High Resolution Anion Exchange Chromatography Purification of Oligonucleotides

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Abstract

The use of oligonucleotides as therapeutics continues to grow each year. These include both RNA and DNA based oligonucleotides. Using high resolution anion exchange chromatography resins, a crude oligonucleotide was purified to greater than 98%. Initial experiments indicated that at a higher pH better selectivity was obtained, thus requiring a strong anion exchange resin. For TSKgel SuperQ-5PW resins (both 20 and 30µm nominal particle size), pH 9.0 gave optimal results. Not surprisingly, a longer column (15 vs 7.5cm) gave better resolution between the main peak and the N-1 (and smaller) moieties. The 1000Å pore size of this resin offered excellent binding kinetics and almost quantitative recoveries. The TSKgel SuperQ-5PW capacities were superior to competitive resins even under saturation conditions. This resin is ideal for use in oligonucleotide purifications at all process scales.

Introduction

Oligonucleotides are short, linear sequences of deoxyribonucleic acid or ribonucleic acid generally made through chemical synthesis. Many oligonucleotide products produced by biopharmaceutical companies are working their way through clinical trials. The interest in using oligonucleotides as therapeutic agents continues to grow each year.

Because of the unique structure of these molecules and the way that they are synthesized, oligonucleotides require special considerations during chromatographic purification. During the synthesis of the oligonucleotide there are a small percentage of "failure" sequences (N-1 is the common nomenclature) that taken collectively may produce measurable amounts of impurity. The similarity in the impurities requires high resolution techniques to adequately purify the final product. Typically reversed phase and high resolution anion exchange chromatography are the two most frequent modes used. This poster describes the use of high resolution anion exchange chromatography for the purification of a 20-base-length synthetic deoxyribonucleotide.

Experimental

Oligonucleotide:

The phosphodiester oligonucleotide (20-mer) used in this study had the following sequence:

5' - GAA TTC ATC GGT TCA GAG AC - 3'

and was purchased unpurified (estimated at 34% purity by HPLC) in lyophilized form from Trilink Biotechnologies, San Diego, CA. The extinction coefficient was 199.9 OD units/μmole and the molecular weight of the free acid was 6140.9 Da. This sequence was chosen to minimize the amount of secondary structure effects during the purification experiments.

Prior to purification the oligonucleotide was reconstituted in 20mmol/L Tris-HCl at pH 9.0 containing 10mmol/L EDTA.

Experimental

Analytical Analysis

Representative samples and fractions were analyzed using a TSKgel DNA-NPR column (4.6mm ID x 7.5cm L) run on an Agilent 1100 HPLC system. The TSKgel DNA-NPR is a 2.5µm non porous resin for the rapid separation of large biomolecules like oligonucleotides and DNA fragments. Oligonucleotide fractions from each of the columns were injected (15µL) onto the analytical column untreated. After injection a linear gradient from Buffer A (20mmol/L Tris-Cl pH 9.0) to B (Buffer A with 1.0mol/L NaCl) was run over 15 minutes and all samples were eluted within 10 minutes time. Quantitative analysis was obtained using Agilent's ChemStation software. Representative chromatograms are shown in the figures.

