Purification of oligonucleotides using anion-exchange chromatography

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Abstract

Synthetic oligonucleotides continue to grow as biotherapeutic agents. Initial experiments described in this poster focused on a one-step purification using small particle size anion exchange chromatography resins. The best selectivity was found at pH=9.0. Not surprisingly, longer columns (up to 20cm) gave better resolution between the product peak, the N-1 and N+1 moieties. A larger particle-size resin exhibited more flexibility when applying simple gradients, whereas, the smaller particle-size chromatography resins performed best when the gradient was modified. A crude (<50% pure) 20-mer oligonucleotide was purified to greater than 98% with >80% recovery in a single step using the 20µm TSK-GEL SuperQ-5PW resin and 95% purity with >75% recovery using the 75µm Toyopearl GigaCapQ-650M resin. Purity was confirmed by HPLC and gel electrophoresis. The 1000Å pore size of these resins offered excellent binding kinetics and less peak tailing. Both resins were tested under low- and high-loading conditions and both exhibited excellent resolution and recovery of the oligonucleotide tested. The conditions presented in this poster indicate that these anion exchange chromatography resins can be efficient in both small and large-scale purification unit-operations.

Introduction

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

Because of the unique structure of these molecules and the way 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 modes used most frequently. This poster describes the use of high resolution anion exchange chromatography for the purification of a 20-base-length single stranded synthetic deoxyribonucleotide.

Experimental

Oligonucleotide:

The phosphodiester deoxyoligonucleotide (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 54% purity by HPLC) in lyophilized form from Trilink Biotechnology, San Diego, CA. The extinction coefficient was 199.9 OD units/µmol 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=7.0 containing 10mmol/L EDTA

Experimental

Analytical Analysis

Representative crude samples and chromatographic 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-HCl pH=8.0) to B (buffer A + 1.0mol/L NaCl) was run over 23 minutes. All samples were eluted in the first 10 minutes of the gradient. Quantitative analysis was obtained using Agilent's ChemStation software. Representative chromatograms are shown in the figures.

Gel electrophoresis

Both crude and purified oligonucleotides were analyzed by gel electrophoresis for further confirmation of purity. Approximately 5µg from the purified oligonucleotide fractions were loaded onto a 20% PAGE gel run in TBE (from Invitrogen) and separated at a constant voltage (150V) until the tracking dye migrated to the bottom of the gel. The gel was then fixed and visualized using silver stain according to the manufacturer's directions.

Experimental

Chromatographic Resins

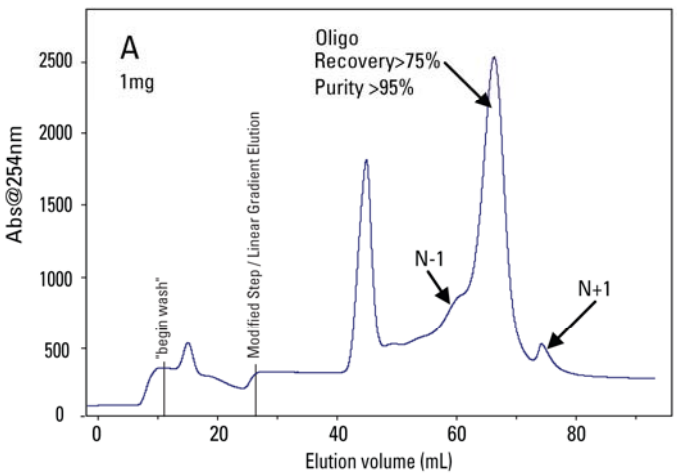
The TSKgel SuperQ-5PW (20) and Toyopearl GigaCap Q-650M strong anion exchange resins were used in this study. They are highly crosslinked methacrylate resins functionalized with quaternary amine groups. These resins have an estimated pore size of 1000Å. All resins were packed into 0.66cm Omnifit columns at the desired length and evaluated for packing efficiency prior to use.

