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# Membrane Purification of an Antisense Oligonucleotide

Ajay R. Lajmi,*,† Larry Schwartz,‡ and Yogesh S. Sanghvi§,⊥

*Scientific and Laboratory Services, Pall Life Sciences, 8780 Ely Road, Pensacola, Florida 32514, U.S.A., Pall Life Sciences, 2200 Northern Boulevard, East Hills, New York 11548, U.S.A., and Development Chemistry, Isis Pharmaceuticals, 2292 Faraday Avenue, Carlsbad, California 92008, U.S.A.*

**Abstract:**

**This contribution describes a membrane chromatography purification of a therapeutically useful antisense oligonucleotide (ASO). Sample self-displacement purification conditions were optimized for a 20-mer ASO on a 10-mL bed volume, high-resolution, strong anion-exchange membrane chromatography unit. The scale-up experiments were performed at 177-g scale on a 1-L bed volume unit that resulted in 84.4% yield and 95.0% (analyzed by LC−MS) pure full-length ASO following the membrane chromatography process. The purified ASO was subsequently significantly concentrated and diafiltered into water using a 650-Da MWCO ultrafiltration membrane. This robust, high throughput, membrane-based, anion-exchange chromatographic (AX) method simplifies the process-scale purification of phosphorothioate oligonucleotides by eliminating the need for column packing and packing validation.**

## 1. Introduction

Antisense therapeutics are an emerging class of drugs with more than two dozen drug candidates in various stages of clinical trials with three in late phase III clinical trials and one ASO on the market.[1] Demand for these synthetic oligonucleotide analogue drugs is expected to reach metric tons per year.[1] Developing cost-effective, high-throughput processes for manufacture of these drugs at the metric ton scale and with the requirement for high purity is an important and substantial challenge for the industry.[1−3]

Achieving good chromatographic selectivity between the phosphorothioate (P═S) product (*n*-mer) and its deletion sequences (*n*−1) as well as sequences containing phosphodiesters $(P{=}O)_n$ is difficult.[4] Higher-molecular weight products referred to herein as *n*+1-mer are often obtained during oligonucleotide synthesis.[5] Among the phosphodiesters $(P{=}O)_n$ that are formed as byproducts during synthesis, the major component is monophosphodiester $(P{=}O)_1$ which has been reported to be separated by AX. Oligonucleotides are synthesized from the 3′- to 5′-end of a nucleoside.[1] The terminal nucleoside is capped at the 5′-end with 4,4′-dimethoxytrityl (DMT) protecting group. The terminal 5′-DMT group may be retained or removed depending, on the choice of subsequent purification.[6] Reversed-phase chromatography (RP-HPLC) and AX are currently the standard purification techniques to manufacture ASO under cGMP conditions. Only RP-HPLC, hydrophobic interaction chromatography (HIC), and AX have been documented to be robust, scaleable, and consistent with cGMP requirements for therapeutic oligonucleotide manufacture.[3,4,7]

With the increasing number of ASO therapeutic candidates progressing through clinical trials, manufacturers have sought to maximize purity of these products using a variety of methods. General strategies for the purification of ASO have been reviewed in the literature.[7−10] For example, DMT-on ASOs could be purified by preparative RP-HPLC due to hydrophobic interactions of DMT groups with the stationary phase, whereas DMT-off ASOs could be separated by AX. Hydrophobic-interaction chromatography (HIC) has also been shown to be a useful technique in the purification of DMT-on ASOs where retention on the stationary phase occurs through interaction with the hydrophobic DMT groups.[8]

In displacement chromatography, a molecule that has higher affinity for the stationary phase than any of the feed components, called a displacer, is used to elute the product. Under optimized displacement conditions, the feed components form adjacent zones of highly concentrated material as the displacer trails the adjacent feed zones in the displacement train. The general mechanism of displacement chromatography as well as its application to the purification of protein, peptides, and other biomolecules has been reported and reviewed in the literature.[11−13] Displacement chroma-

* Corresponding author. Telephone: (850) 316-3504. Fax: (850) 316-3601. E-mail: ajay_lajmi@pall.com.

† Scientific and Laboratory Services, Pall Life Sciences, Pensacola, Florida.

‡ Pall Life Sciences, East Hills, New York.

§ Development Chemistry, Isis Pharmaceuticals, Carlsbad, California.

⊥ Current Address: Rasayan Inc., 2802 Crystal Ridge Road, Encinitas, CA 92024.

(1) Sanghvi, Y. S.; Andrade, M.; Deshmukh, R. R.; Holmberg, L.; Scozzari, A. N.; Cole, D. L. In *Manual of Antisense Methodology*; Hartman, G., Enders, S., Eds.; Kluwer Academic Publishers: Norwell, 1999; pp 3−23.

(2) Bennett, C. F.; Cowsert, L. M. *Biochim. Biophys. Acta* **1999**, *1489*, 19−30.

(3) Brown, K. D. *Genet. Eng. News* **1998**, *18*, 1.