Experimental

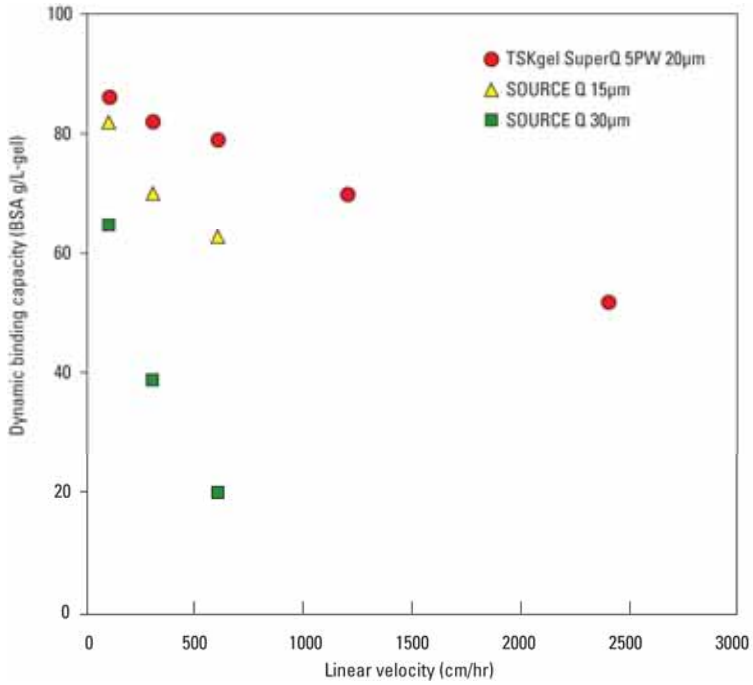
Chromatographic Resins

The TSK-GEL SuperQ-5PW resins used in this study are highly crosslinked methacrylate resins functionalized with quaternary amine groups. This strong anion exchange resin has an estimated pore size of 1000Å. This resin was supplied in bulk containing 20 or 30µm particle sizes and was obtained from Tosoh Bioscience LLC, Montgomeryville, PA. For comparison purposes, Source 15Q, a 15µm quaternary anion exchange resin from GE Healthcare, Piscataway, NJ was also evaluated. All resins were packed into 0.66cm Omnifit columns at the desired length and evaluated for packing efficiency prior to use.

Experimental Conditions

The conditions for running the chromatographic columns are listed in each figure.

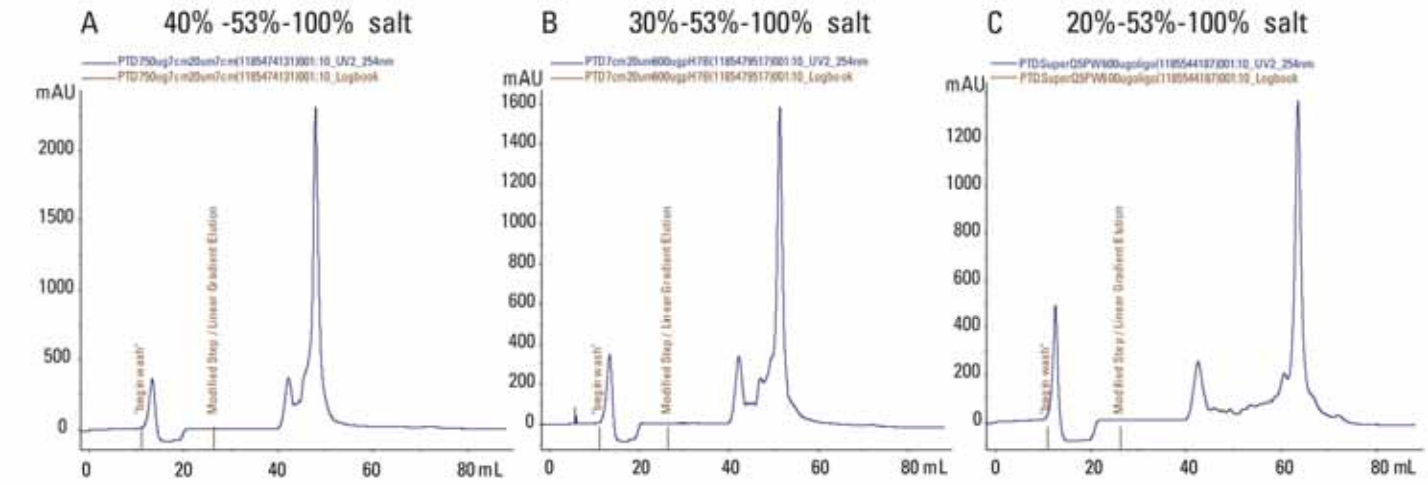
Figure 1: Dynamic Binding Capacity of Commercially Available High Resolution Anion Exchange Resins



