# Analytical Separation Of Oligonucleotides Using Strong Anion Exchange and C18 Reversed Phase Chromatography

Justin Steve[1], ChangPo Chen[2], Eric Wickstrom[2] and Atis Chakrabarti[1]

[1] Tosoh Bioscience LLC, King of Prussia, PA 19406
[2] Laboratory of Molecular Design, Thomas Jefferson University, Philadelphia PA 19107

# Introduction

- Biotherapeutic oligonucleotides are predicted to comprise 50% of top 100 products by 2016.
- DNA-based oligonucleotides, as well as RNA-based oligonucleotides, are among many different kinds of biotherapeutic oligonucleotides.
- Synthetic DNA oligonucleotides, mimicking a pseudo-peptide backbone such as Peptide Nucleic Acids (PNA) and their conjugates, have a great potential as biotherapeutics too.
- So it is imperative to have chromatography resins that are capable of meeting the demands for product purity and recovery.
- Seamless scalability will remain an added advantage for a particular resin if it can be used for both analytical and large scale chromatography.

# Introduction

- Typically reversed phase and anion exchange chromatography are the two modes used most frequently in the separations of oligonucleotides.
- For large scale purification, sometimes both reversed phase and ion exchange chromatography (IEX) are used sequentially.
- The predominant feature of nucleic acids in aqueous solution is the ionic nature of their phosphate groups, making IEX the best mode of separation.
- The effect of pH, salt concentration, temperature, and sample load on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, are important.

# Introduction

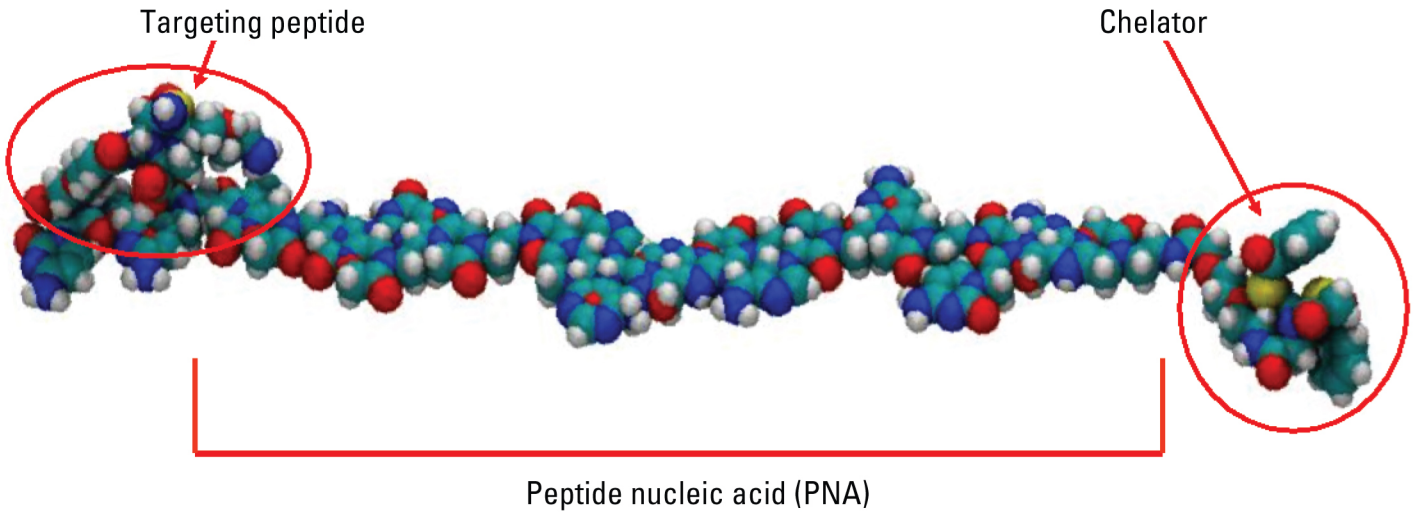
- When purification moves from analytical to manufacturing scale, having the same bonding chemistry is helpful, since the selectivity is expected to remain the same.
- Seamless scalability is important.
- TSKgel SuperQ-5PW analytical columns have the same backbone chemistry and selectivity as the bulk process scale resins *viz.* TSKgel SuperQ-5PW and TOYOPEARL SuperQ-650.
- In this study a TSKgel SuperQ-5PW,10 µm, 7.5 mm ID × 7.5 cm column is used for the one step analysis of a 20-mer DNA-based oligonucleotide.

# Introduction

- Reversed phase chromatographic separations are also used using the weak hydrophobic nature of the nucleic acid bases.
- These interactions are independent of the individual and specific nucleotide base.
- Here we report analyses of
  - A. a DNA-based oligonucleotide
  - B. a PNA-peptide based bioconjugate labeled with visible and near infrared fluorescent dyes, and with radionuclides.
- Methods for their separation was developed using different anion exchange and C18 reversed phase analytical chromatography columns for better selectivity and purity.