(4) Deshmukh, R.; Warner, T. N.; Hutchison, F.; Murphy, M.; Leitch, W. E., II; DeLeon, P.; Srivatsa, G. S.; Cole, D. L.; Sanghvi, Y. S. *J. Chromatogr.* **2000**, *890*, 179−192.

(5) Krotz, A.; Klopchin, P. G.; Walker, K. L.; Srivatsa, G. S.; Cole, D. L.; Ravikumar, V. T. *Tetrahedron Lett.* **1997**, *38*, 3875−3878.

(6) Deshmukh, R. R.; Cole, D. L.; Sanghvi, Y. S. *Methods Enzymol.* **1999**, *313*, 203−226.

(7) Deshmukh, R. R.; Leitch, W. E., II; Cole, D. L. *J. Chromatogr. A* **1998**, *806*, 77−92.

(8) Puma, P. In *HPLC: Practical and Industrial Applications*; Swadesh, J., Ed.; CRC Press: Boca Raton, FL, 1997; pp 81−110.

(9) Crooke, S. T., Ed. *Antisense Drug Technology Principles, Strategies and Applications*; Marcel Dekker: New York, 2001.

(10) Deshmukh, R. R.; Leitch, W. E., II; Sanghvi, Y. S.; Cole, D. L. In *Handbook of Bioseparations*; Ahuja, S., Ed.; Academic Press: New York, 2000, pp 511−534.

(11) Cramer, S. M.; Horvath, C. *Prepr. Chromatogr.* **1988**, *1*, 29−49.

(12) Hodges, R. S.; Burke, T. W. L.; Mant, C. T. *J. Chromatogr.* **1988**, *444*, 349−368.

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tography using high-molecular weight dextran sulfate as the displacer has been shown feasible for purification of phosphorothioate oligonucleotides by AX.[14] High-affinity low-molecular weight amaranth and saccharin displacers have been reported effective in purification of ASO at high column loading by displacement AX.[15−17] Although this elegant displacement method provides high sample loading and a highly concentrated product pool, the need for an additional orthogonal chromatography step and the costs associated with validation for complete removal of displacer preclude its implementation in manufacturing processes.

In sample self-displacement chromatography of phosphorothioate oligonucleotides, the full-length, fully thioated oligonucleotide component in the sample that has the highest charge, hence the highest affinity for the stationary phase, acts as the displacer. Examples of ASO purification by sample self-displacement using AX on beaded media columns have been reported from milligram- to 100-gram scale.[7,18,19] Cost factors related to column packing and packing validation, high operating pressures, low flow rates, and throughput in terms of amount of oligonucleotide processed per unit time per unit volume of AX media makes it less attractive compared to membranes.[20]

Mustang ion-exchange membranes exhibit separation efficiencies better than 15 $\mu$m ion-exchange chromatographic beads.[21] Additionally, an advantage of operating in the displacement mode is the potential for high-resolution separation. Therefore, utilizing sample self-displacement chromatography conditions where the sample component that has the highest affinity for the stationary phase serves as a displacer further enhances the separation.[13]

Purification of ASO by membrane chromatography has been reported to yield purity comparable to conventional beaded AX media.[4] Prepacked reusable membrane chromatography media offer fast, high-resolution separations without any column packing or packing validation. One drawback of AX is that the purified product contains salt at high concentration. Desalting and concentration of the purified product by tangential flow filtration (TFF) using a selective molecular weight cut off (MWCO) ultrafiltration membrane can provide a robust and scaleable alternative.[22]

Herein, we report a sample self-displacement polishing protocol to purify an RP-HPLC purified 20-mer therapeutically useful phosphorothioate oligonucleotide using high-resolution AX on a 177-g scale. The 1-L bed volume unit provided purity greater than that obtained from conventional beaded AX. Oligomeric impurities associated with raw materials and failure sequences introduced during the solid-phase synthesis were either minimized or effectively removed, and the resulting product was subsequently qualified as a primary reference standard. The results indicated that AX membrane chromatography of phosphorothioate oligonucleotides under sample self-displacement conditions separated the target 20-mer AO with both high yield and purity. The membrane chromatography-purified product was concentrated and desalted by 650-Da MWCO polyethersulfone membrane ultrafiltration cassettes by TFF.

## 2. Experimental Section

**2.1. Chromatography.** *2.1.1. Materials.* All solutions were prepared in deionized water (13 MΩ water) that was obtained by running reversed osmosis purified water through a dual-tank mixed-bed water purification system model no. ZWDJ02541067 (US Filter, Lowell, MA). All stock solutions that were used to prepare salt-wash and elution solutions for scale-up runs and the solution used to dilute the sample before loading were filtered through 0.2 $\mu$m Supor Vacucap 60 or Supor Vacucap 90 filters. All storage tanks (20, 40, 100, and 400 L) were made of high-density polyethylene (Nalgene) with needle-type polypropylene spigot. The storage containers and tanks were sanitized with 1 M NaOH followed by deionized water prior to use. Conductivities of all salt-containing solutions were measured using an Orion conductivity meter model 130A (Beverly, MA) equipped with a temperature probe. The conductivity meter was calibrated with 1 mS/cm and 100 mS/cm (±0.25% at 25 °C) calibration standards (YSI Incorporated, Yellow Springs, OH) at room temperature (22 ± 1 °C).