Column:	4.6mm ID x 5cm
Sample:	10g/L BSA in 20mmol/L Tris-HCl buffer pH8.5
Flow rate:	100-1200cm/hr
Temperature:	ambient
Detection:	280nm

The capacities of the resins were determined at 10% breakthrough and at different flow rates using bovine serum albumin (BSA) under the conditions listed. For this protein, the TSKgel SuperQ 5PW (20) gave the highest capacity at all flow rates. Based on this data it was assumed that the binding capacities of the resin for oligonucleotides would follow the same trend, albeit not at the same values. This data was provided by Tosoh Corporation, Tokyo, Japan.

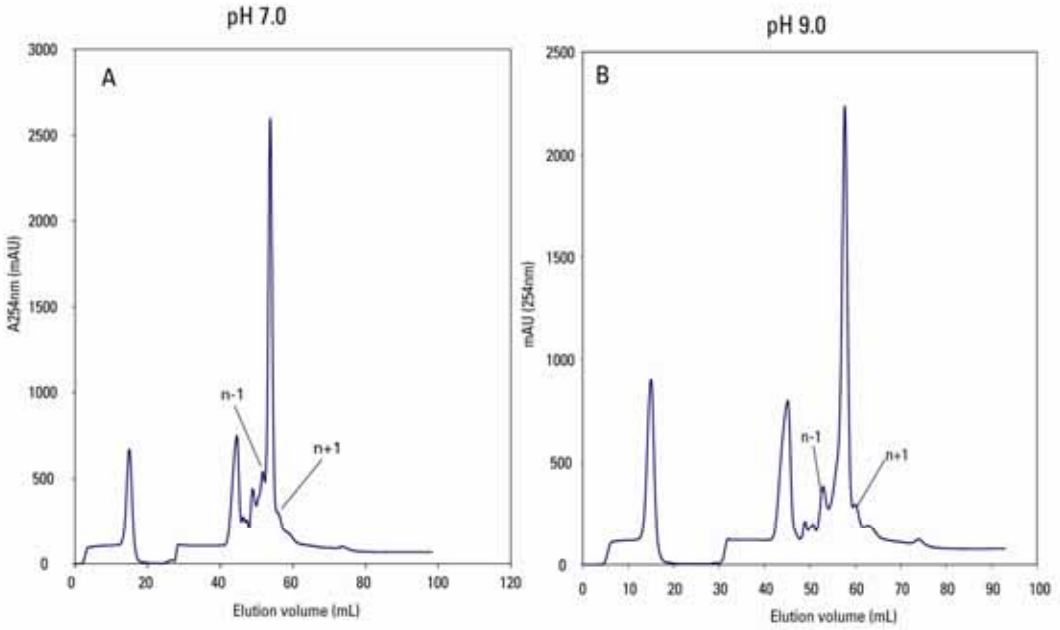
Figure 2: Gradient Optimization on TSKgel SuperQ-5PW (20)



Column:	TSKgel SuperQ-5PW (20) in 0.66cm x 7.5cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 7.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 7.0
Sample:	DNA based oligonucleotide (28mg/mL) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 9.0 (buffer A)
Sample loaded:	1mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11ml injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 40%-50% buffer B (panel A), 30%-53% buffer B (panel B) and 20%-53% buffer B (panel C). Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Abs@254nm

Various modifications of the gradient were attempted to optimize the separation of the closely related species. For example, in the first panel the column was washed with 2 column volumes (CV) of 100% A, followed by a step to 40% B and a linear gradient over 3CV to 53% B and finally a step to 100% B for 5CV. This is represented as a gradient 40 - 53 - 100. The optimized gradient in these experiments was 35 - 53 - 100.