# Introduction

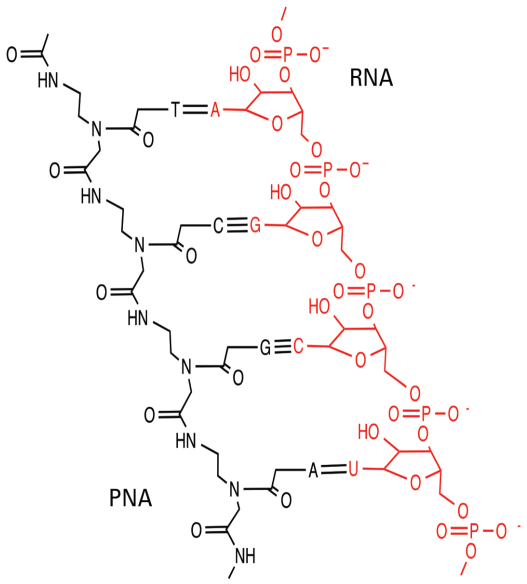
PNA-peptide based oligonucleotide biotherapeutic

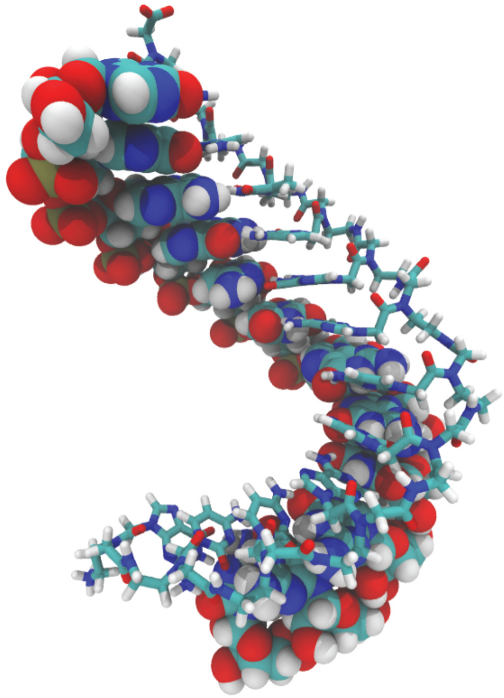



- This figure represents the PNA-peptide bioconjugates.
- These are designed for noninvasive molecular imaging of oncogene expression in cancer cells in cancer imaging research.
- Positron Imaging Tomography (PET) was used to image the tumor in mouse model (data not shown here).

# Introduction

Peptide Nucleic Acid (PNA) and RNA interaction



- Receptor-mediated endocytosis of [$^{64}$Cu] mRNA agent via cognate receptors on malignant cells, then released from endosomes.
- The PNA probes diffuse throughout the cytoplasm, hybridizing to target oncogene mRNAs if present, or effluxing via exosomes if not.

# Introduction

The following are the factors to consider in the purification of PNA bioconjugates:

- Monomer impurities
- Incomplete coupling
- Incomplete deprotection
- Others, such as the $NH_2$ to OH change in the sidechain of PNA cytosine
- Non-specific intramolecular or intermolecular hybridization

# Introduction

Here we report:

- A one-step analysis of a 20-mer DNA-based oligonucleotide using a TSKgel SuperQ-5PW,10 µm, 7.5 mm ID × 7.5 cm column
- Analysis of a 20-mer DNA-based oligonucleotide, using two reversed phase columns: TSKgel ODS-140HTP, 2 µm, 2.1 mm ID × 5 cm and TSKgel OligoDNA-RP, 5 µm, 4.6 mm ID × 15 cm
- Analysis of a chelator-12-mer PNA-tetrapeptide oligonucleotide biotherapeutic using TSKgel ODS-100V, 5 µm, 4.6 mm ID ×25 cm column

# Objectives

1. To show the usefulness of the TSKgel SuperQ-5PW analytical column in the separation of oligonucleotides, particularly in reference to the fact that the TSKgel SuperQ-5PW bulk resin and the TSKgel SuperQ-5PW analytical column both have the same selectivity.
2. To show the usefulness of reversed phase chromatography in the separation of DNA-based, as well as PNA-peptide based, oligonucleotide biotherapeutics.

The report is divided in two separate sections

- Section A – separation of DNA-based oligonucleotides
- Section B – separation of PNA-peptide based oligonucleotides

# Separation of DNA-based Oligonucleotides using Ion Exchange and Reversed Phase Chromatography

# TSKgel Ion Exchange Columns Useful for Oligonucleotide Separations - Product Attributes

TSKgel SuperQ-5PW

| | |
|---|---|
| Matrix: | polymethacrylate |
| Particle size (mean): | 10 µm and 13 µm |
| Pore size (mean): | 100 nm |
| Functional group: | trimethylamino |
| Counter ion: | $Cl^-$ |
| pH range: | 2.0 - 12.0 |
| Capacity: | 100 mg BSA/mL |
| Small ion capacity: | >0.13 meq/mL |
| pKa: | 12.2 |

TSKgel SuperQ-5PW columns have the same backbone chemistry and selectivity as the bulk process scale resins, TSKgel SuperQ-5PW and TOYOPEARL SuperQ-650. These columns are very useful for the separation and analysis of proteins, oligonucleotides and other biologically active molecules.