*2.1.2. Stock Solutions and Working Solutions.* All solutions were filtered through Supor Vacucap 90 filter into sanitized storage tanks and stored at room temperature. All receiving containers were sanitized with 1 M NaOH. The salt concentration of all solutions was measured in terms of conductivity. A 20 mM NaOH was 3.38 mS/cm. A 0.625 M NaCl solution in 20 mM NaOH was 56 mS/cm (at 22 ± 1 °C). A 0.75 M NaCl solution in 20 mM NaOH had a conductivity value of 64 mS/cm; a 1.375 M NaCl solution in 20 mM NaOH was 105 mS/cm (at 22 ± 1 °C). A 2.5 M NaCl in 1 M NaOH was 259 mS/cm (at 22 ± 1 °C). A 0.5 M sodium phosphate, dibasic solution in 0.1 M NaOH (soaking solution) was 47 mS/cm (22 ± 1 °C).

The oligonucleotide sample for loading onto the chromatography module was prepared by dissolving 177.1 g (based on $OD_{260}$ reading 25 $OD_{260}$ units = 1 mg) of ISIS 2302 API[23] in 17.5 L of 20 mM NaOH. The oligonucleotide solution was sterile-filtered through a 0.2 $\mu$m SuporVacucap 90 filter. This was an ASO that was already purified by RP-HPLC and was 87.7% in the full-length oligonucleotide by LC−MS analysis. The LC−MS analytical method for an ASO that was used here has been previously reported.[24]


(13) Shukla, A. A.; Cramer, S. M. In *Handbook of Bioseparations*; Ahuja, S., Ed.; Academic Press: New York, 2000; pp 379−415.
(14) Gerstner, J. A.; Pedroso, P.; Morris, J.; Bergot, B. J. *Nucleic Acids Res.* **1995**, *23*, 2292−2299.
(15) Shukla, A. A.; Deshmukh, R. R.; Moore, J. A.; Cramer, S. M. *Biotechnol. Prog.* **2000**, *16*, 1064−1070.
(16) Tugcu, N.; Deshmukh, R. R.; Sanghvi, Y. S.; Moore, J. A.; Cramer, S. M. *J. Chromatogr. A* **2001**, *923*, 65−73.
(17) Tugcu, N.; Deshmukh, R. R.; Sanghvi, Y. S.; Cramer, S. M. *React. Funct. Polym.* **2003**, *54*, 37−47.
(18) Deshmukh, R. R.; Miller, J. E.; De Leon, P.; Leitch, W. E., II; Cole, D. L.; Sanghvi, Y. S. *Org. Process Res. Dev.* **2000**, *4*, 205−213.
(19) Deshmukh, R. R.; Ericsson, K−O.; Moore, P.; Cole, D. L.; Sanghvi, Y. S. *Nucleosides Nucleotides Nucl.* **2001**, *20*, 567−576.
(20) Warner, T. N.; Nochumson, S. *BioPharm Int.* **2003**, *1*, 58−60.
(21) Teeters, M. A.; Root, T. W.; Lightfoot, E. N. *J. Chromatogr. A* **2002**, *944*, 129−139.
(22) Schwartz, L. *BioProcess Int.* **2003**, *1*, 43−49.

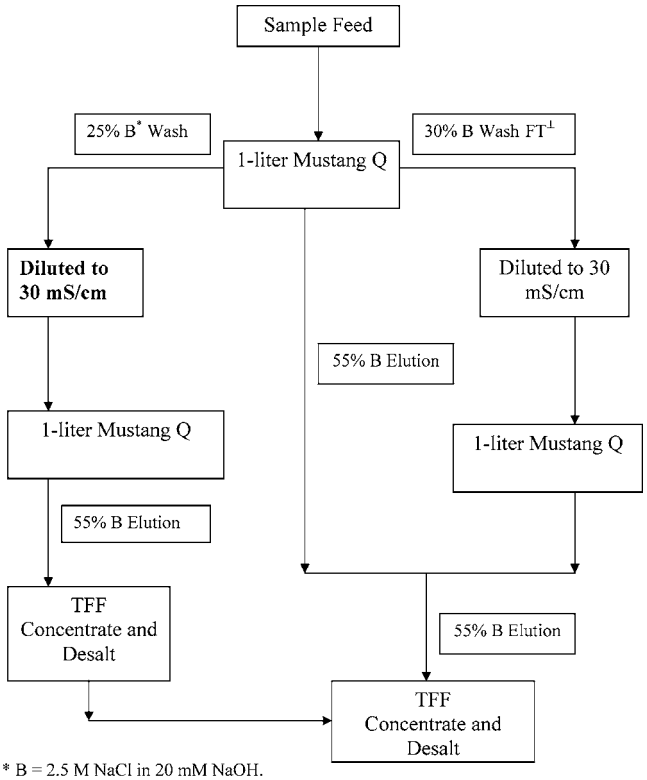

(23) Single lot of ISIS 2302 API [5′-GCC CAA GCT GGC ATC CGT CA-3′] was used for all experiments. This ASO is designed to bind to the 3′ UTR region of the ICAM-1 mRNA and is currently in phase III clinical trials for the treatment of Crohn's disease. ISIS 2302 was synthesized on OligoProcess automated synthesizer and purified by RP-HPLC.

