



Selectivity studies in the analytical separation of oligonucleotides using anion exchange chromatography

Bruce Kempf, R. Christopher Manzari, J. Kevin O'Donnell, Ph.D. and Atis Chakrabarti, Ph.D.

Tosoh Bioscience LLC, King of Prussia, PA 19406



Introduction

- Synthetic oligonucleotides are becoming increasingly popular as biotherapeutic agents.
- When purification moves from analytical to manufacturing scale, having the same bonding chemistry is helpful, since the selectivity is expected to remain the same.
- 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.
- 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 \times 7.5 cm column.



Introduction

- 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 discussed.
- Two other columns: one packed with 5 μm hydrophilic non-porous resin particles of which the surface consists of an open access network of multi-layered anion exchange groups (TSKgel DNA-STAT) and the other column packed with 2.5 μm non-porous, high efficiency, hydrophilic polymer beads which are surface modified with a weak anion exchanger (TSKgel DNA-NPR) are used for reference.



Objective

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.



Characteristics of TSKgel Ion Exchange Columns Used in this Study – Product Attributes

	TSKgel DNA-NPR	TSKgel SuperQ-5PW	TSKgel DNA-STAT
Matrix	hydroxylated methacrylic polymer	polymethacrylate	hydrophilic polymer
Particle size (mean):	2.5 μm	10 μm & 13 μm	5 μm
Pore size (mean):	non-porous	1000 Å	non-porous
Functional group:	proprietary	trimethylamino	quarternary ammonium
Counter ion:	ClO_4^-	Cl^-	Cl^-
pH range:	2-12	2-12	3-10
Capacity (mg BSA/mL):	5 mg	100 mg	
Static binding capacity (mg BSA/g dry gel):			ca. 35 (5 μm)
Small ion capacity:	>0.1 meq/mL	>0.13 meq/mL	270 $\mu\text{g/g}$ dry gel
pKa:	11.2	12.2	10.5
Hardware:			stainless steel tubing & fittings, PEEK frits



Material and Methods

- HPLC System:** Analyses were carried out using an Agilent-1200 HPLC system running Chemstation (ver B.04.02).
- Columns:** TSKgel SuperQ-5PW, 10 μm , 7.5 mm ID \times 7.5 cm (S0082-84NM)
TSKgel DNA STAT, 5 μm , 4.6 mm ID \times 10 cm (R0036-502N)
TSKgel DNA-NPR, 2.5 μm , 4.6 mm ID \times 7.5 cm (R0037-87M)
- Sample:** Phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A
5' - GAA TTC ATC GGT TCA GAG AC – 3'
- purchased unpurified.
 - the extinction coefficient was 199.9 OD units/ μmol
 - the molecular weight of the free acid – 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 (buffer 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 $\mu\text{g}/\mu\text{L}$



Chromatographic Conditions

Mobile phase: TSKgel SuperQ-5PW

- buffer A: 20 mmol/L Tris, pH 9.0
- buffer B: 20 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
- 40-80% B over 30 minutes – an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide

Mobile phase: TSKgel DNA-STAT

- buffer A: 100 mmol/L Tris, pH 9.0
- buffer B: 100 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
- 20-100% B over 30 minutes – an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide

Mobile phase: TSKgel DNA-NPR

- buffer A: 20 mmol/L Tris, pH 9.0
- buffer B: 20 mmol/L Tris, 1 mol/L NaCl, pH 9.0
- 0-100% buffer B (15 minute gradient)
- 100% buffer B (3 minutes)
- 0% buffer B (5 minutes)



Chromatographic Conditions

Flow rate:

- 0.9 mL/min (TSKgel Super Q-5PW)
- 0.5 mL/min (TSKgel DNA-STAT)
- 0.7 mL/min (TSKgel DNA-NPR)

Detection: UV @ 260 nm

Temperature:

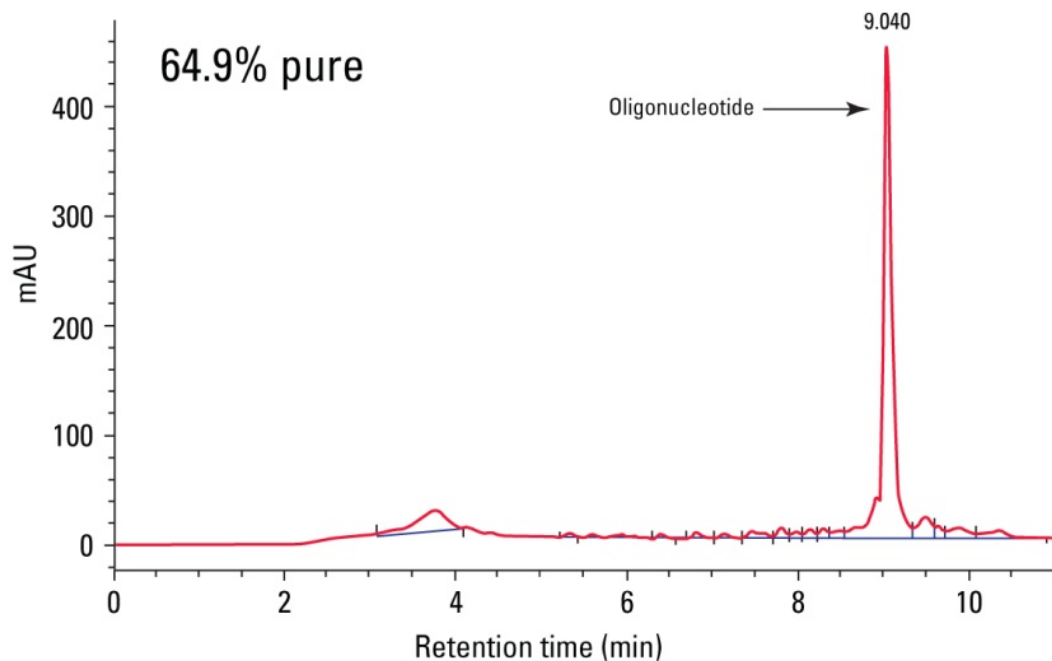
- TSKgel Super Q-5PW - Ambient & 60 °C
- TSKgel DNA-STAT - 60 °C
- TSKgel DNA-NPR - 40 °C

Injection vol.:

- 15 µL (TSKgel SuperQ-5PW)
- 10 µL (TSKgel DNA-STAT)
- 15 µL (TSKgel DNA-NPR)