# TSKgel Reversed Phase Columns Useful for Oligonucleotide Separations - Product Attributes

TSKgel ODS-140HTP

| | |
|---|---|
| Pore size (silica): | 14 nm |
| Endcapped: | yes |
| Particle size: | 2.3 µm |
| pH stability: | 2.0 - 7.5 |
| Functional group: | C18 (polymeric bonding chemistry) |
| % carbon: | 8% |

TSKgel OligoDNA RP

| | |
|---|---|
| Pore size (mean): | 25 nm |
| Exclusion limit: | 500 mer |
| Endcapped | no |
| Particle size (mean): | 5 µm |
| pH stability: | 2.0 - 7.5 |
| Functional group: | C18 (monomeric bonding chemistry) |
| Carbon %: | 10% |

TSKgel ODS-100V

| | |
|---|---|
| Pore size (mean): | 10 nm |
| Molecular weight Limit (Da) | 10,000 |
| Endcapped | yes |
| Particle size (mean): | 3 µm and 5 µm |
| pH stability: | 2.0 - 7.5 |
| Functional group: | Octadecylmethylsilane |
| Carbon %: | 15% |
| Surface area ($m^2/g$) | 450 |

- TSKgel ODS-140HTP columns provide high resolution and short analyses time at moderate pressures, enabling high throughput separations and can be used with either UHPLC (up to 9000 psi) or conventional HPLC systems.
- TSKgel OligoDNA-RP is specifically designed for purification of oligonucleotides, RNA and DNA fragments (up to 500 mer), providing excellent separations of samples with very similar sequences.
- TSKgel ODS-100V, with proprietary monomeric bonded phase chemistry, provides complete wetting and retention stability in 100% aqueous mobile phases and improved efficiency and peak shape.

# Material and Methods (IEX)

| | |
|---|---|
| **HPLC System:** | Analyses were carried out using an Agilent-1200 HPLC system Chemstation (ver B.04.02). |
| **Column:** | TSKgel SuperQ-5PW, 10 µm, 7.5 mm ID × 7.5 cm (S0082-84NM) |
| **Mobile phase:** | A: 20 mmol/L Tris, pH 9.0<br>B: 20 mmol/L Tris, pH 9.0 + 1 mol/L NaCl<br>40-80% B over 30 minutes - an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide |
| **Flow rate:** | 0.9 mL/min |
| **Detection:** | UV @ 260 nm |
| **Temperature:** | ambient & 60 ºC |
| **Injection vol.:** | 15 µL |

# Material and Methods (IEX), Continued

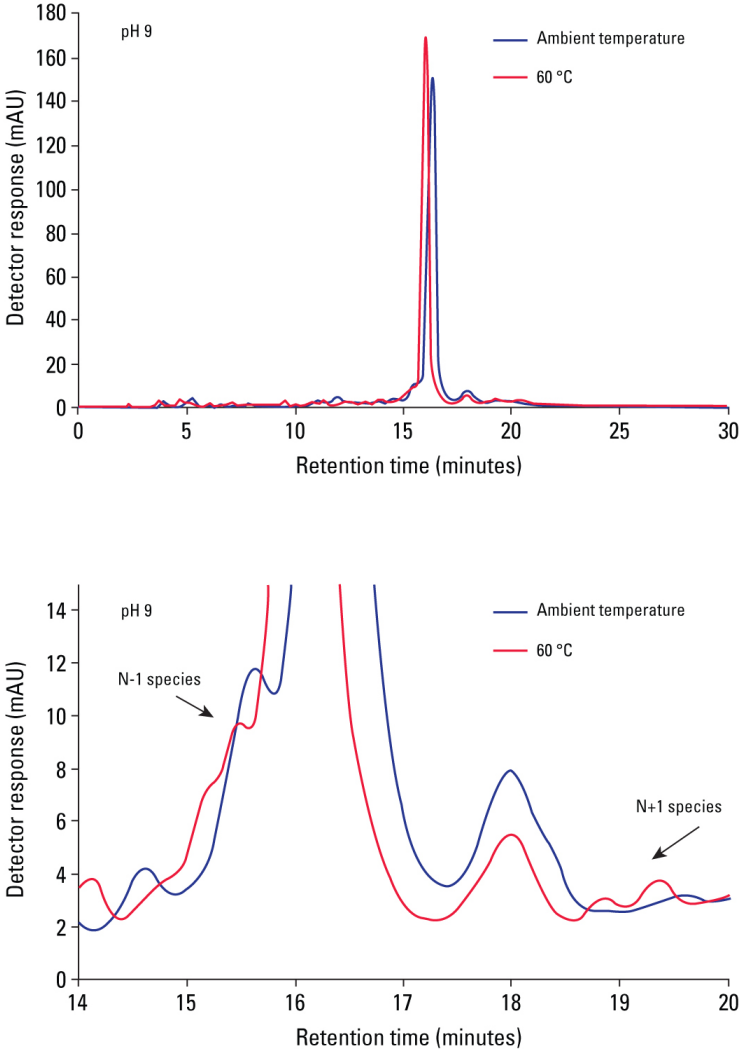
**Sample:** Phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A
5' - GAA TTC ATC GGT TCA GAG AC – 3'

- The oligonucleotide (purchased unpurified) was estimated at 64.9% purity by HPLC in lyophilized form.
- The extinction coefficient was 199.9 OD units/µmol.
- 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.
- Reconstitution of oligonucleotide: For all of the experiments performed, the crude oligonucleotide was diluted into the equilibration buffer (mobile phase A) before loading onto the column.
- stock concentration: 26.6 mg/mL, final dilution: (1:100) in mobile phase A
- final concentration: 0.266 mg/mL = 0.266 µg/µL