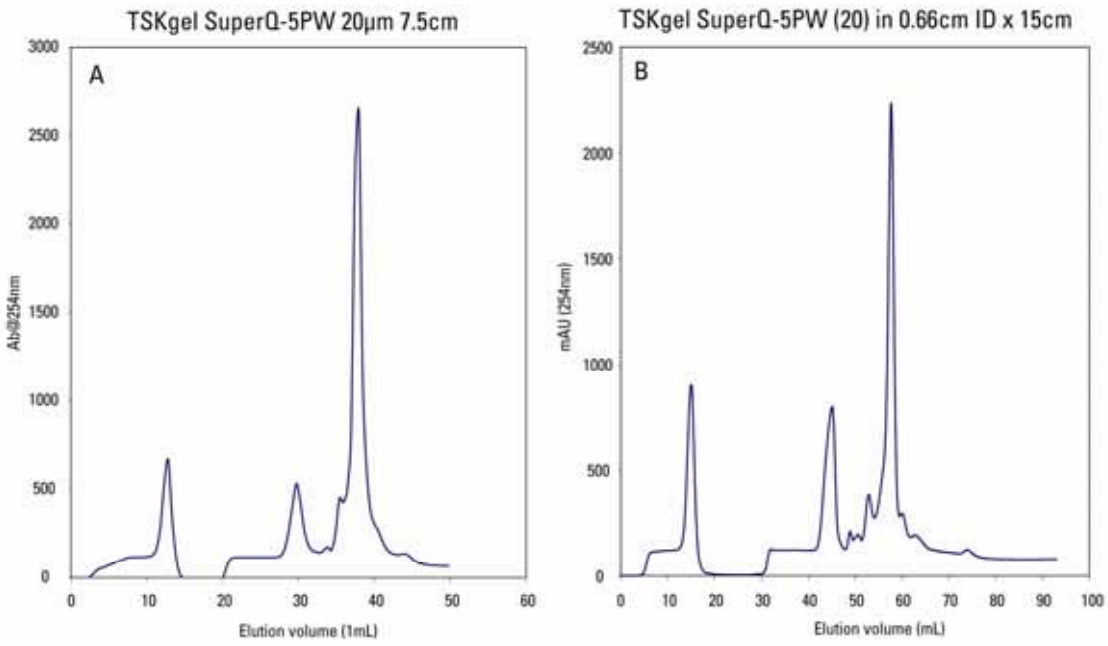
Figure 3: Optimization of pH using TSKgel SuperQ-5PW (20)



Column:	TSKgel SuperQ-5PW (20) in 0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	Fig.A: 20mmol/L Tris-HCl + 10mmol/L EDTA pH 7.0; Fig. B: 20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	Fig.A: 20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 7.0; Fig. B: 20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample:	DNA based oligonucleotide (28mg/ml) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 7.0 (buffer A)
Sample loaded:	1mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Abs@254nm

TSKgel SuperQ-5PW (20) was run at pH 7.0, 8.0 and 9.0. In all cases good selectivity was observed. The gradient conditions were then adjusted to give the best apparent resolution between the desired product, n-1 and the n+1 oligonucleotides. The optimal gradient at pH 7.0 and pH 9.0 are shown and is 35 - 53 - 100 (see next figure).