Figure 1: Analysis of Crude Oligonucleotide Using TSKgel DNA-NPR Column

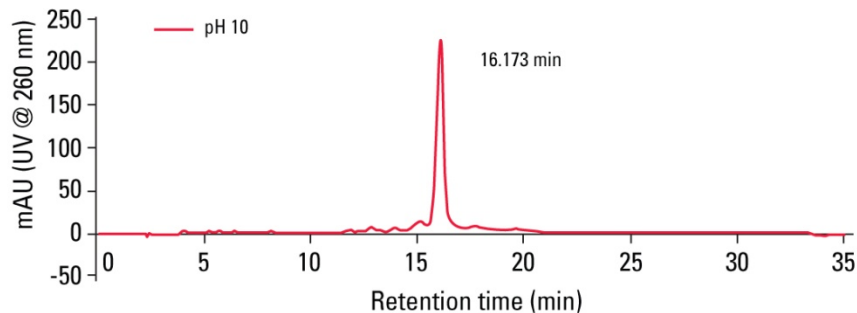
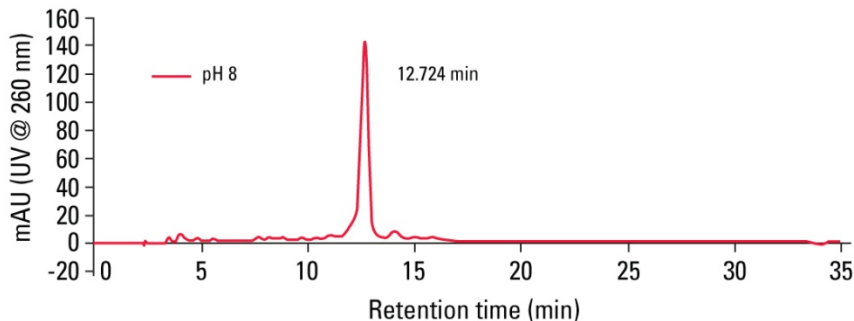
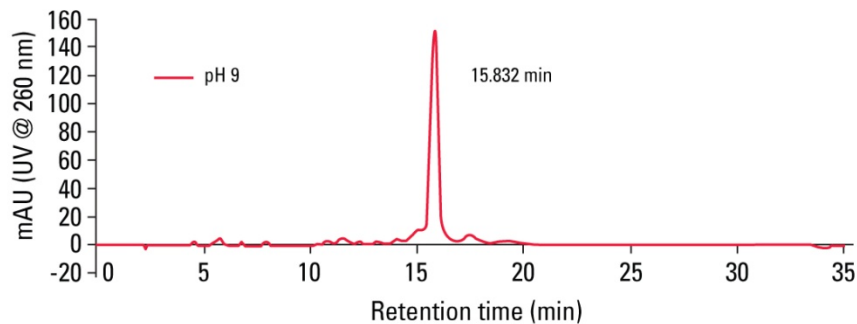
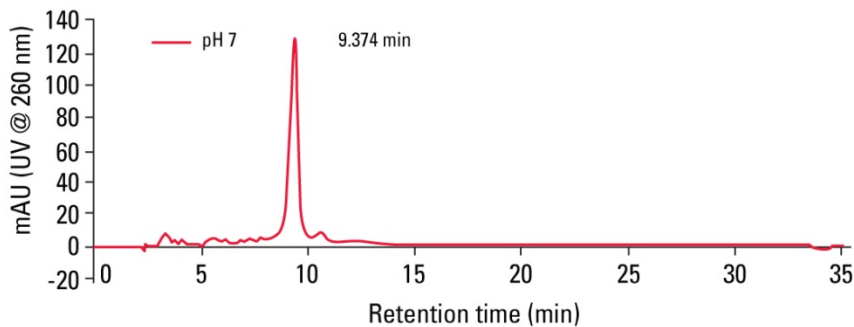


TSKgel DNA-NPR:
buffer A: 20 mmol/L Tris, pH 9.0
buffer B: 20 mmol/L Tris, 1 mol/L NaCl, pH 9.0
15 μ L sample injection
0-100% buffer B (15 minute gradient)
100% buffer B (3 minutes)
0% buffer B (5 minutes)

The oligonucleotide was estimated at 64.9% purity by HPLC in lyophilized form.



Figure 2: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different pH



- 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. (Please see the reference 1 for the method of characterization of the N-1 and N+1 peaks by gel electrophoresis analysis of purified oligonucleotides.)
- The best pH value was found to be pH 9.0.



Figure 3: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different pH (zoomed in view)