# Material and Methods (RPC)

| | |
|---|---|
| **HPLC System:** | All analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.02). |
| **Columns:** | TSKgel ODS-140HTP, 2 µm, 2.1 mm ID × 5 cm<br>TSKgel OligoDNA-RP, 5 µm, 4.6 mm ID × 15 cm |
| **Mobile phase:** | A: 0.1 mol/L ammonium acetate, pH 7.0<br>B: $CH_3CN$ |
| **Gradient:** | See chromatograms |
| **Flow rate:** | 0.5 mL/min (TSKgel ODS-140HTP)<br>1.0 mL/min (TSKgel OligoDNA-RP) |
| **Detection:** | UV @ 260 nm |
| **Temperature:** | 30 ºC |
| **Injection vol.:** | 1 µL (TSKgel ODS-140HTP<br>10 µL (TSKgel OligoDNA-RP) |
| **Sample:** | Phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A<br>5' - GAA TTC ATC GGT TCA GAG AC – 3’ |

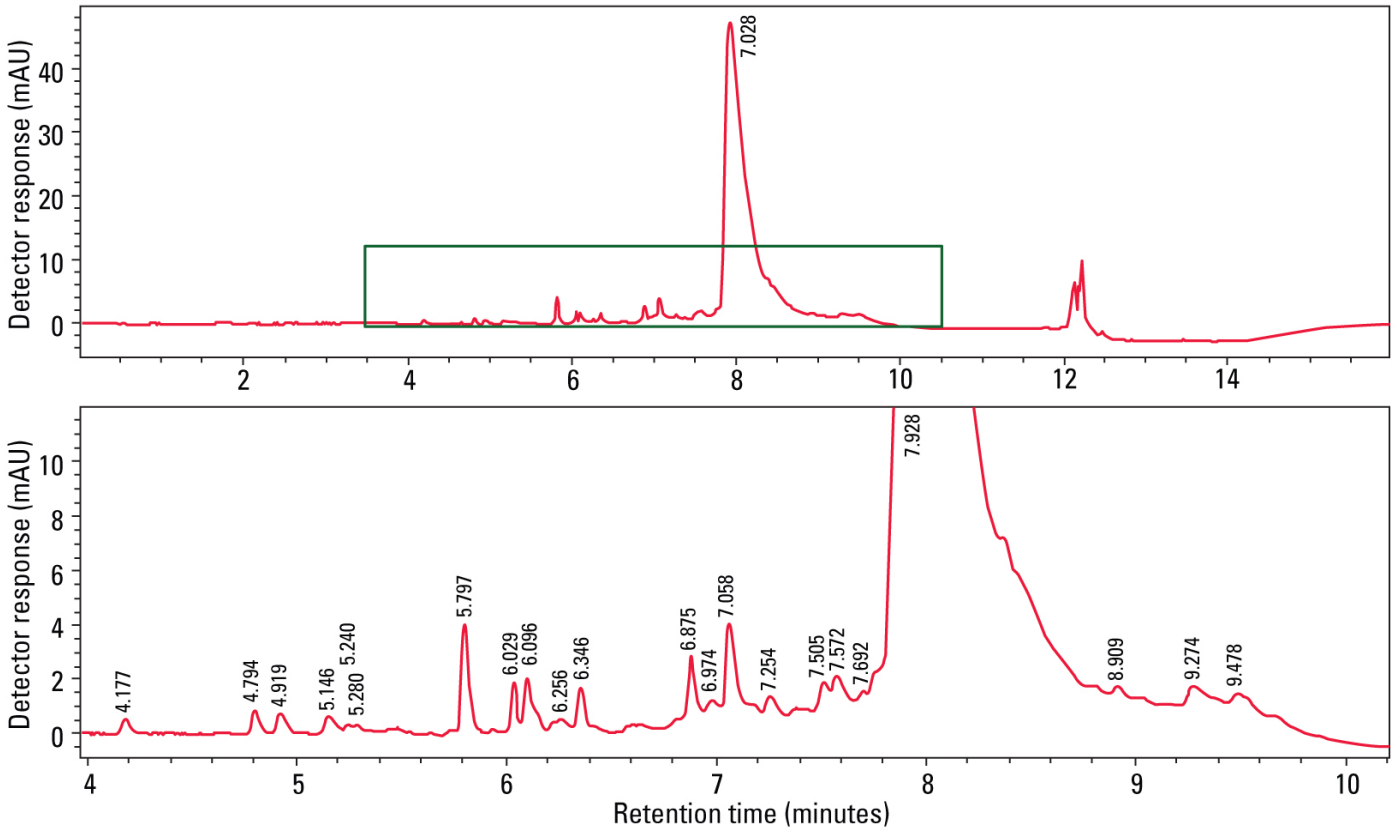
# Figure 1: Analysis of DNA based Oligonucleotide using TSKgel SuperQ-5PW Column at 60 ºC