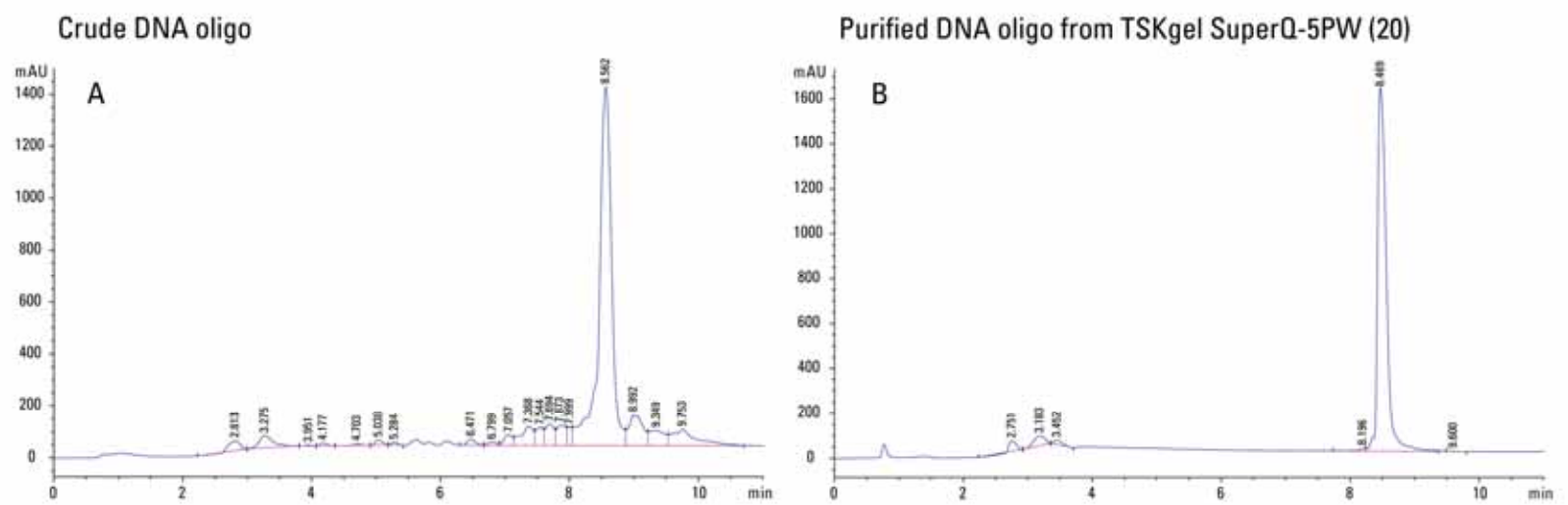
Figure 4: Effect of Column Length on Selectivity



Column:	A: TSKgel SuperQ-5PW (20) in 0.66cm x 7.5cm; B: TSKgel SuperQ-5PW (20) in 0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample:	DNA based oligonucleotide (28mg/mL) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 9.0 (buffer A)
Sample loaded:	1mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Abs@254nm

Not surprisingly increasing the column length from 7.5 to 15cm increased the resolution between the n-1 and n+1 impurities from the main peak. This also increased the pressure (data not shown).

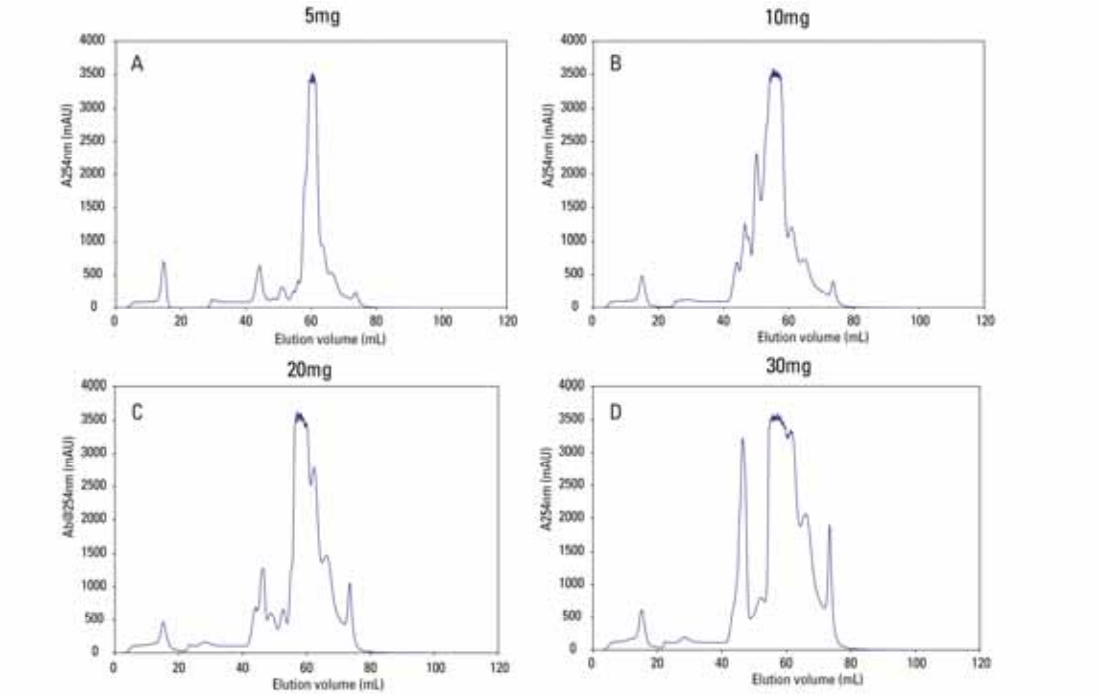
Figure 5: Analysis of fractions from column using TSKgel DNA-NPR Analytical Column