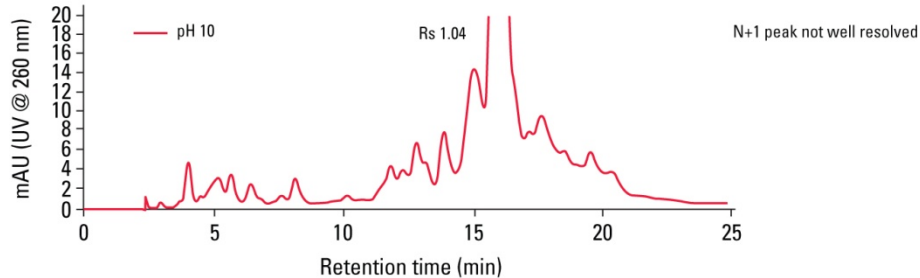
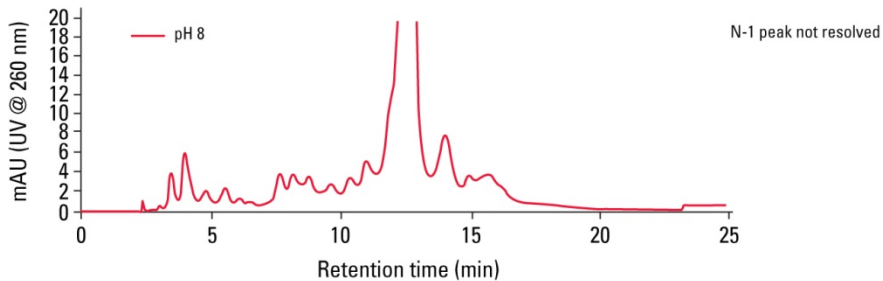
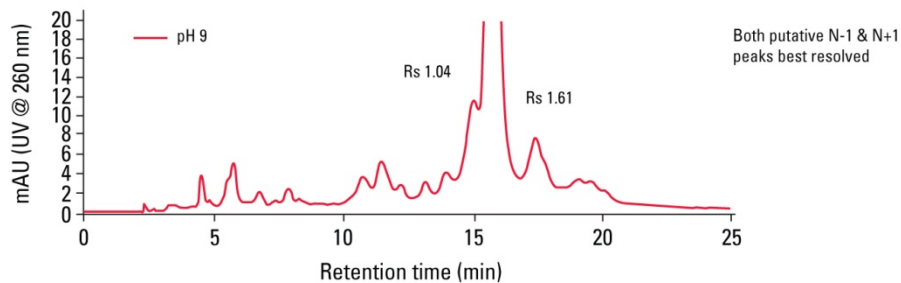
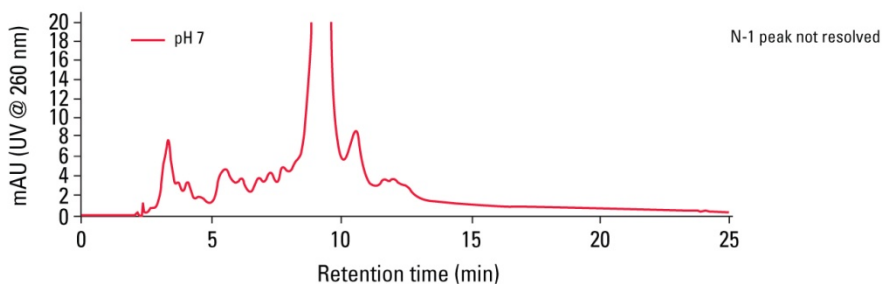
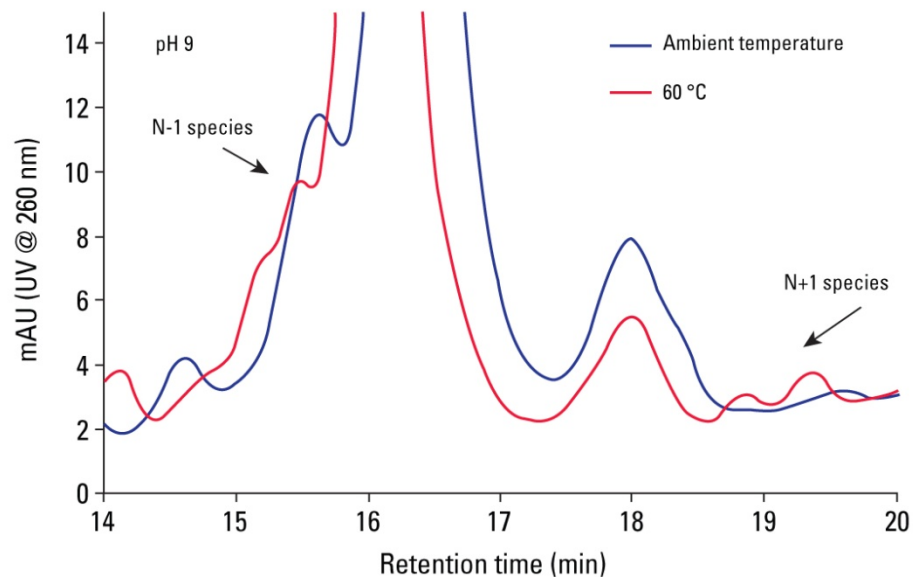
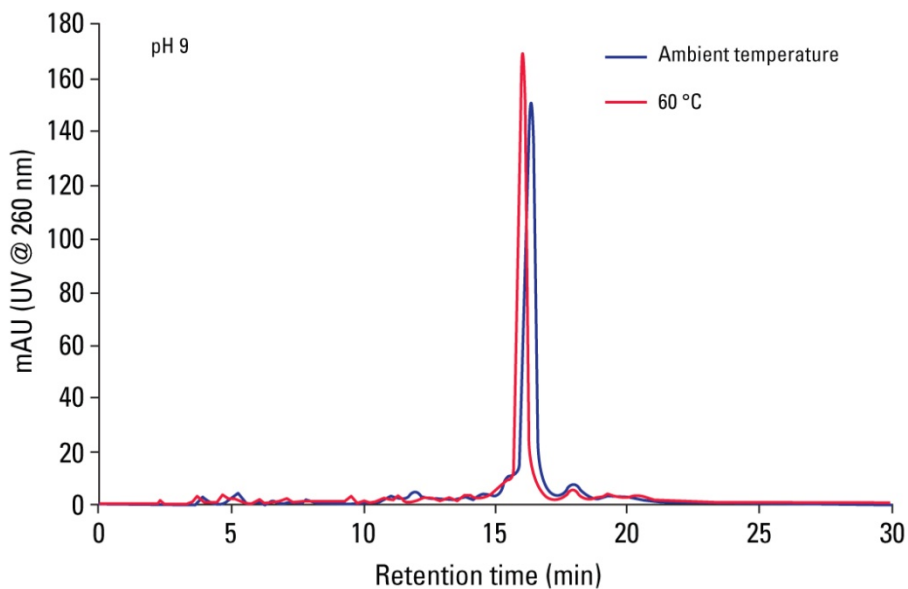




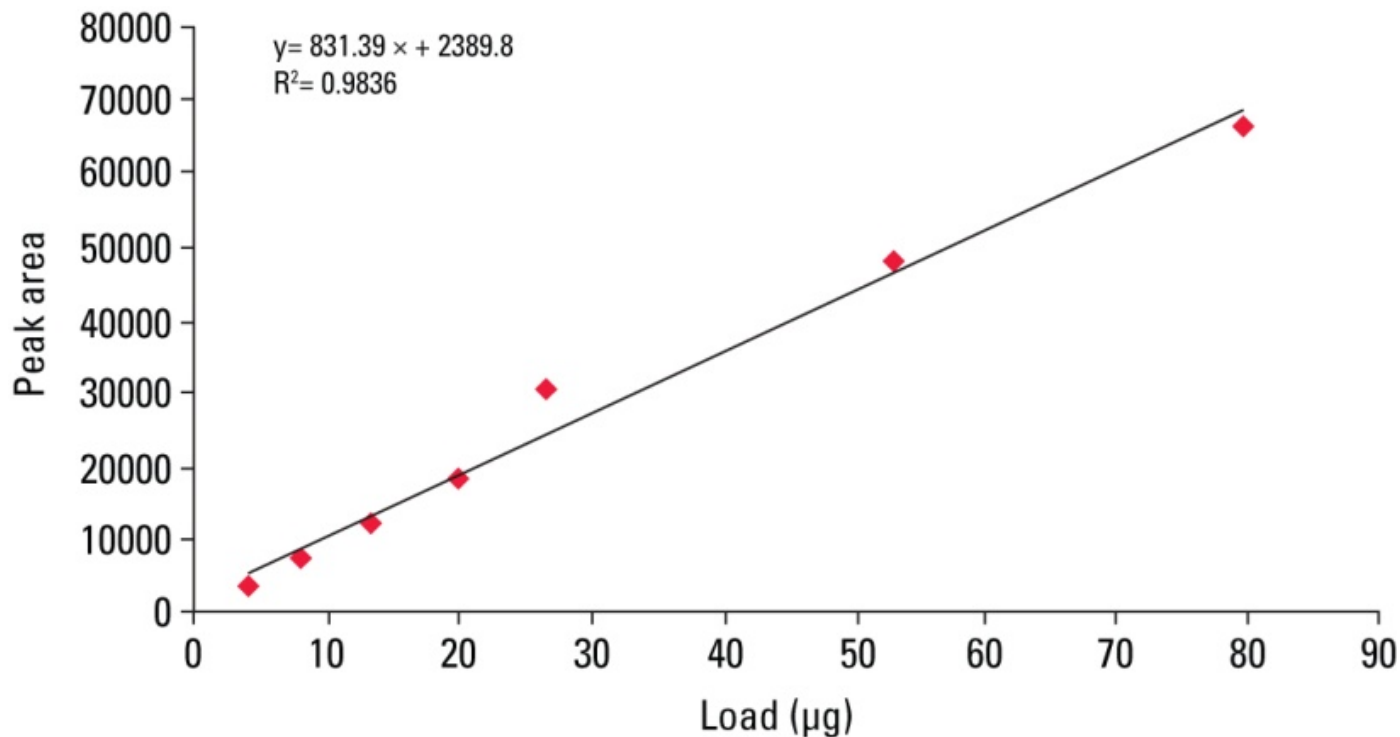
Figure 4: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different Temperature



- Effect of temperature on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, was studied.
- Better resolution could be obtained at 60 °C column temperature compared to the experiment at ambient temperature.
- Both N-1 and N+1 peaks appear to be heterogeneous.



