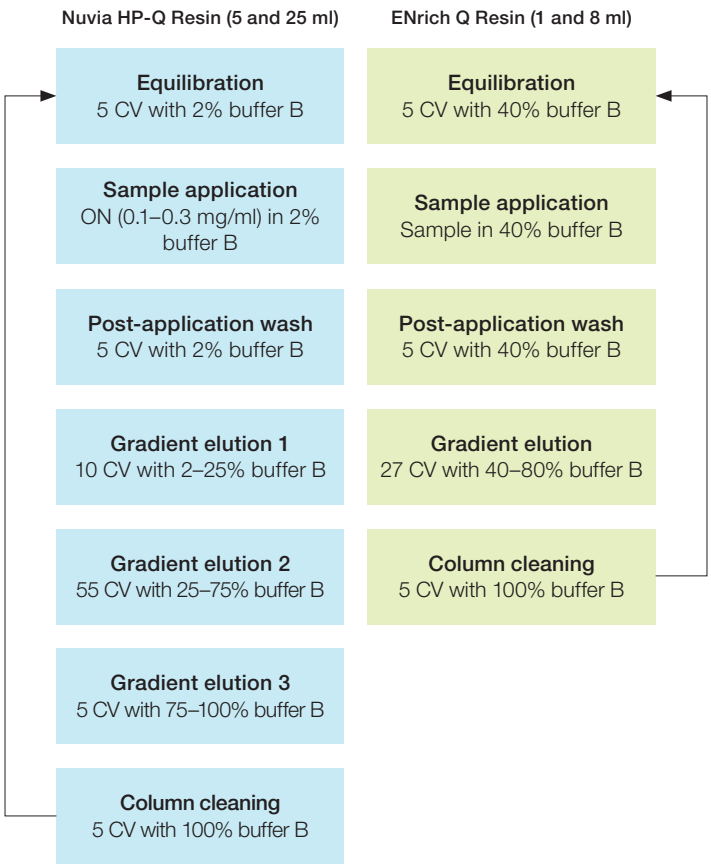


Fig. 2. Chromatography operating conditions. Proposed phases for the preparative purification of phosphorothioate DNA oligonucleotides (ONs) with Nuvia HP-Q Resin and subsequent fraction purity analysis with analytical anion exchange chromatography (ENrich Q Resin). CV, column volumes.

Electrophoresis

The quality of the chromatographic purification experiments in general and ON length-based purity were monitored by 15% Criterion TBE-Urea Polyacrylamide Gels available with different well numbers (12+2-, 18-, and 26-well formats; Bio-Rad #3450091, 3450092, and 3450093, respectively) in conjunction with 1x Tris/boric acid/EDTA (TBE) running buffer (diluted from 10x TBE Buffer, #1610733). Samples for analysis (typical concentration range: 20–200 ng/µl) were dissolved 1:1 in 2x TBE Urea Sample Buffer (Thermo Fisher Scientific Inc., #J60186.AC), which consists of 90 mM Tris base, 90 mM boric acid, 2 mM EDTA, 12% Ficoll solution, 7 M urea, 0.03% bromophenol blue, and 0.03% xylene cyanol. Samples were heated at 70°C for 4 min and the preferred loading volume was 10 µl. After sample loading, gels were run at constant amperage until the bromophenol blue front reached two-thirds of the gel length (for example, 30 min at 10 mA and 30–40 min at 20 mA). After the run, the gels were washed twice for 2 min each time in water and stained in the dark for 30 min with GelRed Nucleic Acid Gel Stain (Biotium, #41003) diluted to 1x in electrophoresis buffer or water. The stained gel was washed again in the dark with water twice for 2 min each time, then imaged with the GelDoc Go Gel Imaging System (Bio-Rad, #12009077) using the preset acquisition settings for ethidium bromide. Quantitative gel analysis was performed with Image Lab Software, version 6.1.0 (Bio-Rad, #12012931).

Analytical assays

For rapid PS-ON quantification, 2 µl of sample were applied to a NanoQuant Plate (Tecan Trading AG, #30033939) and absorbance at 260 nm was measured with an Infinite 200 Microplate Reader (Tecan, #30016056). The fraction of the full-length target ON in contaminated samples or side fractions obtained after preparative chromatographic purification with Nuvia HP-Q Resin was measured after further separation with analytical AEX columns (ENrich Q 5 x 50 or 10 x 100 Columns) (see Table 2). Peak integration and quantification were done with ChromLab Software, version 6.1.27 (Bio-Rad, #12009390).