Experimental Conditions

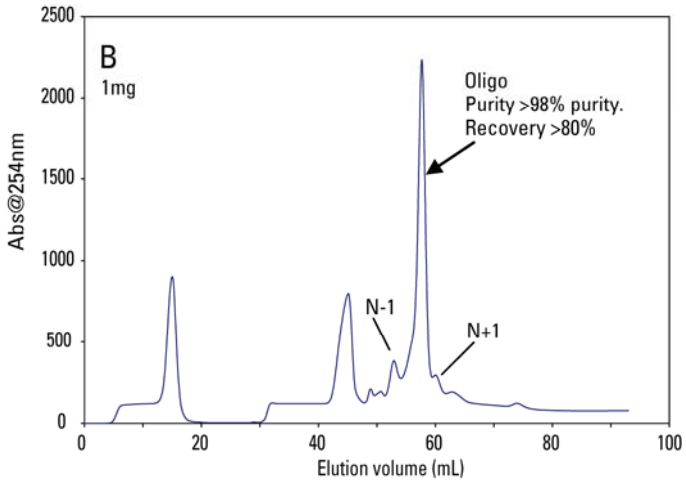
The conditions for running the chromatographic columns are listed in each figure.

Figure 1: Purification of oligonucleotides using Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) under optimal linear gradients and pH conditions

Toyopearl GigaCap Q-650M
35-73% 1mol/L NaCl, pH=8.0



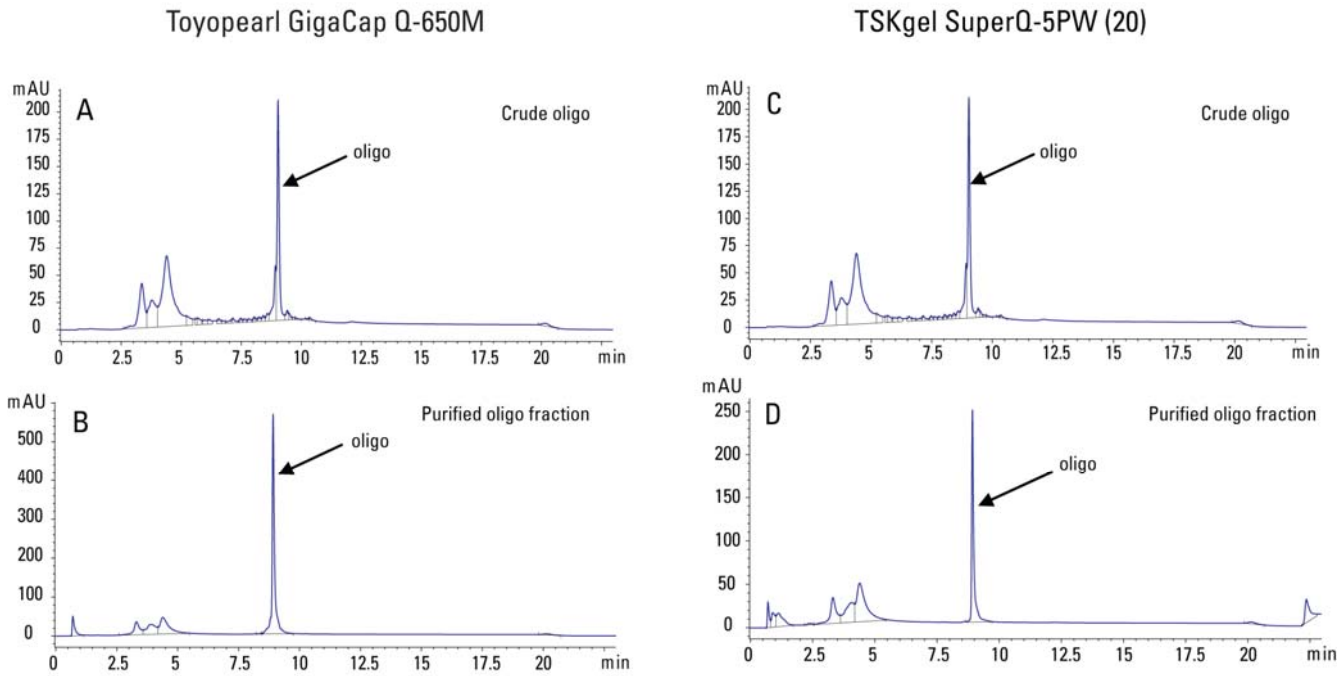
TSKgel SuperQ-5PW (20)
35-53% 1mol/L NaCl, pH=9.0