Figure 5: Effect of Higher Loading on the HPLC Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column



- The peak area analysis was linear within the experimental range of 3.98 µg to 79.68 µg (based on single injection data).
- Higher loadings of crude oligonucleotide within this experimental range did not affect the peak purity percentage.



Figure 6: Analysis of Crude Oligonucleotide Using TSKgel DNA-STAT Column

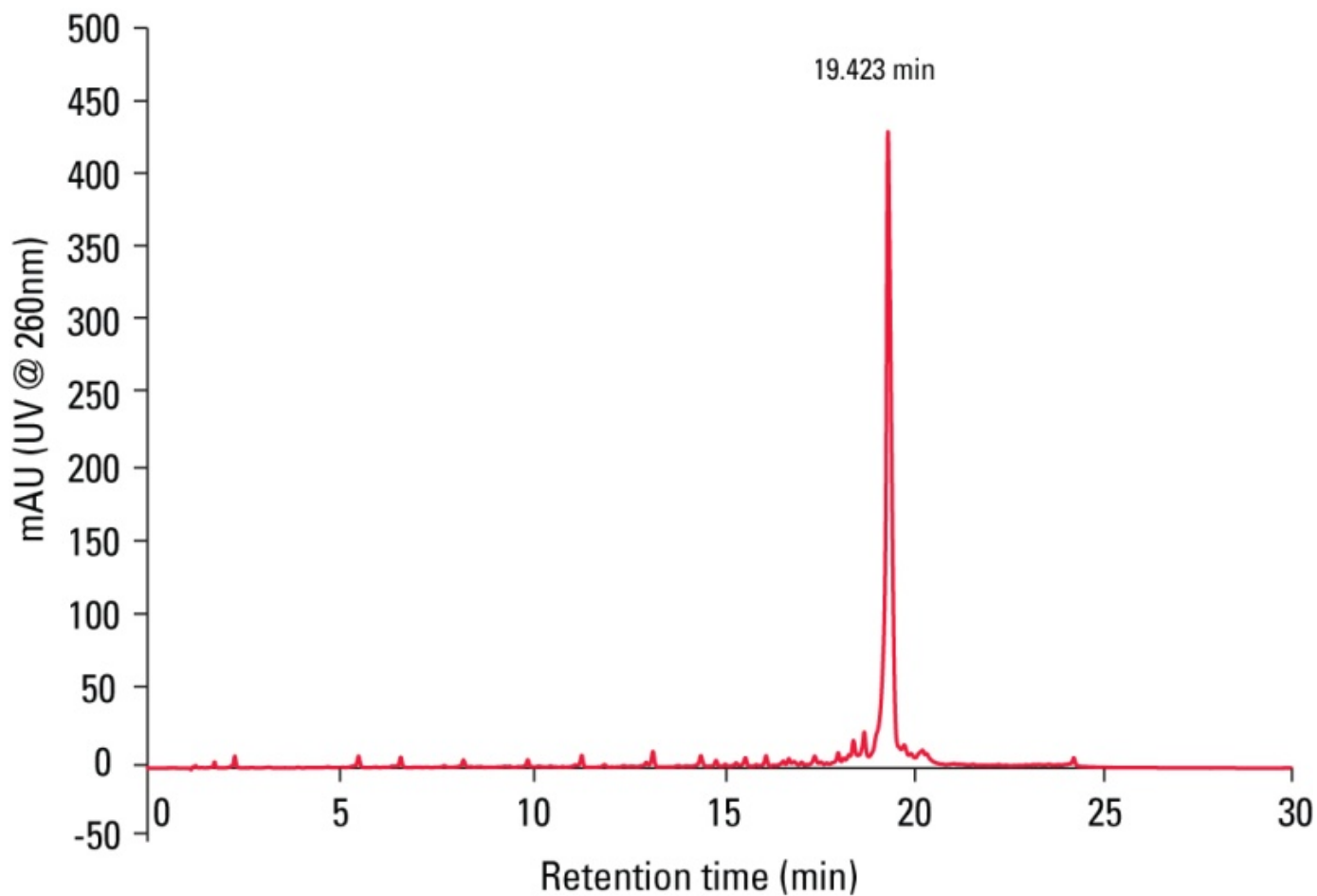
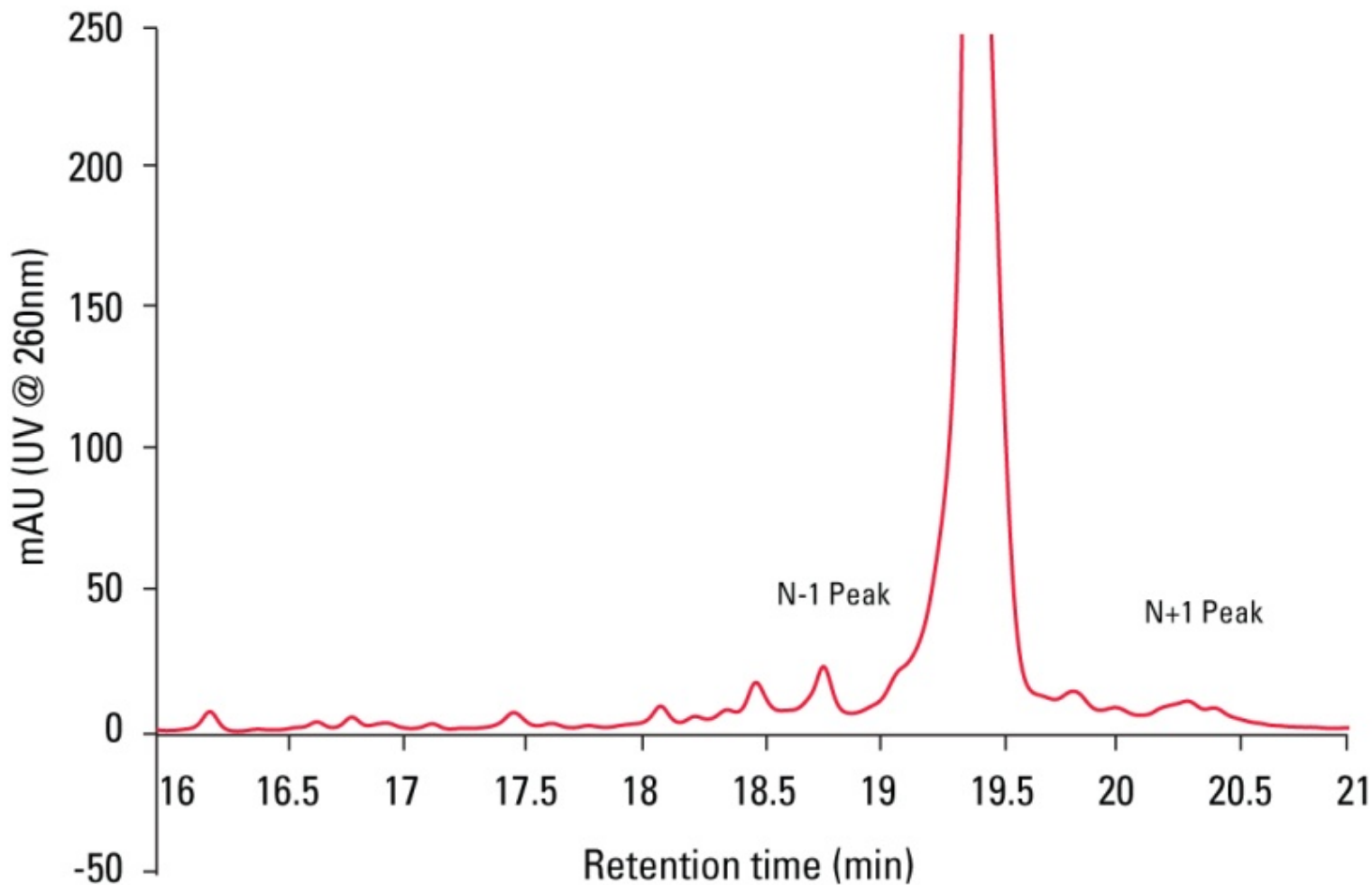