The quaternary amine membrane AX devices used were 10-mL Mustang Q module and 1-L Mustang Q module (Pall Corp., East Hills, NY) with effective membrane diameter of 3.7 and 37 cm, respectively. The process-development experiments on the 10-mL Mustang Q membrane chromatography module were performed using a peristaltic Masterflex LS pump (Cole Parmer, model 77250-62) that was connected to a BioRad dual wavelength detector (EM-1 Econo UV monitor) set to monitor at 260-nm wavelength along with a software-driven data collection system. The tubings used were Tygon Masterflex4S 16 (Cole Parmer). The scale-up runs were performed on a 1-L Mustang Q module that was connected to a rotary displacement Waukesha Cherry Burrell pump (model 018, Delavan, WI) using 1.27 cm 316 stainless steel sanitary fittings and 1.27 cm reinforced PVC sanitary tubing (Cole Parmer). The detector that was used with the 1-L module was Optek UV detector model AF46-B (Essen, Germany) and was set to monitor at 260-nm wavelength.

*2.1.3. Methods.* The AX purification conditions were optimized for yield and purity on a 10-mL Mustang Q module with respect to the gradient conditions while maintaining the amount of oligonucleotide loaded per injection (200−300 mg), sample loading (at a concentration of 10 mg/mL), and flow rate (3 membrane volumes (MV)/min) at 1 MPa total system pressure on the AKTA Explore 100 workstation. The low-salt-wash fractions were recycled once through the 10-mL Mustang Q module using the optimized gradient conditions to improve the product yield. The optimized conditions were then used to purify the 177.1-g ASO in six injections and two recycle runs on a 1-L Mustang Q module.

*2.1.4. 10-mL Mustang Q Chromatography.* The chromatography module was rinsed with 50 mL, 5 MV (membrane volumes) of solution B, 2.5 M NaCl in 20 mM NaOH followed by equilibration with 5−10 MV of solution A (20 mM NaOH). Between 200 and 300 mg was pumped onto the module. All unbound oligonucleotides were washed from the module with 5−10 MV of solution A. Three salt-wash conditions were analyzed for product yield in the elution. The bound oligonucleotide was washed with (a) 30 MV of 25% B (mobile phase B = 2.5 M NaCl in 20 mM NaOH), 38 and 40% B, (b) 25, 35, and 40% B, and (c) 25 and 30% B. The full-length oligonucleotide was eluted with 20 MV of 55% B.

*2.1.5. 1-L Mustang Q Chromatography.* The system including the module was sanitized with 3 L of 1 M NaOH for 20 min and rinsed with 10 L of 2.5 M NaCl in 20 mM NaOH. The chromatography process is summarized in Figure 1. The ASO (250−300 mL) was pumped onto the 1-L module at 10.1 mg/mL for purification. This protocol was repeated a total of six times to process the entire batch. The module was rinsed with 5 L of B followed by 6−8 L of solution A or until the conductivity declined to baseline. A = 20 mM NaOH and B = 2.5 M NaCl in A. The flow rate was adjusted to 3 L/min at 0.31 MPa, 30 g of the oligonucleotide was loaded, and the flow-through was collected and pooled with the flow-through from the subsequent five injections and saved for rechromatography with all of the other wash fractions after readjusting the conductivity to 30 mS/cm with solution A. The unbound oligonucleotide was washed off the chromatography module with 5−6 L of solution A or until $OD_{260}$ stabilized at the baseline. The chromatography module was then washed with 25−30 L of 25% B until the $OD_{260}$ stabilized to baseline. This fraction was combined with the flow-through and other 25% B wash fractions for rechromatography. The chromatography module was washed with 25−30 L of 30% B or until $OD_{260}$ stabilized near the baseline. This fraction was combined with other 30% B wash fractions for rechromatography. The full-length oligonucleotide was eluted with 25−30 L of 55% B or until $OD_{260}$ stabilized near the baseline. Product purity in the eluate was confirmed by analytical reversed-phase, ion-pairing (RP−IP) HPLC. All of the elution pools from the six 1-L Mustang Q module runs were concentrated and then desalted by TFF. At the end of each run, the module was washed with 5 L of 100% B followed by 5 L of 2.5 M NaCl in 1 M NaOH. The chromatography module was cleaned with 4 mM phosphoric acid for 30 min at 0.2 L/min and regenerated with 5 L of 100% B. The 1-L module was washed with 5−10 L of storage solution (0.5 M $Na_2HPO_4$ in 0.1 M NaOH) and stored by submersion.