Results and Discussion

Previously, we have shown that the AEX Nuvia HP-Q Resin is well-suited for the length-based purification of standard ONs with phosphodiester backbones (Posch et al. 2021). In that study, key process parameters such as flow rate, gradient slope, buffer pH, and dynamic binding capacity (DBC) were explored in small scale and the resulting protocol optimized for yield was successfully applied to a self-packed 25 ml column. This dataset was used as a starting point to analyze the purification potential of Nuvia HP-Q Resin for fully phosphorothioated ONs, which are often used as therapeutics. In the current study, the focus of our resin characterization (or protocol development) strategy was the detection of differences in elution conditions and the optimization of gradient slope. Buffer composition, buffer pH, and flow rate remained unchanged and data evaluation was again accompanied by an analysis strategy based on two complementary techniques: denaturing polyacrylamide gel electrophoresis (PAGE) and analytical AEX chromatography. This analysis strategy allows yield and purity calculations for the full-length ON, but the relative abundance of any oxidized analogs cannot be determined and requires high-resolution analytical chromatography techniques coupled to mass spectrometry or ^{31}P NMR analysis.

Sample characterization by analytical AEX chromatography

The influence of PS chemistry on the elution conditions in analytical AEX chromatography was investigated with ON 2 and PS-ON 2: two sequence-identical 20-mers, one with a regular phosphodiester backbone and one fully phosphorothioated. ON 2 required lower conductivity conditions (~85 mS/cm or 0.65 M NaCl) for elution, while increased salt concentrations (~160 mS/cm or 1.25 M NaCl) were needed for PS-ON 2 detachment from the resin (Figure 3). The weak release of phosphorothioates from strong anion exchange resins using a standard salt gradient with sodium chloride can be explained by the hard-soft acid-base (HSAB) theory. The negatively charged sulfur anion is regarded as a soft base and forms a strong ion pair with quaternary ammonium (+) exchangers such as ENrich Q beads. Consequently, phosphorothioated DNA is most efficiently eluted

with buffers containing soft competitive anionic species such as bromide or thiocyanate (Bergot and Zon 1992). This approach is used as an analytical technique and allows the separation of fully phosphorothioated ONs from P = O defect species but is not applied in large-scale purification schemes for therapeutic ONs. In addition, the PS-ON 2 sample shows significant peak broadening, which represents and is caused by the diastereoisomeric nature of the material, but a decline in separation performance due to the specific structural characteristics of PS-ONs or a combination thereof cannot be excluded.

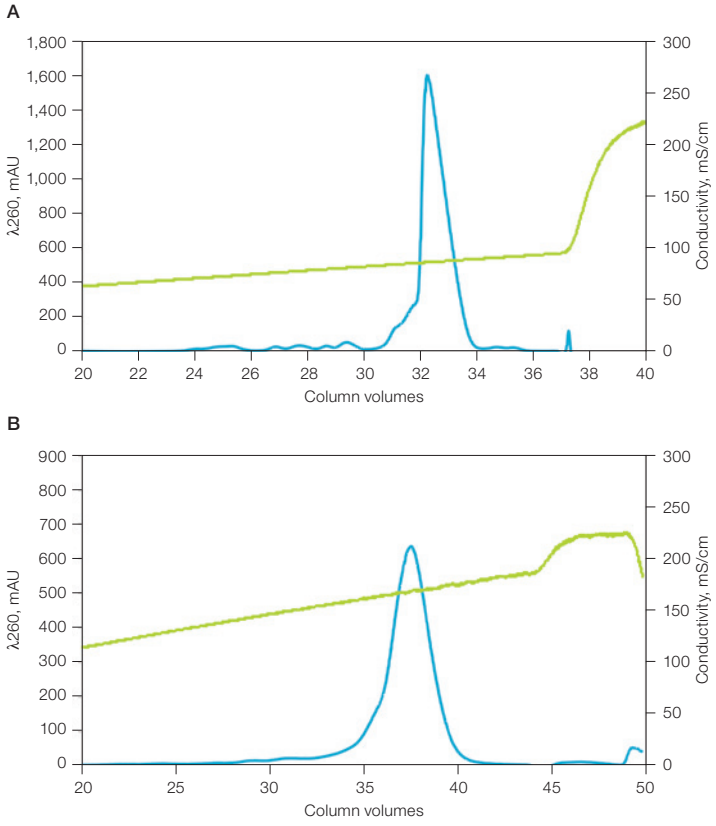


Fig. 3. Elution profiles of two sequence-identical 20-mers obtained with a 1 ml anion exchange column (ENrich Q Column) run at 300 cm/h. Approximately 150 µg of unpurified sample was loaded and the absorbance at 260 nm (—) and conductivity (—) measured. **A**, elution profile of ON 2 (phosphodiester backbone), eluted in 20 column volumes (CV) of buffer containing 400–700 mM NaCl, pH 11.7. **B**, elution profile of PS-ON 2 (fully thioated), eluted in 27 CV of buffer containing 800–1,600 mM NaCl, pH 11.7. PS-ON 2 requires higher conductivity conditions for elution and shows peak broadening. ON, oligonucleotide; PS-ON, phosphorothioate oligonucleotide.