# Figure 1: Conclusions

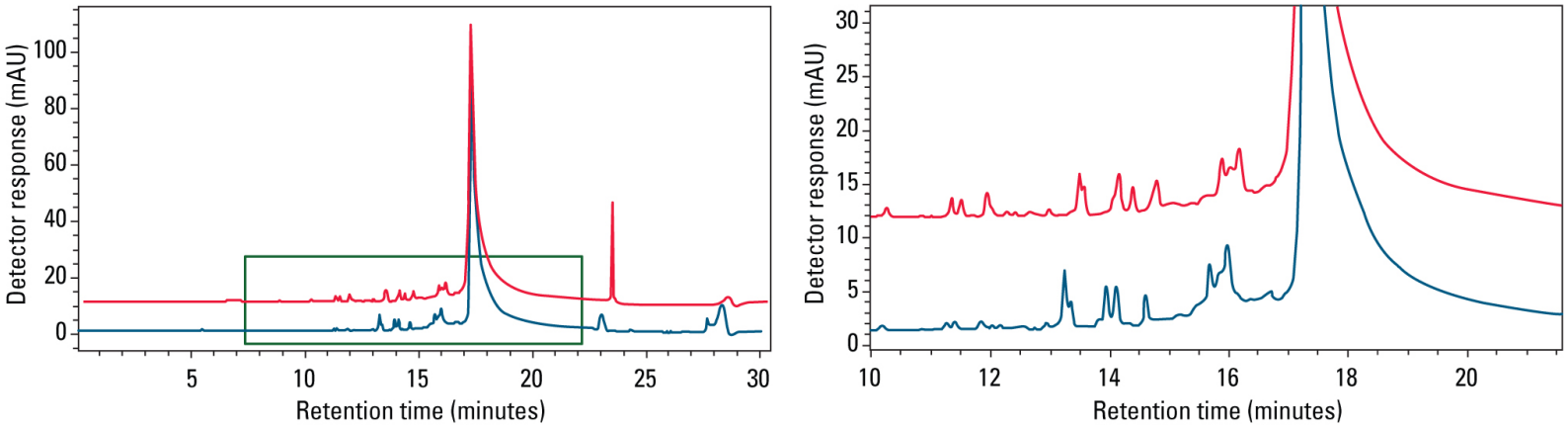
- The effect of pH on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, was studied[1].
- The best pH value was found to be pH 9.0.
- Better resolution could be obtained at 60 ºC column temperature compared to ambient temperature.
- Both N-1 and N+1 peaks appear to be heterogeneous.
- The peak area analysis was linear within the experimental range of 3.98 µg to 79.68 µg (based on single injection data)[2].
- Higher loadings of crude oligonucleotide within this experimental range did not affect the peak purity percentage[2].

# Figure 2: Separation of Oligonucleotides by RPC using TSKgel ODS-140HTP Column



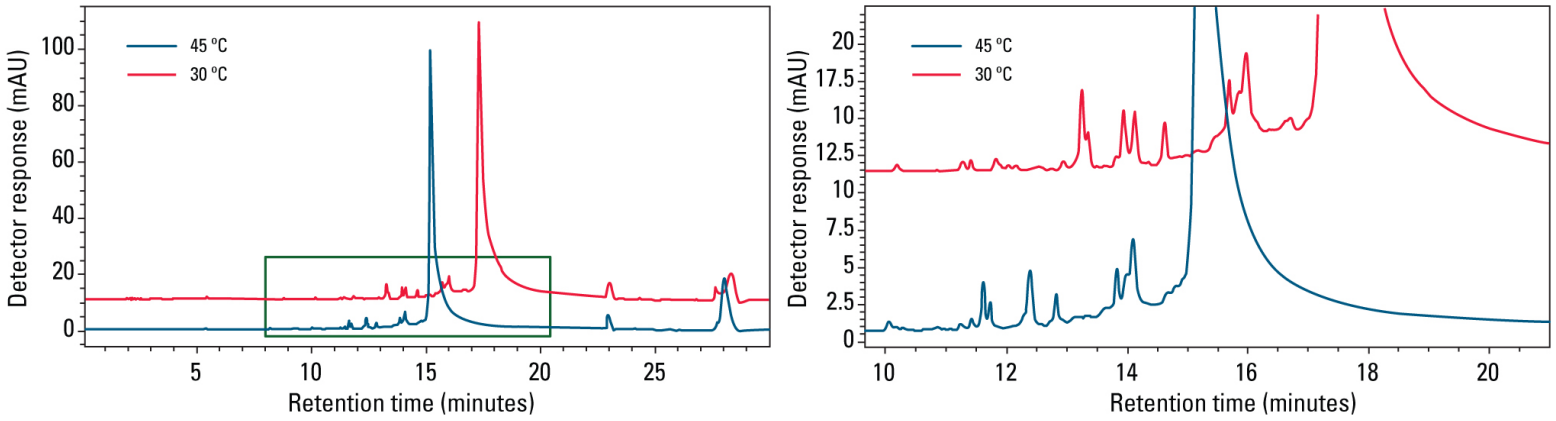

A shallow gradient of 0-15% B in 10 minutes run provides the separation and resolution of oligonucleotide impurities species within a short run time.