* B = 2.5 M NaCl in 20 mM NaOH.

⊥ FT = Flow-through.

***Figure 1.*** **One-liter strong anion-exchange membrane chromatography process scheme.**

**2.2. Ultrafiltration and Diafiltration.** *2.2.1. Materials.* The TFF unit consisted of a LV Centrasette Housing (Pall Corp., East Hills, NY) with three gauges, two valve-fitting

(24) For details see: Ravikumar, V. T.; Kumar, R. K.; Capaldi, D. C.; Turney, B.; Rentel, C.; Cole, D. L. *Org. Process Res. Dev.* **2003**, *7*, 259−266.

***Table 1.*** **Comparison of yields and LC−MS purity of a 20-mer phosphorothioate oligonucleotide in the elution pool from a 10-mL high-resolution strong anion-exchange membrane chromatography purification**

| sample load[a] (mg) by $OD_{260}$ | salt-wash steps | amount of oligo in elution[a] (mg) by $OD_{260}$ | % yield | % *n*-mer | % (*n*−1) mer | % (*n*+1) mer | % total P═O |
|---|---|---|---|---|---|---|---|
| feed | | | | 87.7 | 2.0 | 0.3 | 2.47 |
| 207.0 | 25% B, 38% B, 40%B | 39.6 | 19.1 | 91.0 | 1.9 | 0.3 | 6.8 |
| 207.0 | 25% B, 35% B, 40%B | 52.7 | 25.5 | 90.1 | 1.6 | 0.3 | 3.4 |
| 251.9 | 25% B, 38% B, 40%B | 59.1 | 23.7 | 93.9 | 1.1 | 0.2 | 2.1 |
| 249.3 | 25% B, 30% B | 126.5 | 50.2 | 94.0 | 1.0 | 0.3 | 1.1 |

[a] Amount of oligonucleotide was based on the purity of the full-length oligonucleotide in the sample by LC−MS analysis. [*n*-mer: full-length 20-mer phosphorothioate oligonucleotide; *n*−1: one residue short 19-mer phosphorothioate oligonucleotides; *n*+1: 21-mer phosphorothioate oligonucleotides with an additional residue.]

packages, and two 0.5 $m^2$, 650-Da molecular weight cut off (MWCO) Omega membrane, Centrasette medium screen channel cassettes (Pall Corp., East Hills, NY). The pumping system and plumbing consisted of a Quattroflow 1000 (Pall Corp., East Hills, NY) quaternary diaphragm pump with 1.9 cm sanitary elbows to connect pump with feed port and 1.3 cm i.d. reinforced PVC sanitary tubing (Cole Parmer). A peristaltic Masterflex LS pump (Cole Parmer, model 77250-62) and tubing (0.6 cm i.d.) for diafiltration.

*2.2.2. Methods.* The air integrity test, nominal water permeability (NWP), and water flush were performed on the ultrafiltration membrane cassettes as per manufacturer's guidelines prior to use.[25] The TFF system was flushed with deionized water to remove air bubbles and any preservatives from the membrane cassettes. The feed pressure was adjusted to 0.28 MPa by closing the retentate valve with the filtrate valve open. The retentate flow rate was set to 6.5 L/min by adjusting the feed pump. The system contained 1.5 L of water including the volume in the reservoir prior to adding the sample from the first chromatography elution. This concentration procedure was followed for each addition of Mustang Q elution fraction. The product was concentrated to 3.5 L including the hold-up volume. Diafiltration was performed against deionized water immediately following concentration while maintaining the same operating conditions. A diafiltration cycle consisted of addition of 3.5 L of deionized water to the sample reservoir and concentrating the sample to 3.5 L. Conductivity of the filtrate was measured before and after each diafiltration cycle. The diafiltration process was stopped when conductivity of the filtrate remained unchanged with two successive diafiltration cycles. The final concentrated and desalted product was recovered by slowly pumping the sample through the system into a polypropylene collection bottle. The hold-up volume was displaced and collected by pumping air through the system. Any product that remained on the cassette in the form of a gel layer was recovered by recirculating 200 mL of deionized water through the TFF system for 5 min and collected by pumping air through the system. Samples were taken before and after the TFF process for RP−IP HPLC analysis.[25] The product purity was confirmed by electrospray LC−MS analysis.

(25) Pall Corp., Membrane Cassette Care and Use Procedure, 2001, 00640 rev. 0.01.

(26) Gilar, M. *Anal. Biochem.* **2001**, *298*, 196−206.