Column:	TSKgel DNA-NPR (4.6mm ID x 7.5cm)
Flow rate:	0.7mL/min
Buffer A:	20mmol/L Tris-HCl pH 9.0
Buffer B:	20mmol/L Tris-HCl + 1.0mol/L NaCl pH 9.0
Sample:	Panel A: 2mg crude DNA oligo in 20mmol/L Tris-HCl at pH 9.0; Panel B: 2mg purified DNA oligo in 20mmol/L Tris-HCl at pH 9.0
Sample loaded:	15µL
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Detection:	Abs@260nm

Either crude oligonucleotide or fractions from the various columns were run on a TSKgel DNA-NPR column. The injection volumes were 15µL and were untreated. The software was used to calculate peak purity. Recoveries were calculated by pooling fractions and checking the total volume and oligonucleotide present in the sample, compared to the quantity of oligonucleotide injected onto the TSKgel SuperQ-5PW column.

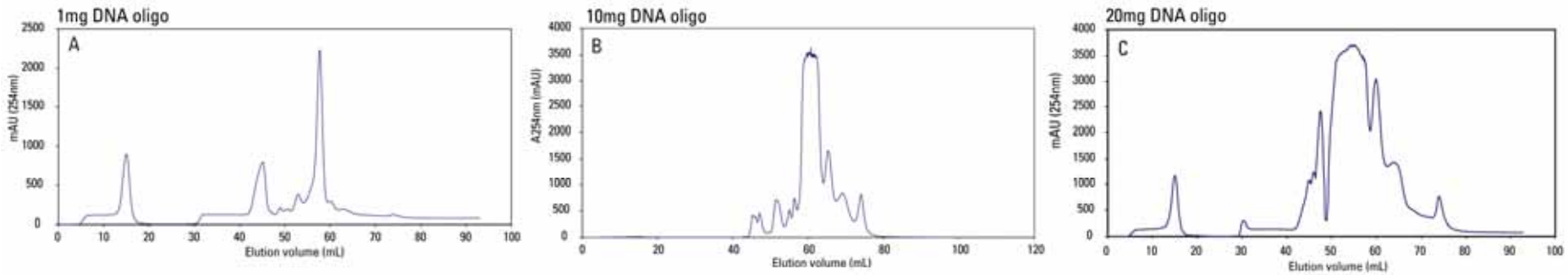
Figure 6: TSKgel SuperQ-5PW (30) Loading Study



Column:	TSKgel SuperQ-5PW (30) in 0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample:	DNA based oligonucleotide (28mg/mL) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 9.0 (buffer A)
Sample loaded:	A: 5mg/column, B: 10mg/column; C: 20mg/column; D: 30mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Ab@254nm

Increasing amounts of oligonucleotide were injected onto the columns. Although the peak quickly went off-scale at 254nm, the 30mm TSKgel SuperQ-5PW column was able to maintain good selectivity. By carefully selecting the fractions good purity and yield were obtained (see below).