Figure 7: Analysis of Crude Oligonucleotide Using TSKgel DNA-STAT Column (zoomed in view)





Reversed Phase Separation of Oligonucleotides

- The high throughput silica-based reversed phase UHPLC column, TSKgel ODS-140HTP, 2.3 μm , 2.1 mm ID \times 5 cm, and one conventional HPLC column, TSKgel ODS-100V, 5 μm , 4.6 mm ID \times 15 cm, are being compared for their use in orthogonal analysis of oligonucleotides.
- Preliminary study shows these two columns are useful for the separation of the oligonucleotides.
- Optimization of the best chromatographic conditions are in progress and will be published at a later date.



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.
- Different chromatographic conditions (pH, salt, and temperature) were tried.
- The best result was obtained at pH 9.0 for the TSKgel SuperQ-5PW column.



Conclusions

- 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.
- 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.



Reference

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



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

Justin Steve¹, ChangPo Chen², Eric Wickstrom² and Atis Chakrabarti¹

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 \times 7.5 cm column is used for the one step analysis of a 20-mer DNA-based oligonucleotide.

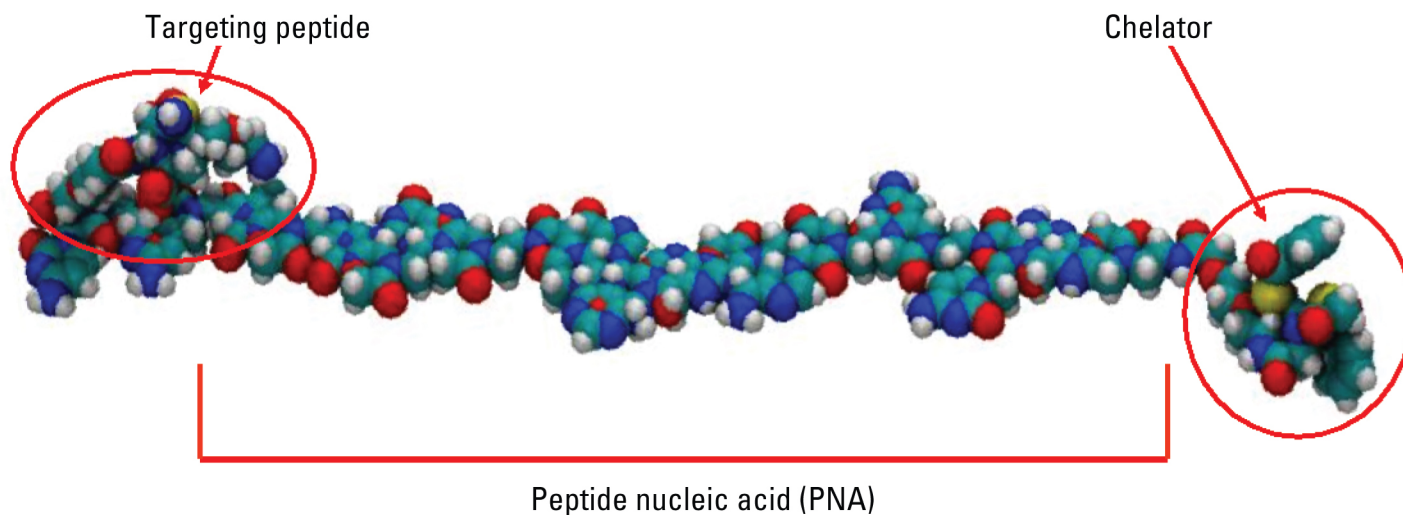


Introduction

- 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

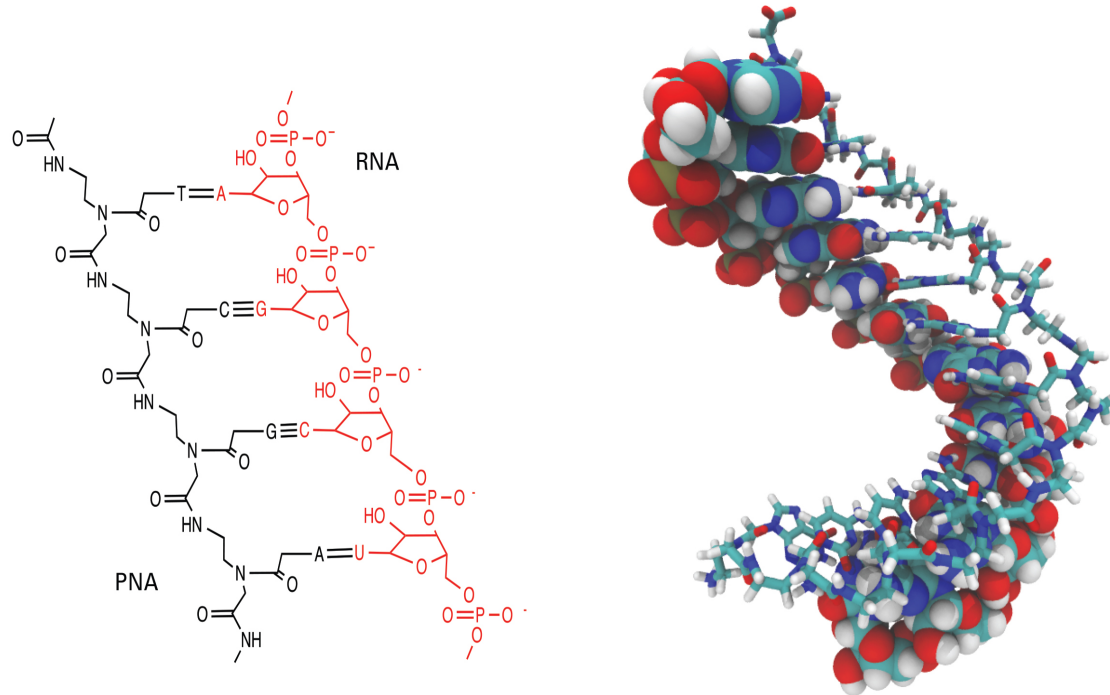
PNA-peptide based oligonucleotide biotherapeutic