Initial sample characterization of PS-ON 2 was continued with a 10 x 100 mm analytical ENrich Q Column, to which 1.6 mg of raw material was applied for analysis (Figure 4A). The goal of this experiment was to visualize the n–x to n transition zone by PAGE of individual consecutive chromatography fractions (1 ml) in the first half of the main peak. The eluted material between column volumes (CV) 24 and 26 corresponds to 17 fractions and was size-separated by PAGE (Figure 4B). In previous chromatography experiments with the unpurified standard 20-mer, we have seen a length-based elution order with a linear ratio change of ON pairs differing by one nucleotide in length. Here, the full-length PS-ON 2 (20-mer) is present in all fractions, even in fractions without clearly visible 18- or 19-mer contaminant (for example, see lanes 1 and 2). As discussed above, crude PS-ONs contain full-length analogs with phosphoric acid diester linkages (P = O). According to Yang et al. this type of modification changes the behavior of these impurities in AEX chromatography so that they elute at slightly lower conductivity conditions than the unmodified full-length product does (Yang et al. 2022). For example, in lanes 8–11, the 20-mer is still present in an equal ratio to contaminating species, which is very unusual compared to results obtained with standard ONs. It can be hypothesized that lanes 1–9 contain the full-length ON, but with one $[(P = O)_1]$ or more $[(P = O)_n]$ phosphodiester linkages anywhere in the sequence.

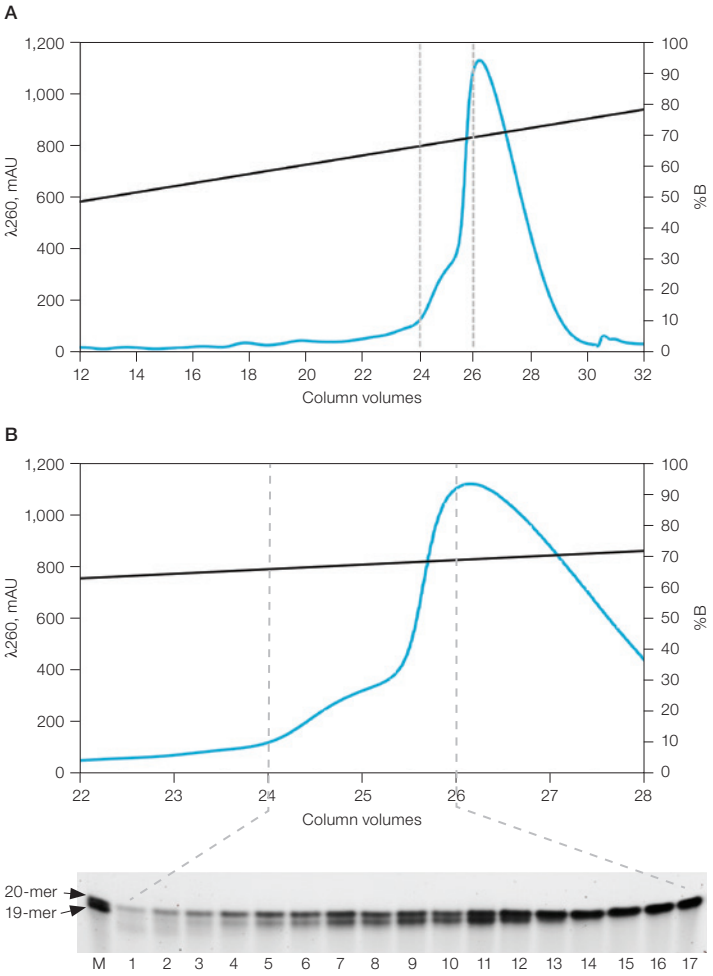


Fig. 4. Elution profile and denaturing PAGE analysis of a fully thioated 20-mer oligonucleotide (PS-ON 2). Absorbance at 260 nm (—); %B (—). The portion of the elution profile subjected to PAGE analysis is shown (– – –) for reference. **A**, elution profile of PS-ON 2 obtained with an 8 ml anion exchange column (ENrich Q Column) run at 300 cm/h. Approximately 1,600 µg of unpurified sample was loaded and 1 ml fractions were collected. See Figure 2 for chromatography conditions. **B**, 17 consecutive fractions between CV 24 and 26 were subjected to denaturing PAGE to visualize the n–x to n transition zone. Lane M represents a purified mixture of PS-ON 1 and PS-ON 2. CV, column volumes; PAGE, polyacrylamide gel electrophoresis; PS-ON, phosphorothioate oligonucleotide.