# Figure 3: Separation of Oligonucleotides by RPC using TSKgel OligoDNA-RP Column




- A shallow gradient of 0-15% B in 20 minutes run provides the separation and resolution of oligonucleotide from impurities.
- The separation yields highly reproducible results from day-to-day.

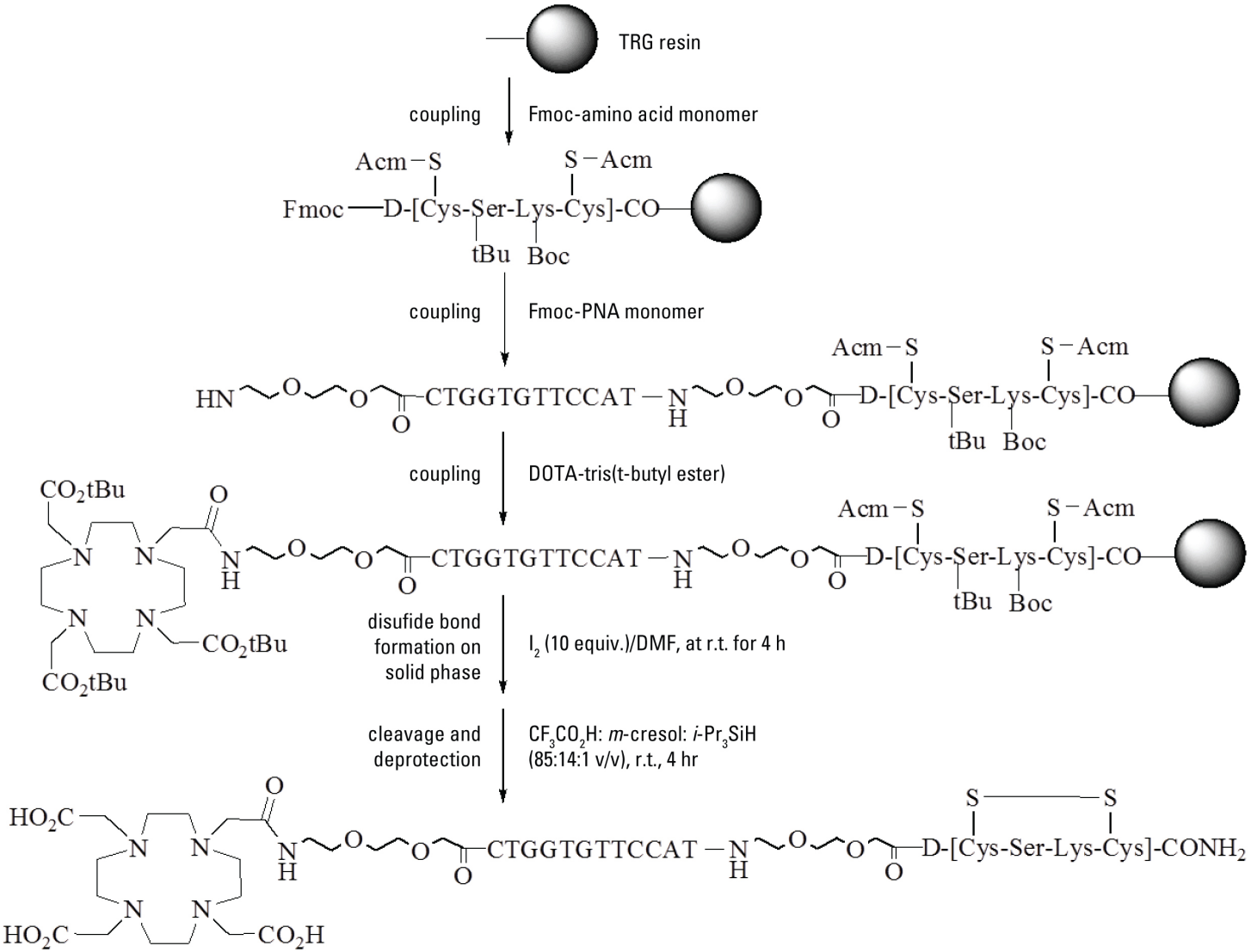
# Figure 4: Separation of Oligonucleotides by RPC using TSKgel OligoDNA-RP Column at 45 ºC




- The use of  elevated temperature leads to (a) moderately decreased tailing (b) doubling of column efficiency and (c) shorter run time.
- Day-to-day reproducibility was achieved.