- 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 [⁶⁴Cu] 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 \times 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 \times 5 cm and TSKgel OligoDNA-RP, 5 μm , 4.6 mm ID \times 15 cm
- Analysis of a chelator-12-mer PNA-tetrapeptide oligonucleotide biotherapeutic using TSKgel ODS-100V, 5 μm , 4.6 mm ID \times 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 μ m and 13 μ m
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 μ m
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 μ m
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 μ m and 5 μ m
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 \times 7.5 cm (S0082-84NM)
- Mobile phase:** A: 20 mmol/L Tris, pH 9.0
B: 20 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
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 °C
- 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 $\mu\text{g}/\mu\text{L}$



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 μ m, 2.1 mm ID \times 5 cm
TSKgel OligoDNA-RP, 5 μ m, 4.6 mm ID \times 15 cm
- Mobile phase:** A: 0.1 mol/L ammonium acetate, pH 7.0
B: CH₃CN
- Gradient:** See chromatograms
- Flow rate:** 0.5 mL/min (TSKgel ODS-140HTP)
1.0 mL/min (TSKgel OligoDNA-RP)
- Detection:** UV @ 260 nm
- Temperature:** 30 °C
- Injection vol.:** 1 μ L (TSKgel ODS-140HTP)
10 μ L (TSKgel OligoDNA-RP)
- Sample:** Phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A
5' - GAA TTC ATC GGT TCA GAG AC – 3'



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

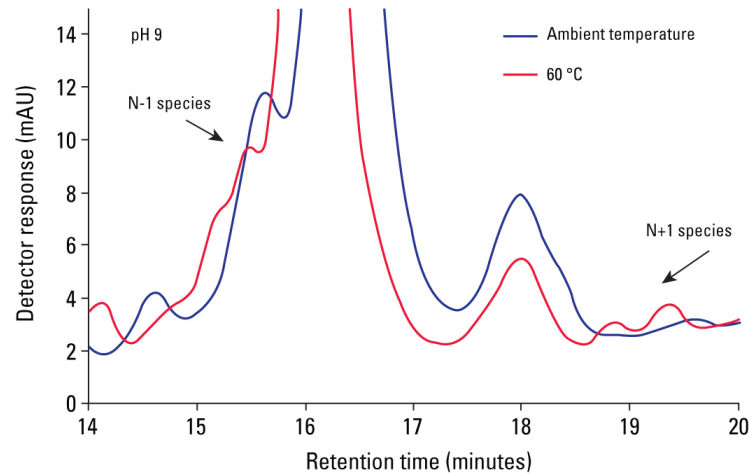
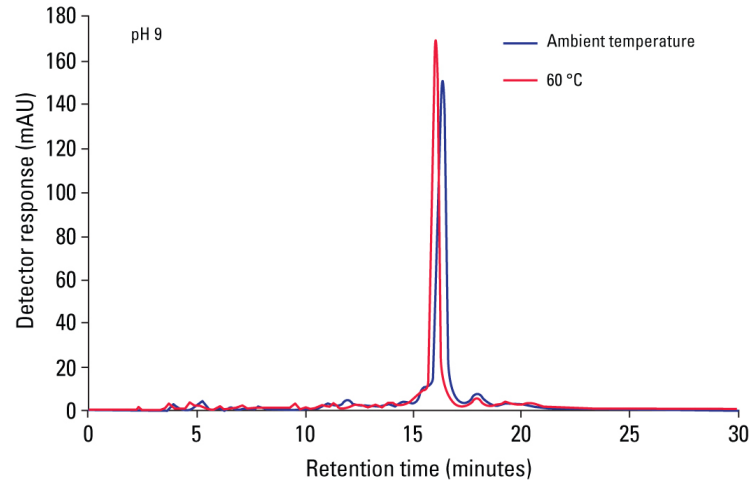