Purification of PS-ON 2 with a 5 ml Nuvia HP-Q Column

In previous chromatography experiments with a 20-mer standard ON, the DBC of Nuvia HP-Q Resin at 10% breakthrough was about 20 mg of crude ON 2 per ml of resin, measured at a flow rate of 120 cm/h (Posch et al. 2021). This evaluation was repeated with PS-ON 2 and was confirmed to be unchanged (data not shown). This allows operation of the Nuvia HP-Q Resin again at about 75–80% of its maximum DBC. According to Puma it is also possible to run preparative AEX columns for PS-ON purification in a well-defined overload mode to achieve mixed-mode displacement conditions (Puma 2000). This approach requires careful optimization of the loading conditions but can lead to a very robust and effective purification method.

Next, a few test runs were conducted to verify stronger binding of PS-ONs to Nuvia HP-Q Resin, as was observed with the analytical ENrich Q Columns. As expected, the conductivity conditions for elution from the Nuvia HP-Q Resin with sodium chloride had to be adjusted to higher levels. These findings were considered during method development. In addition, to address the increased impurity complexity of PS-ONs, the elution volume was increased from 60 to 70 CV, in comparison to ON 2, and the corresponding gradient slope was changed accordingly. The elaborated purification protocol is described in Figure 2 and was applied for the length-based purification of ~80 mg PS-ON 2 with a 5 ml Nuvia HP-Q Column at 120 cm/h. The corresponding chromatogram is displayed in Figure 5. Fractions containing n–x or n+x species in the presence of full-length PS-ON 2 were identified by denaturing PAGE (data not shown) and fraction pools were generated for further downstream analysis:

- Pool 1: n–x contaminants, without 20-mer
- Pool 2: 20-mer, contaminated with n–x
- Pool 3: purified 20-mer (including possible oxidized forms)
- Pool 4: 20-mer, contaminated with n+x
- Pool 5: n+x contaminants, without 20-mer

Pools 2 and 4 can be regarded as transition zones, where the full-length PS-ON is present in significant amounts, but considerably contaminated by shortmers and to a much less extent by n+1 or n+2 longmers. To better understand the effects of fraction pooling or yield on full-length PS-ON purity, two variations of pool 3 were generated. Fraction pool 3.1 reflects moderate pooling stringency, whereas pool 3.2 was created with elevated pooling stringency. The pooling start was different for pools 3.1 and 3.2, but they share an identical pooling endpoint (see Figure 5). The difference in starting points for pools 3.1 and 3.2 also necessitated creation of two variations of pool 2, designated similarly as pool 2.1 and 2.2.

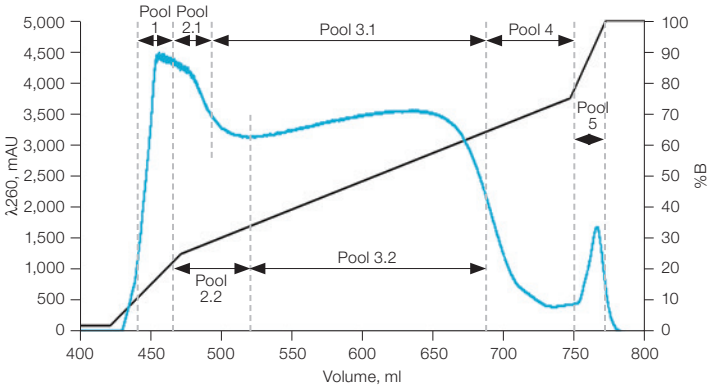
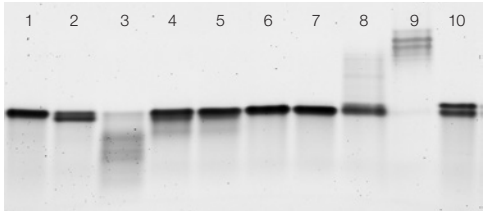


Fig. 5. Purification of a fully thioated 20-mer oligonucleotide (PS-ON 2) with AEX chromatography. Approximately 80 mg of raw material was purified with a 5 ml Nuvia HP-Q Column operated at pH 11.7 and a flow rate of 120 cm/h. Different fraction pools were generated for yield and purity analysis by analytical AEX and PAGE. Absorbance at 260 nm (—); %B (—). AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis.

Throughout the project, ON concentration was measured in all fraction pools and the mass balance recovery of this, and further separations that utilized the 25 ml column, was calculated to be ~98%. The seven fraction pools, together with the starting material and two ON pairs (PS-ONs 1/2 and 2/3) were analyzed using denaturing PAGE (Figure 6) to monitor the quality of the chromatographic run. The data show that short- and longmers were effectively removed from the crude 20-mer PS-ON 2 sample. Furthermore, pools 3.1 and 3.2 show no significant contamination with length-based impurities.