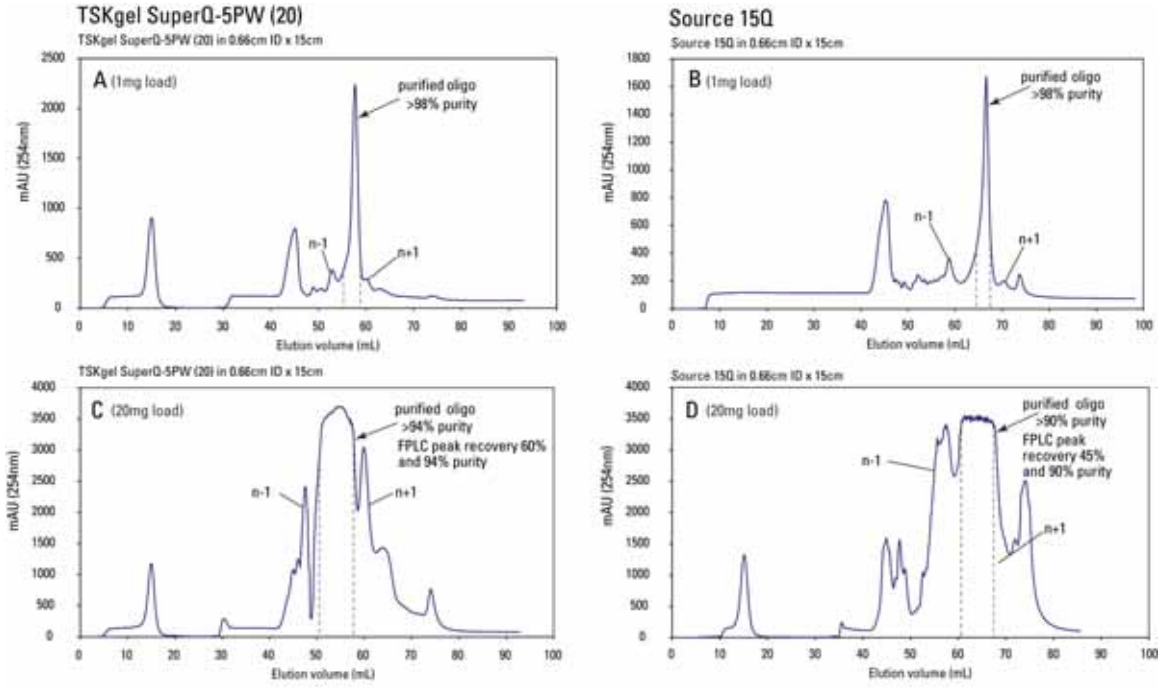
Figure 7: TSKgel SuperQ-5PW (20) Loading Study



Column:	TSKgel SuperQ-5PW (20) in 0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample:	DNA based oligonucleotide (28mg/ml) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 9.0 (buffer A)
Sample loaded:	A: 1mg/column; B: 10mg/column; C: 20mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Abs@254nm

Increasing amounts of oligonucleotide were injected onto the columns. Although the peak quickly went off-scale at 254nm, the 20µm TSKgel SuperQ-5PW column was able to maintain excellent selectivity. By carefully selecting the fractions excellent purity and yield were obtained (see below).

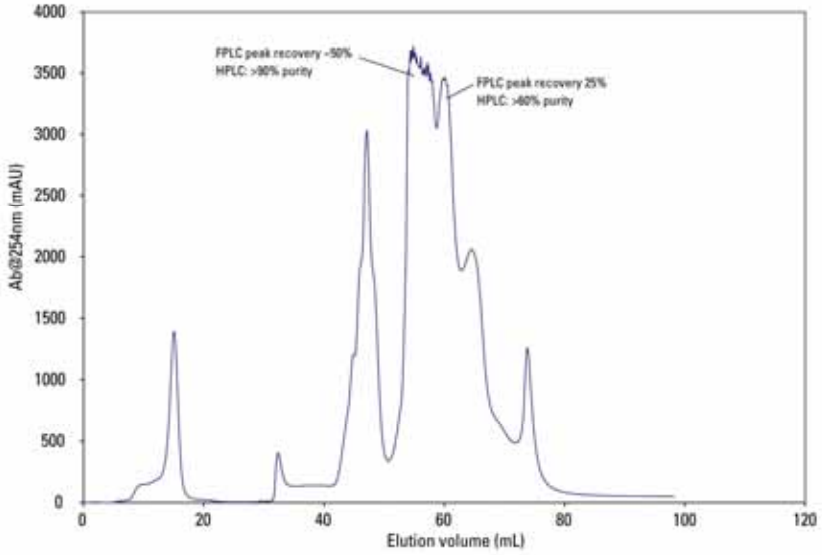
Figure 8: Comparison of High-Resolution Anion Exchange resins