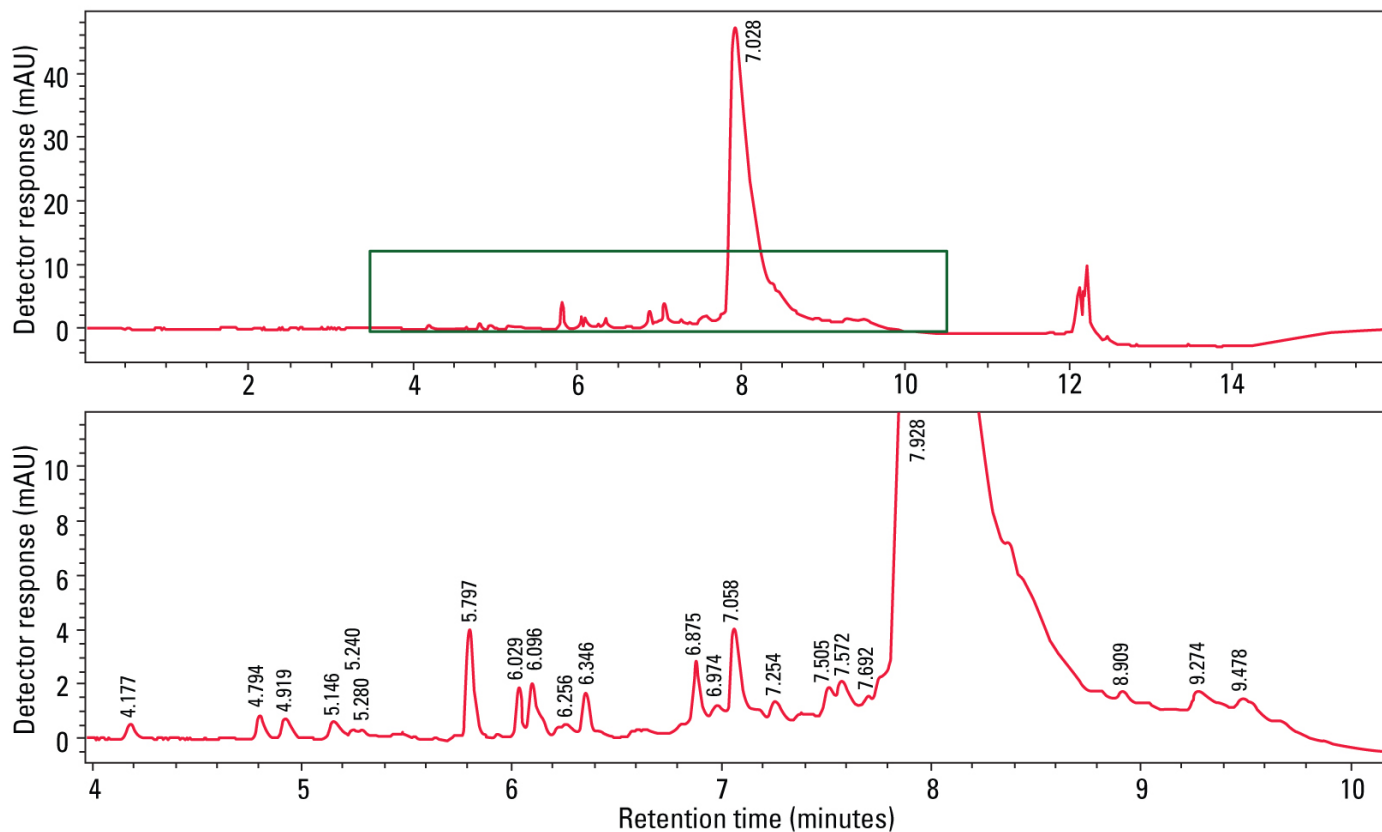


Figure 1: Conclusions

- 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 studied¹.
- The best pH value was found to be pH 9.0.
- Better resolution could be obtained at 60 °C 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 percentage².



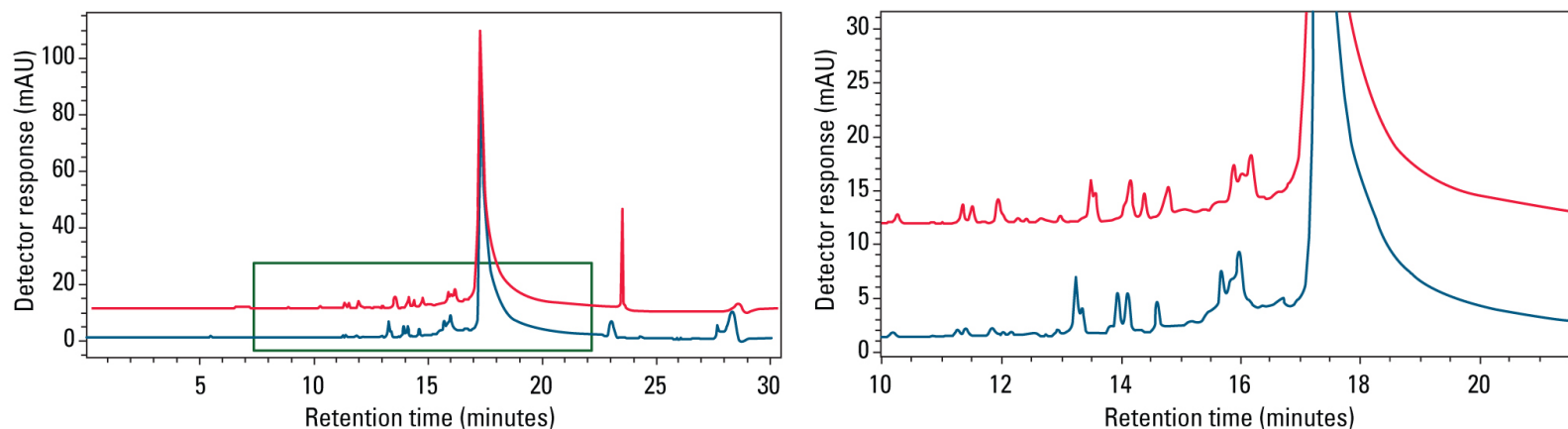
Figure 2: Separation of Oligonucleotides by RPC using TSKgel ODS-140HTP Column



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.



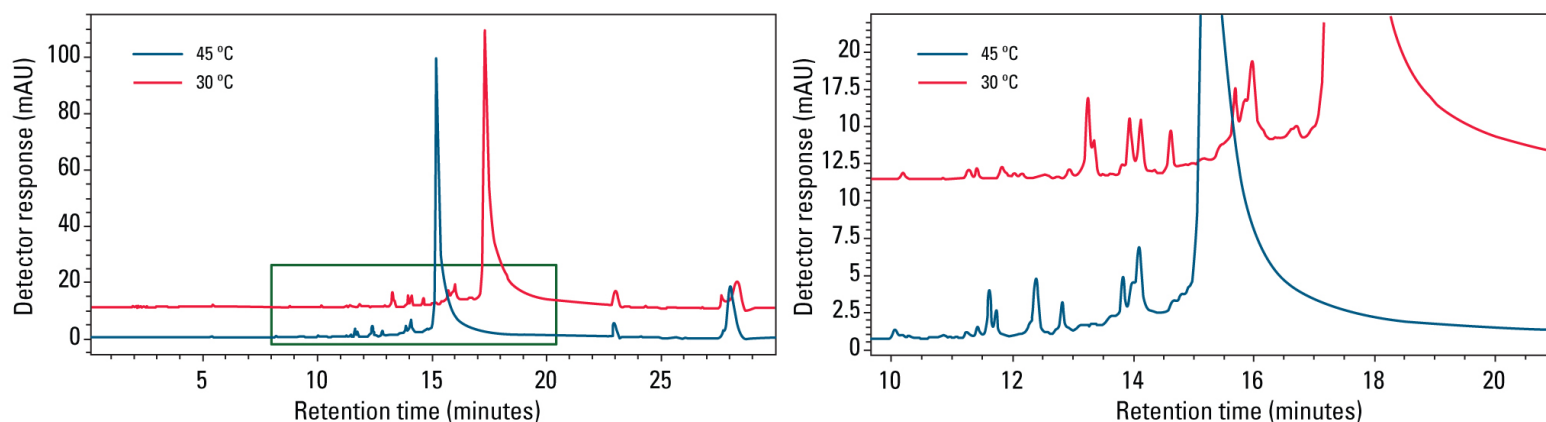
Figure 3: Separation of Oligonucleotides by RPC using TSKgel OligoDNA-RP Column