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (212 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA
Buffer B:	buffer A + 1.0mol/L NaCl
Sample loaded:	1mg/column (in 11mL)
Operating conditions:	Column is washed with 5CV 100% buffer A followed by sample injection. Column is then washed with 3CV 100% buffer A followed by a 6CV linear gradient from 35-73% or 35 - 53% buffer B. Finally, the column is washed with 5CV of 100% buffer B.
Detection:	Abs@254nm

Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) were run at different pH and gradient conditions. In all cases, good selectivity was observed. The pH and gradient conditions were adjusted to give the best apparent resolution between the desired product, N-1 and N+1 oligonucleotides. The optimal gradient and pH are shown for both resins.

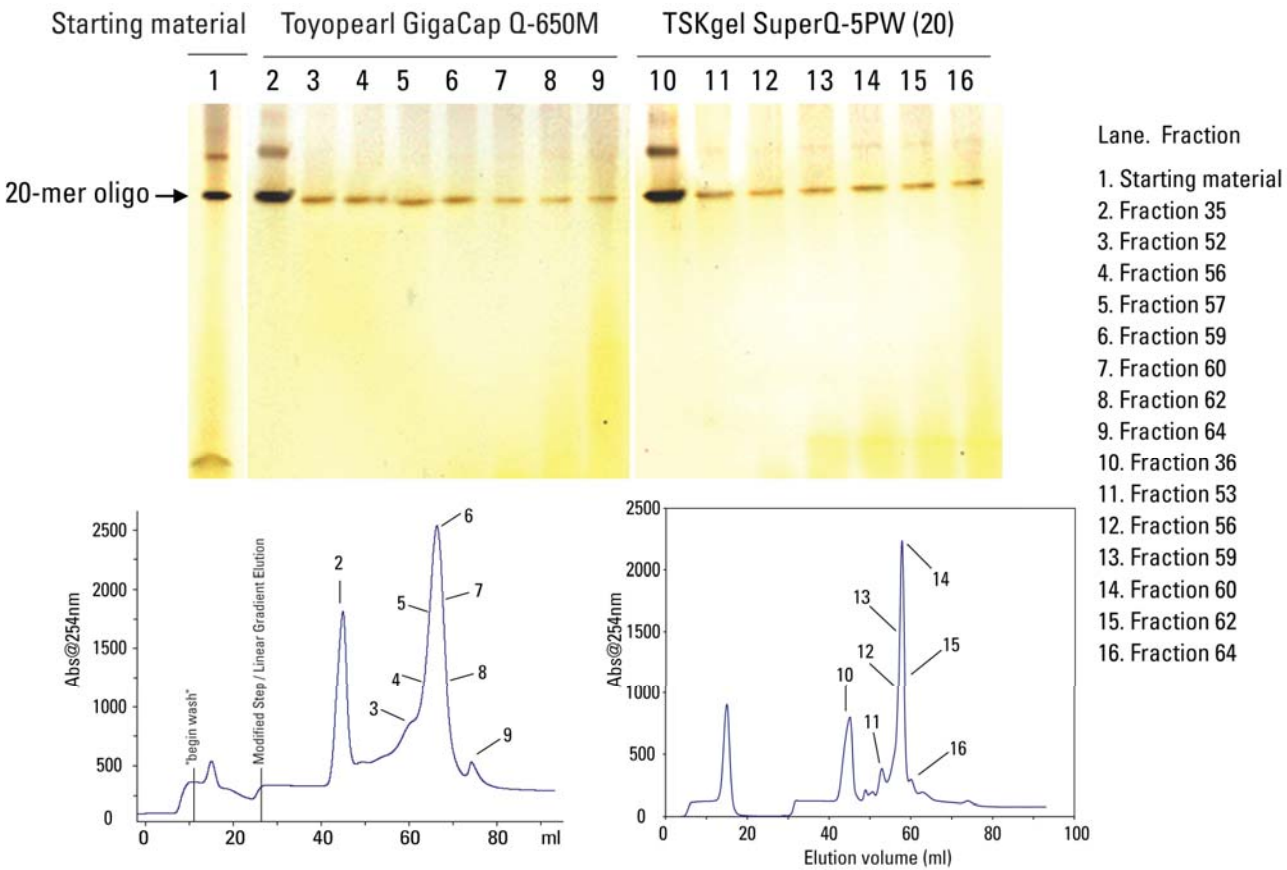
Figure 2: HPLC analysis of purified oligonucleotide from Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) using TSKgel DNA-NPR column