Lane	Contents
1	PS-ON, unpurified
2	M1
3	Pool 1
4	Pool 2.1
5	Pool 2.2
6	Pool 3.1
7	Pool 3.2
8	Pool 4
9	Pool 5
10	M2

Fig. 6. Denaturing PAGE purity analysis of fraction pools obtained after the purification of a fully thioated 20-mer oligonucleotide (PS-ON 2) with a 5 ml Nuvia HP-Q Column. See Figure 5 for pooling information. M1, PS-ON pair 1/2; M2, PS-ON pair 2/3.

For in-depth length-based yield and purity calculations, the respective fraction pools were re-chromatographed on analytical ENrich Q Columns (1 ml and 8 ml sizes) followed by PAGE analysis of selected meaningful fractions. Typical examples of this project-wide strategy are presented in Figures 7 and 8. Table 3 summarizes the evaluation of the various datasets. Pool 3.1, which represents low stringency pooling, showed a length-based purity of 93% at a yield of 88%, whereas pool 3.2, which represents high-stringency pooling, had a calculated length-based yield of 82% but an increased purity level, at 97%.

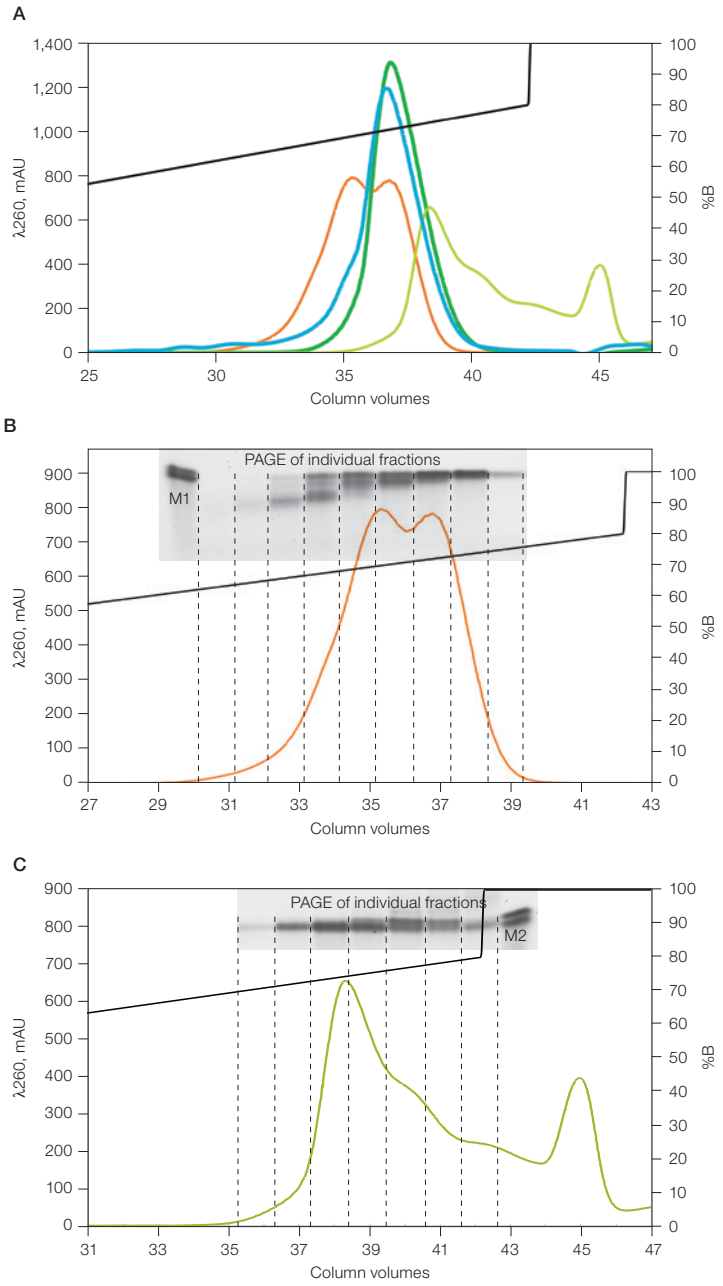


Fig. 7. Analytical AEX chromatography (using a 1 ml ENrich Q Column) for yield and purity calculations of different fraction pools obtained after purification with Nuvia HP-Q Resin. See Figure 5 for pooling information. Sample load was approximately 200 µg. **A**, overlay chromatogram of fraction pools 2.1 (—), 3.1 (—), 4 (—), and the unpurified 20-mer (—), as well as %B (—). **B**, PAGE analysis (inset) of fraction pool 2.1 after AEX chromatography. Absorbance at 260 nm (—); %B (—). **C**, PAGE analysis (inset) of fraction pool 4 after AEX chromatography. Absorbance at 260 nm (—); %B (—). AEX, anion exchange; M1, PS-ON pair 19/20; M2, PS-ON pair 20/21; PAGE, polyacrylamide gel electrophoresis.