## 3. Results and Discussion

The oligonucleotide purification protocol was optimized at a 200−250-mg scale using a 10-mL strong anion-exchange membrane chromatography unit. The sample load was optimized with respect to the purity of the full-length oligonucleotide (the desired product) in the elution as shown in Table 1. The LC−MS analysis of the phosphorothioate oligonucleotide allowed good estimation of *n*-mer, *n*−1-mer, *n*+1-mer, and $(P{=}O)_n$; details of this method have recently been reported.[24] An increase in the sample load from 207.0 to 249.3 and 251.9 mg resulted in the product purity of 94.0% in the full-length oligonucleotide in the elution, suggesting a sample self-displacement effect. Less than 10% of the loaded sample was observed in the flow-through, indicating that the full-length oligonucleotide dynamic binding capacity of the membrane unit was between 230 and 240 mg. The product analysis of the elution fraction shown in Table 1 indicated that the *n*−1 and phosphodiester impurities were reduced by more than half compared to the feed. Reducing the salt concentration in the wash step from 38 to 35% B improved the product yield in the elution to 25.5% with purity in the 90−91% range (Table 1). Analysis of the two salt washes indicated that they contained substantial amount of product. In an attempt to further improve the yield, an additional reduction in the salt concentration of the wash step was considered. Thus, a reduction in the number of salt-wash steps from three to two and decreasing the salt concentration of the wash step from 40 to 30% B prior to product elution with 55% B maintained high product purity of 94.0% (Table 1) but resulted in a 2-fold increase in the yield to 50.2% (Table 1). Increasing the sample load from 207.0 to 249.3 mg under identical salt-wash conditions as shown in Table 2 not only improved the oligonucleotide yield to 50.2% in elution, but it also resulted in higher product purity of 94% (Table 1). The reduction in the number of salt-wash steps was implemented to save buffer volume during scale-up chromatography, as well as process time for desalting and concentration. A step-elution protocol was preferred over gradient elution to obtain a concentrated elution pool of the purified product and to develop a robust scale-up process. The chromatogram in Figure 2 illustrates purification of a 249.3-mg oligonucleotide load using step elution. The analytical RP−IP HPLC chromatogram of the elution pool from the 249.3-mg load on the 10-mL strong anion-exchange membrane chromatography purification shown

***Table 2.*** **One-liter strong anion-exchange membrane chromatography process yield and LC−MS purity of the full-length 20-mer phosphorothioate oligonucleotide**

| | amount of full-length oligonucleotide | % yield or purity |
|---|---|---|
| yield from pooled main elution fractions from six first-pass cycles | 106.6 g | 68.6 |
| yield from single recycling of flow-through and all wash pools from six first-pass cycles | 24.5 g | 15.8 |
| total chromatography yield | 131.1 g | 84.4 |
| Purity of full-length oligonucleotide by LC−MS | | 95.0 |

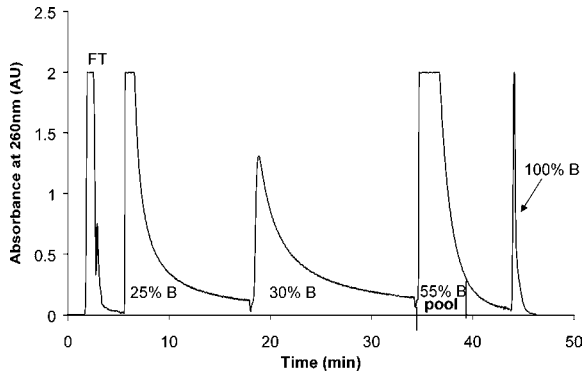



***Figure 2.*** **Chromatogram of 284 mg (249.3 mg based on 87.7% purity in full-length oligonucleotide) load of a 20-mer phosphorothioate oligonucleotide purified by step elution on a 10-mL strong anion-exchange membrane chromatography module. A: 20 mM NaOH, B: 2.5 M NaCl in A, loaded sample at 10 mg oligonucleotide/mL, flow rate: 30 mL/min, unbound oligonucleotide was washed with 8 membrane volumes (MV) of solution A, elution: step to 25% B and hold for 30 MV, step to 30% B and hold for 30 MV, step to 55% B and hold for 20 MV, step to 100% B and hold for 6 MV.**

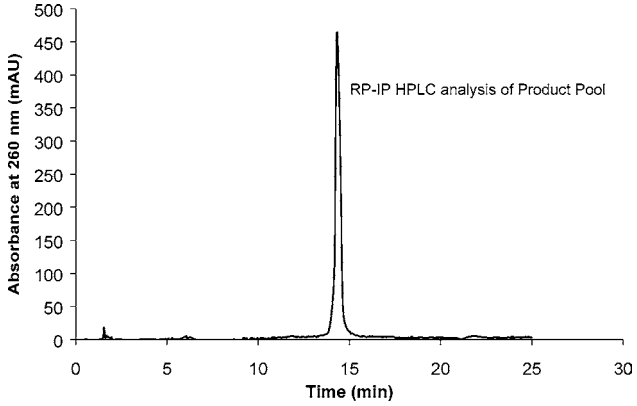



***Figure 3.*** **Analytical RP−IP HPLC of the product pool in elution from 284 mg of oligonucleotide load on a 10-mL strong anion-exchange membrane chromatography module.**

in Figure 3 indicates that the product contains greater than 99.0% full-length oligonucleotide. The low-salt washes comprising the 25% B and 30% B washes effectively removed the short-mer and phosphodiester impurities in the sample. This is illustrated in the RP−IP HPLC analytical chromatogram of the 30% B wash pool shown in Figure 4. Since the impurities are closely related to the product with respect to ionic charge, minor product losses may be