Column:	TSKgel SuperQ-5PW (20) in 0.66cm x 15cm (5.1mL)
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample loaded:	DNA based oligonucleotides were loaded as followed: 1mg/column panels A & B, 20mg/column panels C & D
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Detection:	Ab@254nm
Fractions:	0.5mL fractions were taken from peak of interest and analyzed on TSKgel DNA-NPR column (HPLC system)

At low loading conditions the Source 15Q gave as good if not better selectivity when compared to the TSKgel SuperQ-5PW 20µm resin. However as the load was increased to at least 20mg per column the TSKgel SuperQ-5PW surpassed the Source 15Q material for peak recovery and purity.

Figure 9: TSKgel SuperQ-5PW (20) Loaded with 30mg Crude Oligonucleotide



Column:	TSKgel SuperQ-5PW (20) in 0.66cm x 15cm
Flow rate:	1.43mL/min (250 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA pH 9.0
Buffer B:	20mmol/L Tris-HCl + 10mmol/L EDTA + 1.0mol/L NaCl pH 9.0
Sample:	DNA based oligonucleotide (28mg/mL) in 20mmol/L Tris-HCl with 10mmol/L EDTA at pH 9.0 (buffer A)
Sample loaded:	30mg/column (sample was diluted with buffer A to a final volume of 10mL)
Separation conditions:	Column is washed with 5CV 100% buffer A followed by 11mL injection. Column is then washed with 3CV 100% buffer A followed by 6CV of linear gradient 35-53 buffer B. Finally, column is washed with 5CV 100% buffer B.
Fraction:	0.5mL fractions are taken over gradient containing peak of interest and analyzed on a TSKgel DNA-NPR column.
Detection:	Abs@254nm

At the highest loads tested in this study, the TSKgel SuperQ-5PW (20) still exhibited good selectivity and resolution. Peak purity decreased slightly as well as recoveries. The purity and recovery are still adequate for most clinical trial requirements.

Table 1: Summary of optimal conditions for purifying crude 20-mer oligonucleotide on TSKgel SuperQ-5PW resins

Column	TSKgel Super-5PW 20µm, 15cm	TSKgel Super-5PW 30µm, 15cm
Optimal pH	9	9
Optimal gradient	35-53-100	35-53-100
Recovery	Up to 75%	Up to 75%
Peak purity	>94%	>94%
Loading amount (per column)	<30mg	<30mg

Conclusions

- Crude oligonucleotide can be applied directly to a TSKgel SuperQ-5PW 20 and 30µm resin with excellent results.
- The pH optimum for purification of a 20-mer DNA-based oligonucleotide was determined to be at pH = 9.0.
- The best conditions for gradient elution were a 2 column volume wash after injection; a step to 35% B (0.34 mol/L NaCl) followed by a linear gradient to 53% B (0.53 mol/L NaCl) over 3 column volumes and finally a wash at 100% B.
- By judiciously selecting fraction collection points, high purity (>94%) and good yield (>50%) were obtained on a 0.66cm ID X 15cm (5.13mL) column.
- Although Source 15Q gave excellent selectivity at low load conditions, at higher loads, the usefulness deteriorated quickly.
- A TSKgel DNA-NPR analytical column was a useful tool for rapid analysis of each fraction (untreated fractions) giving quantitative analysis in under 10 minutes.