Column:	4.6mm x 7.5cm
Flow rate:	0.7mL/min
Buffer A:	20mmol/L Tris-HCl, pH=8.0
Buffer B:	buffer A + 1.0 mol/L NaCl
Sample loaded:	15µL of 0.4mg/mL collected fraction
Separation conditions:	Column is washed with 5CV 100% buffer A followed by a 15µL injection. Column was then run with a linear gradient from 1-100% buffer B for 15min and washed with 100% A to 23min.
Detection:	Abs@254nm

Both crude and purifed oligonucleotide fractions from the Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) columns were analyzed on a TSKgel DNA-NPR column to assess purity. Data (see Fig. 2 panels B and D) indicated that the oligonucleotide was purified to >96% purity.

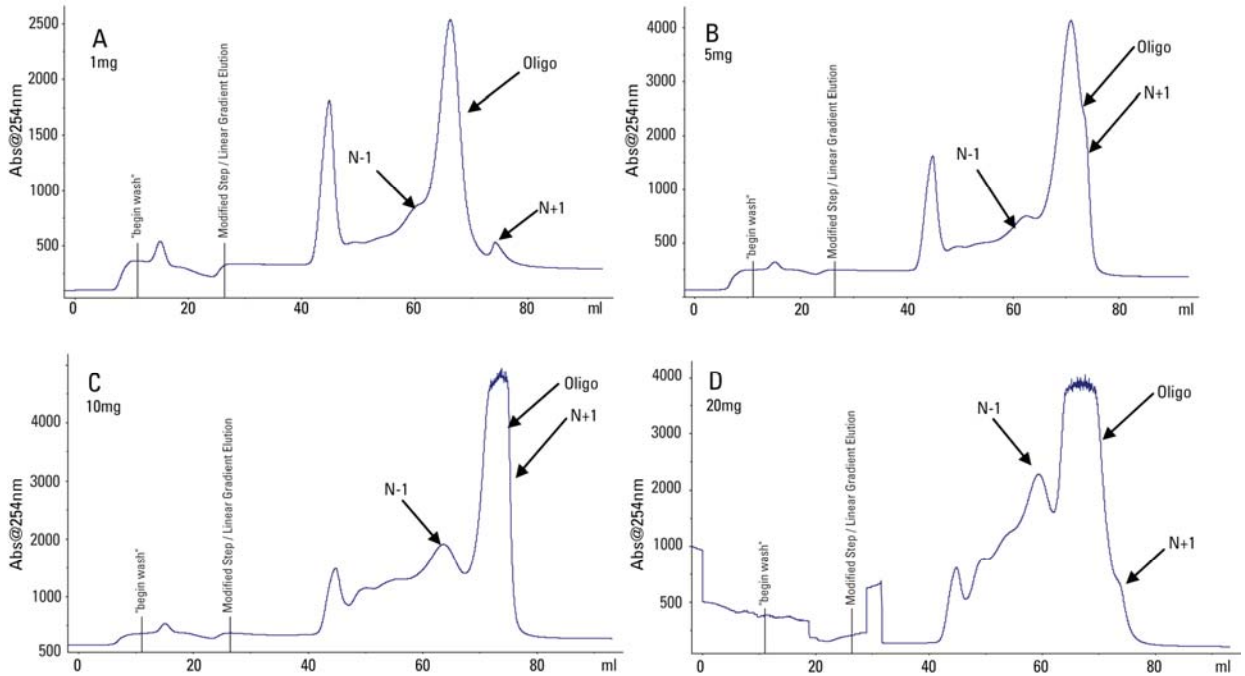
Figure 3: Gel electrophoresis analysis of purified oligonucleotides from Toyopearl GigaCap-650M and TSKgel SuperQ-5PW resin