# Separation of PNA-Peptide based Oligonucleotides using Reversed Phase Chromatography

# PNA Bioconjugate Typical Synthetic Route



# Material and Methods (PNA-Peptide on RPC)

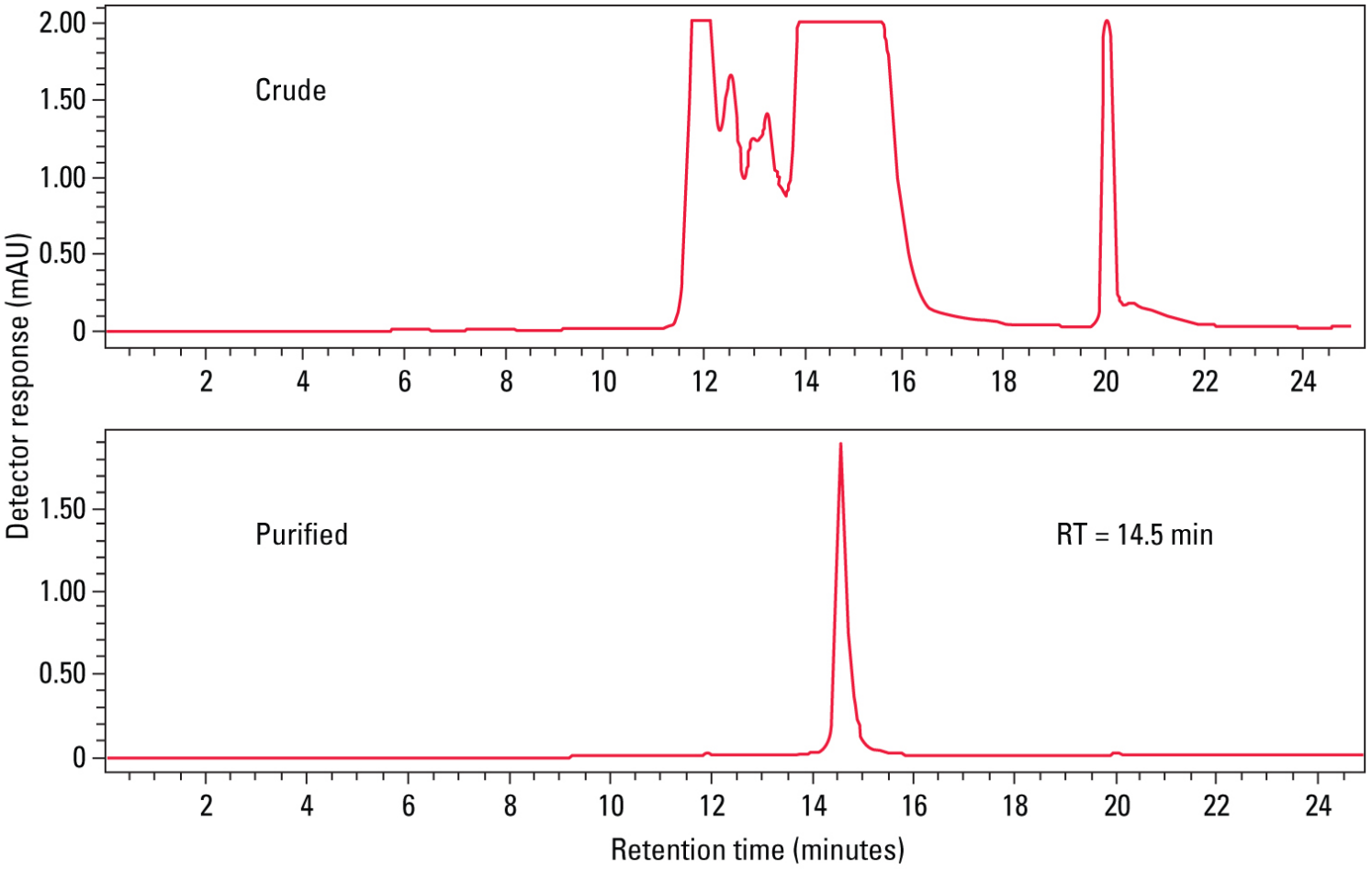
| | |
|---|---|
| **HPLC System:** | Waters HPLC system with 600E pump controller and 486 detector |
| **Column:** | TSKgel ODS-100V, 5 µm, 4.6 mm ID × 25 cm |
| **Mobile phase:** | A: 0.1% TFA in $H_2O$ |
| | B: 0.1% TFA in ACN |
| **Gradient:** | 5-70% B over 25 minutes |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV @ 215 nm |
| **Temperature:** | 50 ºC |
| **Injection vol.:** | 200 µL of crude, 10 µL of purified |