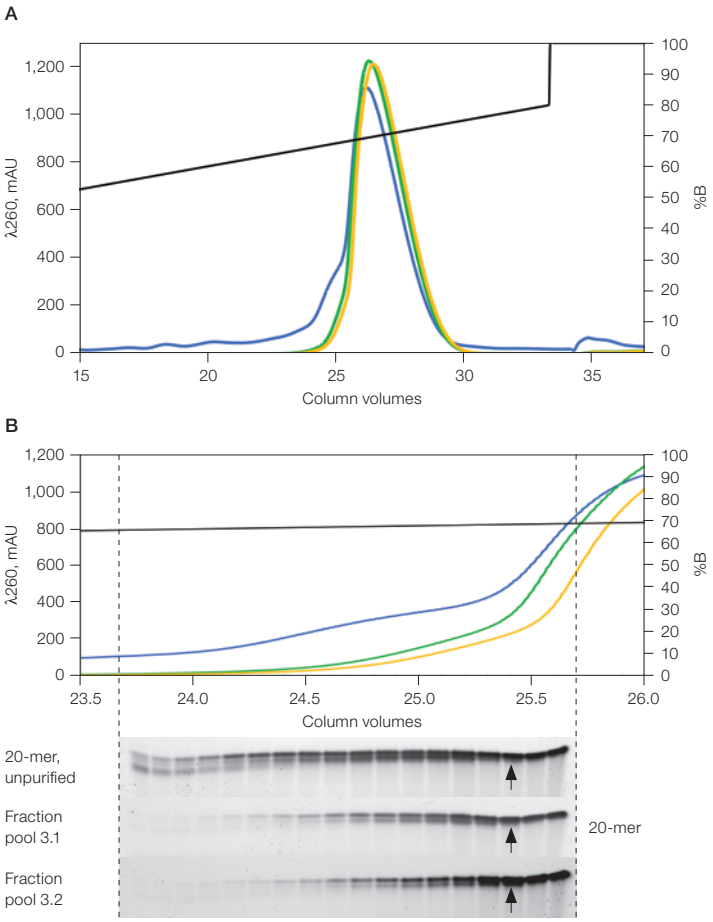


Fig. 8. AEX chromatography (using an 8 ml ENrich Q Column) for yield and purity calculations of different fraction pools obtained after purification with Nuvia HP-Q Resin. See Figure 5 for pooling information. Sample load was approximately 1,600 µg. Chromatograms show %B (—) and the absorbance at 260 nm for fraction pools 3.1 (—), 3.2 (—), and unpurified PS-ON 2 (—). **A**, full overlay chromatogram of selected fraction pools. **B**, PAGE analysis after AEX chromatography. The arrows indicate the first appearance of n–1 shortmers. A zoomed-in view of the AEX chromatogram from panel A is provided for orientation. AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis.

Table 3. Yield and purity data for the purification of 80 mg of PS-ON 2 with a 5 ml Nuvia HP-Q Column.

Sample	PS-ON Content, mg	PS-ON 2 Purity, %
PS-ON 2, unpurified	80	76
Pool 1	8.0	0
Pool 2.1	9.7	52
Pool 2.2	15.3	60
Pool 3.1	56.0	93
Pool 3.2	50.0	97
Pool 4	2.8	68
Pool 5	1.4	0
PS-ON 2 yield	88% Pool 3.1	82% Pool 3.2

See Figure 5 for pooling information.
Data are based on analytical AEX chromatography and PAGE analysis (see Figures 7 and 8). AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis; PS-ON, phosphorothioate oligonucleotide.

In 2021, we reported a length-based purity of 97% for the corresponding standard 20-mer (ON 2) after preparative purification with Nuvia HP-Q Resin, but at a higher yield of 90% (Posch et al. 2021). It is clear that the length-based purification of thioated ONs is challenging, but the provided dataset with Nuvia HP-Q Resin is in good concordance with published data obtained with other AEX resins.