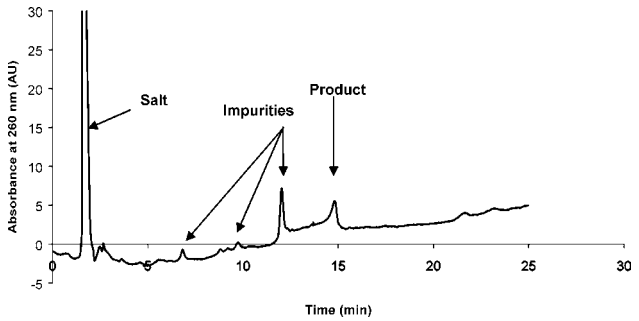



***Figure 4.*** **Analytical RP−IP HPLC of a pool of the 30% B low-salt wash.**

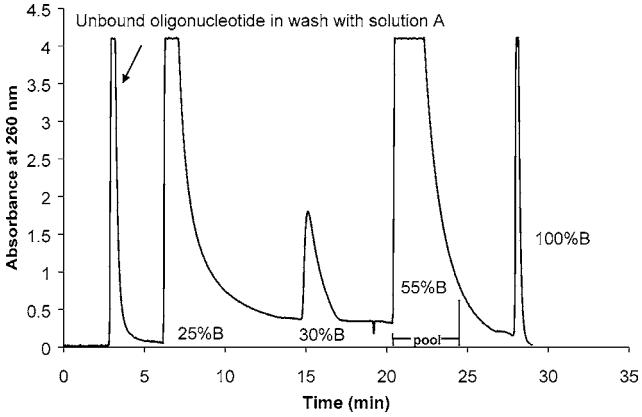



***Figure 5.*** **Chromatogram of 30-g load (26 g of full-length oligonucleotide based on 87.7% product purity) of a 20-mer phosphorothioate oligonucleotide purified by step elution on a 1-L strong anion-exchange membrane chromatography module. A: 20 mM NaOH, B: 2.5 M NaCl in A, loaded sample at 10 mg oligonucleotide/mL, flow rate: 3 L/min, unbound oligonucleotide was washed with 8 membrane volumes (MV) of solution A, elution: step to 25% B and hold for 30 MV, step to 30% B and hold for 30 MV, step to 55% B and hold for 30 MV, step to 100% B and hold for 6 MV.**

recovered by recycling the two low-salt wash pools. The 177.1 g (155.3 g of product based on 87.7% LC−MS purity of the full-length oligonucleotide) was purified in six separate chromatography cycles of between 28 and 30 g per load. A typical chromatogram from the 1-L strong anion-exchange membrane chromatography purification is shown in Figure 5. The yield from pooled elution fractions from six first-pass cycles on the 1-L strong anion-exchange membrane chromatography unit was 68.6% (Table 2). The two salt-wash fractions were recycled separately with the same chromatographic conditions as listed in the caption of Figure 5. This recycle resulted in the recovery of an additional 15.8% of the full-length oligonucleotide. The purified product was pooled for concentration and desalting by tangential flow filtration. The overall chromatography process yield was 84.4% (Table 2). The total chromatography time (including cleaning, regeneration, and equilibration with loading buffer) with the six ∼30 g loads and two recycle runs was 9 h. The key to maintaining high resolution during scale-up with the 1-L strong anion-exchange membrane chromatography was self-displacement and development of robust step gradients. Implementation of AX in the manufacture of therapeutic ASO has the potential to significantly reduce the drug development and manufacturing costs. The results from the

***Table 3.*** **Ultrafiltration and diafiltration of a 20-mer phosphorothioate oligonucleotide purified by 1-L strong anion-exchange membrane chromatography module**

| process | cross-flow rate (L/min/m$^2$) | TMP (MPa) | feed volume (L) | final oligo concentration (mg/mL) | diafiltration volumes | process time (h) |
|---|---|---|---|---|---|---|
| concentration[a] | 6 | 0.28 | 103 | – | – | 11 |
| diafiltration[b,c] | 6 | 0.28 | 3.5 | 35.2 | 5 | 3 |

[a] Concentration: 2 × 0.5 m$^2$ 650-Da MWCO Omega membrane cassettes, temperature 25 °C at an average filtration flow rate of 9.5 L/m$^2$/h. [b] Diafiltration: Same filtration conditions as in concentration. The conductivity decreased from 110 to 7 mS/cm in a total diafiltration volume of 18 L. [c] 98.2% combined recovery of oligonucleotide from concentration and diafiltration.