*20% PAGE-TBE gel 1.0mm with 12 wells. Oligo was stained using manufacturer's silver staining method.

Purity of oligonucleotide from both resins was confirmed by gel electrophoresis. Results showed that purified oligonucleotides from both resins were very clean. Numbers represent the indicated lane on the PAGE gel.

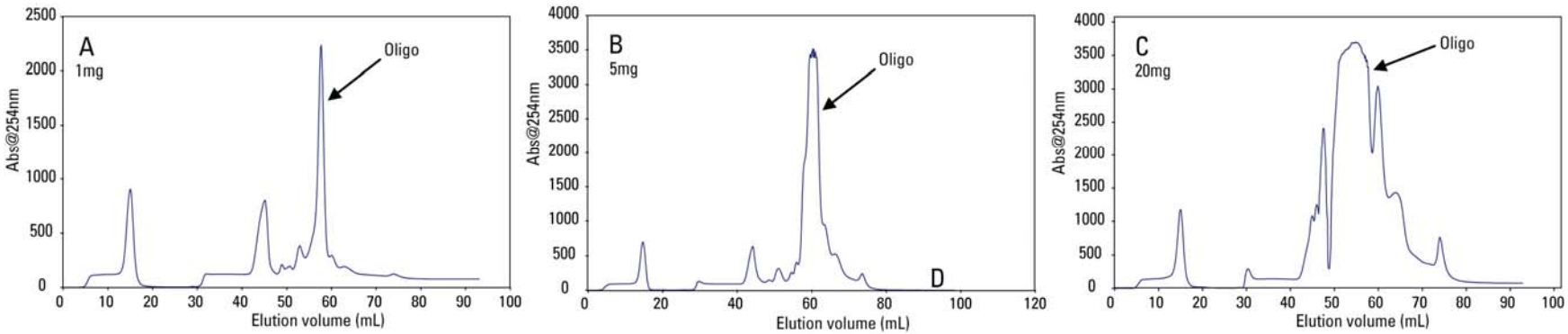
Figure 4: Loading capacity study for Toyopearl GigaCap Q-650M at pH=8.0 and gradient 35-73% gradient of 1mol/L NaCl



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (212 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA
Buffer B:	buffer A + 1.0mol/L NaCl
Sample loaded:	11mg/column (in 11mL)
Operating conditions:	Column is washed with 5CV 100% buffer A followed by sample injection. Column is then washed with 3CV 100% buffer A followed by a 6CV of linear gradient of 35-73% buffer B. Finally, the column is washed with 5CV of 100% buffer B.
Detection:	Abs@254nm

Increasing amounts of oligonucleotide were injected onto the columns. Although the peak quickly went off-scale at 254nm, the highest load (20mg) (Fig. 4, panel D) still exhibited good selectivity and resolution. The identification of the N-1 and N+1 peaks/shoulders were confirmed by analytical HPLC analysis.