Large-scale purification of PS-ON 2 with a 25 ml Nuvia HP-Q Column

For large-scale purification of PS-ON 2 (20-mer), a glass Econo Alpha Column (25 x 100 mm) was packed with 25 ml of Nuvia HP-Q Resin at a flow rate of 120 cm/hr and later operated at 100 cm/hr. 380 mg of raw material was applied to a five-fold scale-up purification run using the chromatography conditions described in the previous section for the prepacked 5 ml Nuvia HP-Q Column. The corresponding AEX chromatogram is displayed in Figure 9. Every fourth fraction (fraction volume: 8 ml) was analyzed using denaturing PAGE to identify the distribution of full-length PS-ON 2 across the main peak (data not shown). The chromatogram looked very similar to the one obtained with the 5 ml Nuvia HP-Q Column, although the column configurations were very different: the height-to-diameter ratios of the 5- and 25-ml column are 12.5:1 and 2:1, respectively. Interestingly, Puma reported that the column configuration in preparative AEX can have a significant impact on the conductivity conditions required for product elution — higher conductivity conditions are usually needed if the column is longer in relation to the diameter (Puma 2000).

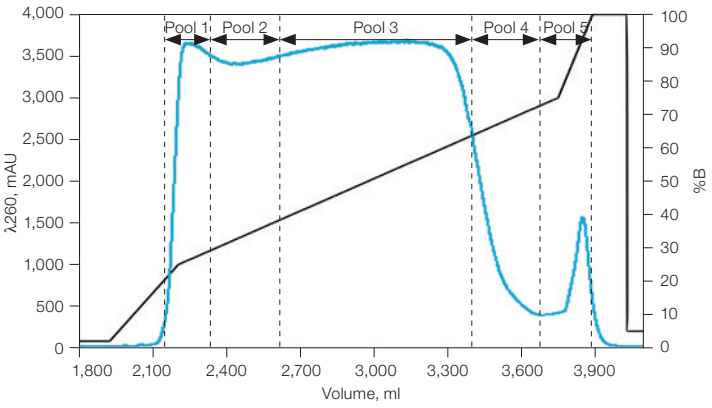
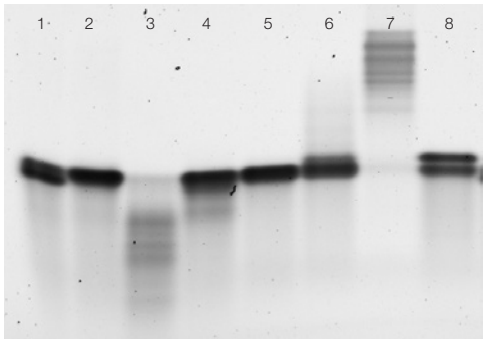


Fig. 9. Purification of a fully thioated 20-mer oligonucleotide (PS-ON 2) with AEX chromatography. Approximately 380 mg of raw material was purified using a column packed with 25 ml Nuvia HP-Q Resin at pH 11.7 and 120 cm/h. Different fraction pools were generated for further yield and purity analysis by analytical AEX chromatography and PAGE. Absorbance at 260 nm (—); %B (—). AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis; PS-ON, phosphorothioate oligonucleotide.

Five fraction pools (1–5) were generated. Pool 3 mainly contains the full-length 20-mer, whereas in pools 2 and 4, the 20-mer is contaminated with either n–x or n+x ON species. Fraction pools 1 and 2 are solely composed of contaminating species without the full-length ON. The quality of the pooling process was assessed by denaturing PAGE (Figure 10) and as expected, pool 3 shows no major contamination with length-based impurities. It is also important to mention that pool 3 was generated in a way such that a length-based process yield of 82% was obtained. This value reflects the pooling conditions of fraction pool 3.2 for the purification run done with the 5 ml Nuvia HP-Q Column and enables a fair side-by-side comparison of the respective purity levels. Using the same analysis strategy as before, fraction pools 2–4 were re-chromatographed on analytical ENrich Q Columns followed by PAGE analysis (PAGE data are not shown). Length-based yield and purity data for pools 2 and 4 were obtained with a 1 ml ENrich Q Column (Figure 11), while pool 3 was evaluated with an 8 ml ENrich Q Column (Figure 12). Data analysis for fraction pool 3 revealed a length-based yield and purity of 82% and 96%, respectively, and the total dataset is presented in Table 4.

In summary, the data support this as a successful scale-up strategy, as the yield and purity levels for PS-ON 2 are very comparable to the results obtained by the small-scale 5 ml Nuvia HP-Q Column.



Lane	Contents
1	M1
2	PS-ON 2, raw
3	Pool 1
4	Pool 2
5	Pool 3
6	Pool 4
7	Pool 5
8	M2

Fig. 10. Denaturing PAGE purity analysis of fraction pools obtained after the purification of a fully thioated 20-mer oligonucleotide (PS-ON 2) with a column packed with 25 ml Nuvia HP-Q Resin. See Figure 9 for pooling information. M1, PS-ON pair 19/20; M2, PS-ON pair 20/21. PAGE, polyacrylamide gel electrophoresis; PS-ON, phosphorothioate oligonucleotide.