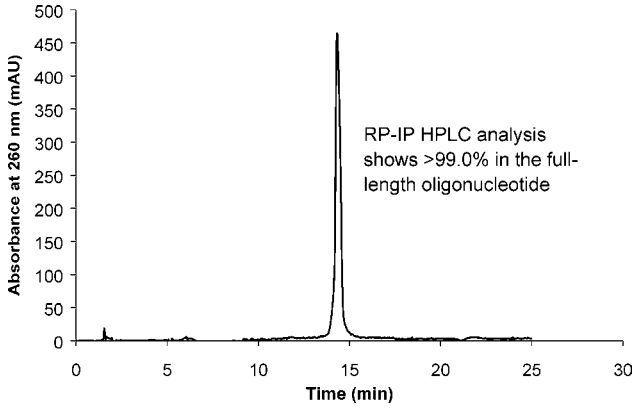



***Figure 6.*** **RP−IP HPLC analysis of the strong anion-exchange membrane chromatography-purified 20-mer phosphorothioate oligonucleotide after desalting by tangential flow filtration.**

1-L strong anion-exchange membrane chromatography process indicates that it provides an aqueous-based, high-resolution, high-throughput purification method for phosphorothioate oligonucleotides.

Ultrafiltration of the 1-L strong anion-exchange membrane chromatography purified product in 103 L was concentrated to 3.5 L to a final concentration of 35.2 mg/mL in 11 h using two 0.5 m$^2$ 650-Da MWCO Omega membrane cassettes at a cross-flow filtration rate of 6 L/min/m$^2$ with a transmembrane pressure (TMP) of 0.28 MPa and average filtrate flow rate of 9.5 L/m$^2$/h (Table 3).

Desalting was performed in the continuous diafiltration mode immediately following ultrafiltration using the same experimental setup. At a cross-flow filtration rate of 6 L/min/m$^2$, with TMP of 0.28 MPa and average filtrate flow rate of 9.5 L/m$^2$/h (Table 3), it took 5 diafiltration volumes (diafiltration volume = 3.5 L) for the conductivity of the product to decrease from 110 to 7 mS/cm in 3 h. The diafiltration was stopped when the conductivity of the filtrate stream remained unchanged with two successive diafiltration volumes. The purity of the final product did not change after the diafiltration step. This was confirmed by HPLC analysis (Figure 6 and Table 4). The scale-up chromatography and tangential flow filtration were operated simultaneously for paucity of refrigerated storage space. The sizing of ultrafiltration and diafiltration system (1 m$^2$) was chosen on the basis of the expected volume and process time for membrane chromatography and to maximize recovery. The chromatography process time could be reduced to under an hour with a 5-L bed volume membrane unit. Similarly, increasing the membrane area could have reduced the tangential flow filtration process time. Using fully automated chromatography and TFF systems could also further reduce the process time.

***Table 4.*** **Process yield and purity of the full-length 20-mer phosphorothioate oligonucleotide from scale-up of ultrafiltration and diafiltration by tangential flow filtration**

| | results |
|---|---|
| total recovery from ultrafiltration and diafiltration process | 98.2% |
| LC−MS purity of the full-length oligonucleotide in the final product | 95.0% |
| RP−IP HPLC purity in the full-length oligonucleotide | > 99.0% |
| concentration of the oligonucleotide in the final product | 35.2 g/L |
| conductivity of the final product | 7 mS/cm |

## 4. Conclusions

A 177-g sample of a 20-mer phosphorothioate oligonucleotide was enriched in full-length oligonucleotide product from 87.7 to 95.0% using a high-resolution 1-L membrane AX unit. The purified product was concentrated from 103 to 3.5 L and desalted in deionized water with 5 diafiltration volumes. The overall chromatography process yield was 84.4% of the full-length oligonucleotide in the final product pool. Membrane ion-exchange chromatography proved to be a scaleable, robust, and high-throughput method for the purification of a therapeutic phosphorothioate oligonucleotide. To the best of our knowledge this work represents the first large-scale demonstration of a membrane-based AX purification protocol that is useful for polishing step and generation of a reference standard with significantly increased product purity (~7%).

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## List of Abbreviations

| | |
|---|---|
| ASO | antisense oligonucleotide |
| AX | anion-exchange chromatography |
| cGMP | current good manufacturing practices |

| | |
|---|---|
| DMT | dimethoxy trityl |
| HIC | hydrophobic interaction chromatography |
| mRNA | messenger RNA |
| MV | membrane volume |
| MWCO | molecular weight cutoff |
| *n*-mer | 20-mer full-length oligonucleotide |
| *n*−1 mer | 19-mer full-length oligonucleotide |
| *n*+1 mer | 21-mer full-length oligonucleotide |
| NWP | nominal water permeability |
| (P═O) | phosphodiester |
| $(P{=}O)_n$ | phosphodiesters |
| (P═S) | phosphorothioate |
| $(P{=}S)_n$ | phosphorothioates |
| Q | quaternary ammonium |
| RP-HPLC | reversed-phase HPLC |
| RP−IP HPLC | reversed phase−ion-pairing HPLC |
| TFF | tangential flow filtration |
| TMP | transmembrane pressure |

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