# Material and Methods (PNA-Peptide on RPC), Continued

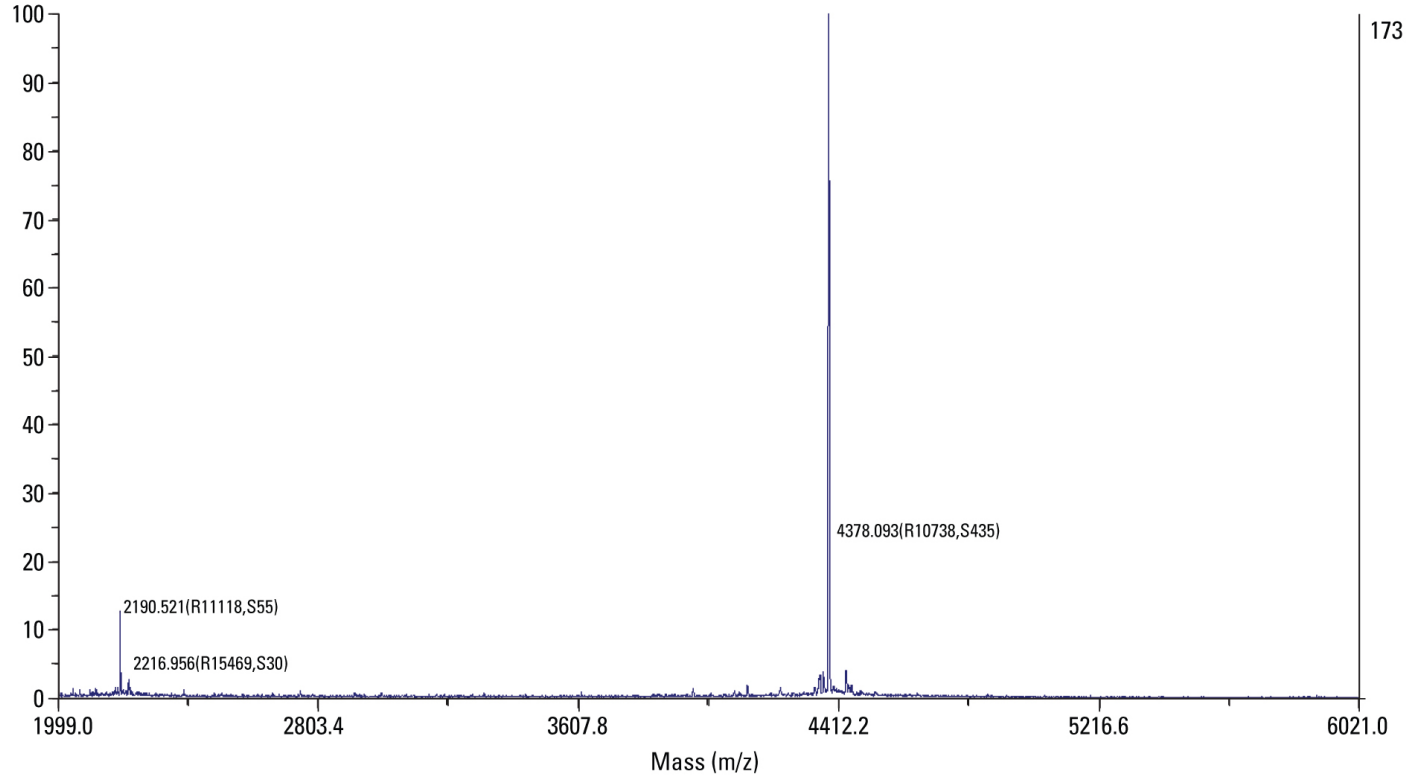
**Sample:** Chelator-spacer-peptide nucleic acid (12-mer)-spacer-peptide N-SBTG2-DAP-AEEA-GCCAHHAGCTCC-AEEA-cyclo[d(Cys-Ser-Lys-Cys)]-C

- Specific for mutant KRAS2 mRNA and IGF1 receptor.
- Sanders, et al. (2013) J. Phys. Chem. B 117:11584–11595.
- Assembled by solid phase synthesis.
- Crude purity 85% by HPLC.
- Extinction coefficient was 96 $A_{260}$ units/µmol.
- Calculated molecular mass was 4,378 Da
- Reconstitution of oligonucleotide: The crude chelator-PNA-peptide was dissolved into the equilibration buffer (mobile phase A) before loading onto the column.

# Figure 5: Analysis of the PNA-Peptide using TSKgel ODS-100V Column



# Figure 6: MALDI TOF MS of Purified PNA-Peptide based Oligonucleotide Biotherapeutic




RPC of crude yielded a purified peak with the calculated mass.

# Conclusions

- TSKgel SuperQ-5PW analytical columns have the same backbone chemistry and selectivity as the TSKgel SuperQ-5PW bulk process scale resins.
- TSKgel SuperQ-5PW could separate impurities containing modified products from the pure product.
- The best result was obtained at pH 9.0 for the TSKgel SuperQ-5PW column.
- Each of the three main species of oligonucleotide (N-1, N=20 and N+1) were separated under the optimum chromatographic conditions using both TSKgel SuperQ-5PW (20) resin and the TSKgel SuperQ-5PW column[1].
- Selectivity of the TSKgel SuperQ-5PW analytical column was found to be the same as that of the TSKgel SuperQ-5PW (20) resin in the separation of oligonucleotides[2].

# Conclusions

- The study shows that the TSKgel SuperQ-5PW analytical column can be used in the separation of oligonucleotides and the method can be useful for scaling up using TSKgel SuperQ-5PW bulk resin, because of the same selectivity of both.
- Reversed phase chromatographic columns could be used for the separation of DNA-based oligonucleotides from the impurity species.
- Reversed phase chromatography columns could also be used for the separation of PNA-peptide based oligonucleotides as well.
- Ion exchange can be coupled with purification by RP-HPLC, adding a second dimension to the separation process.
- Anion exchange HPLC is limited by length (usually up to 40 mers).
- The longer the oligonucleotide, the lower the resolution on the anion exchange HPLC column and thus the purity of the target oligo.
- In overall, this study shows the use of anion exchange and reversed phase chromatography columns in the separation of both DNA-based as well as PNA-peptide based oligonucleotides.

# References

[1]"One-step oligonucleotide purifications using anion exchange chromatography resins" – Phu T. Duong, Shigeru Nakatani and J. Kevin O'Donnell – Poster presentation at ACS, August 2008, Philadelphia, PA – for the method of characterization of the N-1 and N+1 peaks by gel electrophoresis analysis of purified oligonucleotides

[2] TP169, as listed in the literature library of the Tosoh Bioscience website (www.tosohbioscience.com)