Figure 5: Loading capacity study for TSKgel SuperQ-5PW (20) at pH=9.0 and gradient 35-53% gradient of 1mol/L NaCl



Column:	0.66cm x 15cm
Flow rate:	1.43mL/min (212 cm/hr)
Buffer A:	20mmol/L Tris-HCl + 10mmol/L EDTA
Buffer B:	buffer A + 1.0mol/L NaCl
Sample loaded:	1mg/column (in 11mL)
Operating conditions:	Column is washed with 5CV 100% buffer A followed by sample injection. Column is then washed with 3CV 100% buffer A followed by a 6CV of linear gradient of 35-53% buffer B. Finally, column is washed with 5CV 100% of buffer B.
Detection:	Abs@254nm

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

Table 1: Binding capacity of oligonucleotides on Toyopearl GigaCap Q-650Mand TSKgel SuperQ-5PW (20) resins after exposure to caustic

Resin	Toyopearl GigaCap Q-650M	TSKgel SuperQ-5PW (20)
After 50 cycle*	>55mg oligo/mL resin	>45mg oligo/mL resin

*CIP study: column cycled 50 times including 1.0mol/L NaOH cleaning (exposure time at least 1h/cycle)

The capacities of the resins were determined by using oligonucleotide under the condition listed above. Data indicated both Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) have very high binding capacities after exposure to 50 cycles of 1.0mol/L NaOH.

Table 2: Summary of optimal conditions for TSKgel SuperQ-5PW (20) and Toyopearl GigaCap Q-650M resins for oligonucleotide purification

Optimal conditions	TSKgel SuperQ-5PW (20)	Toyopearl GigaCap Q-650M
Sample	20-mer oligonucleotide	20-mer oligonucleotide
pH	9.0 - 9.5	8.0 – 8.5
Modified gradient (1.0mol/L NaCl)*	35-53-100%	35-63-100% and 35-73-100%
Binding capacity (mg/mL resin)**	>45mg	>55mg
Sample purity (%)***	peak fractions >98%	peak fractions >95%
Sample recovery (%)	>80%	>75%

* The linear gradient is accomplished by using 1.0mol/L NaCl.
** Dynamic binding capacity was established after exposing the resin to resins in 1.0mol/L NaOH for 50 cycles and by using > 98% pure oligonucleotide.
***Sample purity was calculated by pooling the highest purity fractions from the column.

Conclusions

- Crude oligonucleotide can be applied directly to Toyopearl GigaCap Q-650M and TSKgel SuperQ-5PW (20) resins with minimal sample preparation.
- The optimal pH for good selectivity on Toyopearl GigaCap Q-650M was between pH 8.0 and 9.0. For TSKgel SuperQ-5PW (20) the optimal pH was 9.0 and above.
- Toyopearl GigaCap Q-650M resin can separate oligonucleotides utilizing a wide-range of gradients (35-55% salt, 35-63% salt and 35-73% salt). TSKgel SuperQ-5PW (20) works well under one specific gradient condition.
- Both resins easily resolve the N+1 and N-1 oligonucleotides.
- Both resins, when used in a single step purification, resulted in >96% purity on the Toyopearl GigaCap Q-650M and >98% purity on the TSKgel SuperQ-5PW (20) resin.
- Recoveries of oligonucleotide on both resins were greater than 75%.
- The binding capacity for the oligonucleotide used in this study on Toyopearl GigaCapQ-650M was at least 55mg/mL of resin and the binding capacity of TSKgel SuperQ-5PW (20) was at least 45 mg/mL.

Future study

- Different cations will be used to determine the effect different salts have on the resin performance.
- The impact of different flow rates on the oligonucleotide purification will be studied.
- RNA based oligonucleotides will be tested on these resins.