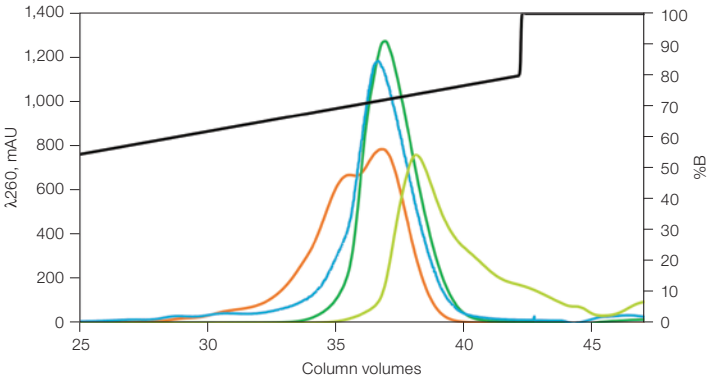


Fig. 11. Analytical AEX chromatography (using a 1 ml ENrich Q Column) for yield and purity calculations of different fraction pools obtained after purification with Nuvia HP-Q Resin. See Figure 9 for pooling information. Sample load was approximately 200 µg. Overlay chromatogram of %B (—) and absorbance at 260 nm for fraction pools 2 (—), 3 (—), 4 (—), and the unpurified 20-mer (—). PAGE data are not shown. AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis.

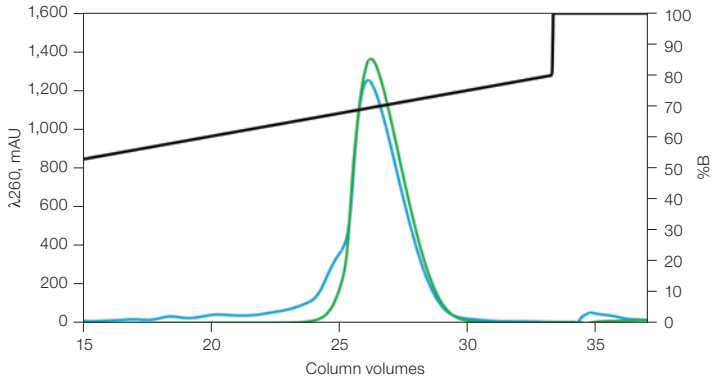


Fig. 12. Analytical AEX chromatography (using an 8 ml ENrich Q Column) for yield and purity calculations of different fraction pools obtained after purification with Nuvia HP-Q Resin. See Figure 9 for pooling information. Sample load was approximately 1,700 µg. An overlay chromatogram of %B (—) and the absorbance at 260 nm for fraction pool 3 (—) and the unpurified 20-mer (—) is given. PAGE data are not shown. AEX, anion exchange; PAGE, polyacrylamide gel electrophoresis.

Table 4. Yield and purity data for the purification of 380 mg PS-ON 2 using a column packed with 25 ml of Nuvia HP-Q Resin.

Sample	PS-ON Content, mg	PS-ON 2 Purity, %
PS-ON 2, unpurified	380	77
Pool 1	31.1	0
Pool 2	72.2	55
Pool 3	242.3	96
Pool 4	16.3	75
Pool 5	9.8	0
PS-ON 2 yield	82% (Pool 3)	

See Figure 9 for pooling information.
Data are based on analytical AEX chromatography (see Figures 11 and 12) and PAGE analysis (not shown). PS-ON, phosphorothioate oligonucleotide.

Conclusion

The production of pure therapeutic DNA ONs requires efficient, scalable, and cost-effective chromatographic purification methods applied after chemical synthesis. Key impurities in crude samples are length-based and are best removed by AEX chromatography. We have demonstrated the preparative-scale purification of a single-stranded, fully thioated 20-mer DNA ON using AEX chromatography with Nuvia HP-Q Resin. Thioated ONs, in contrast to their phosphodiester analogs, are very challenging to purify and require the careful evaluation of a given AEX resin for its applicability in a preparative purification process.

Key process parameters were explored with a 5 ml Nuvia HP-Q Column. The resulting protocol, optimized for length-based yield (82%) and purity (97%), was successfully applied to a self-packed 25 ml column. The DBC of the resin at 10% breakthrough and a flow rate of 120 cm/h is ~20 mg crude 20-mer material per ml of resin. The 25 ml column was operated at 78% of its maximum DBC and 380 mg of a fully thioated 20-mer was efficiently purified with similar length-based yield (82%) and purity (96%) as in the smaller-scale study.

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