- 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 °C

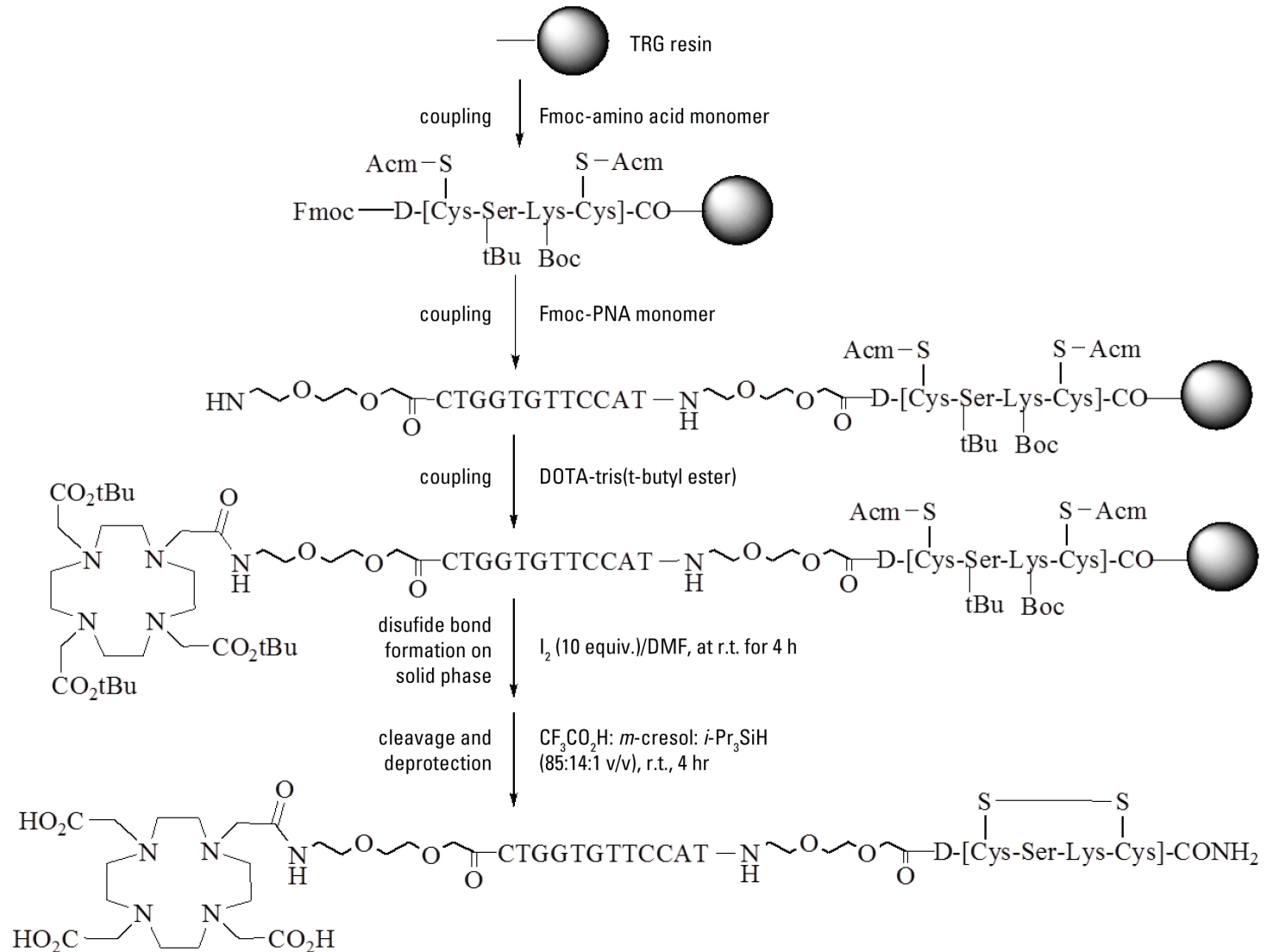


- The use of elevated temperature leads to (a) moderately decreased tailing (b) doubling of column efficiency and (c) shorter run time.
- Day-to-day reproducibility was achieved.



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 \times 25 cm
- Mobile phase:** A: 0.1% TFA in H₂O
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 °C
- 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

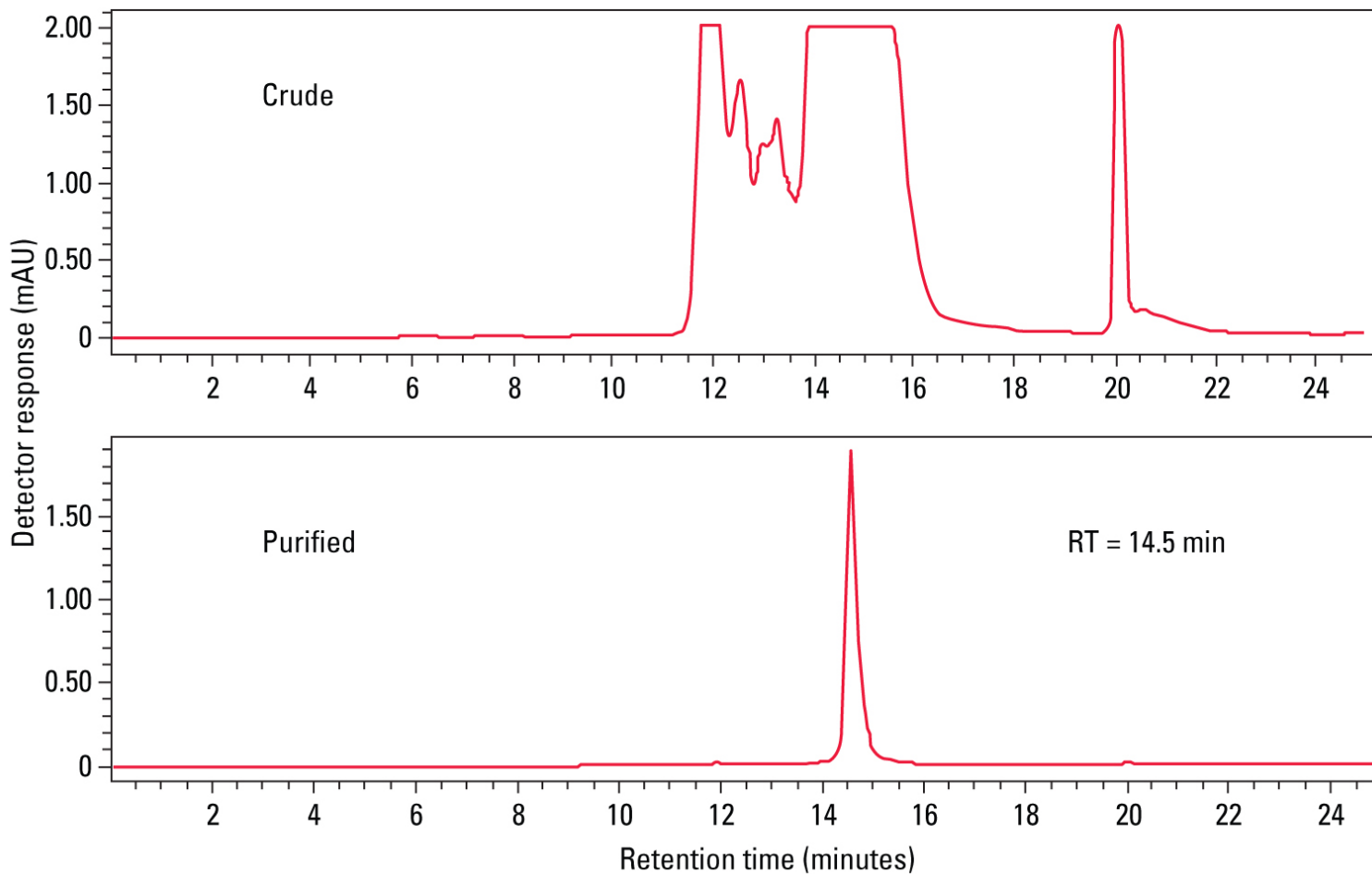
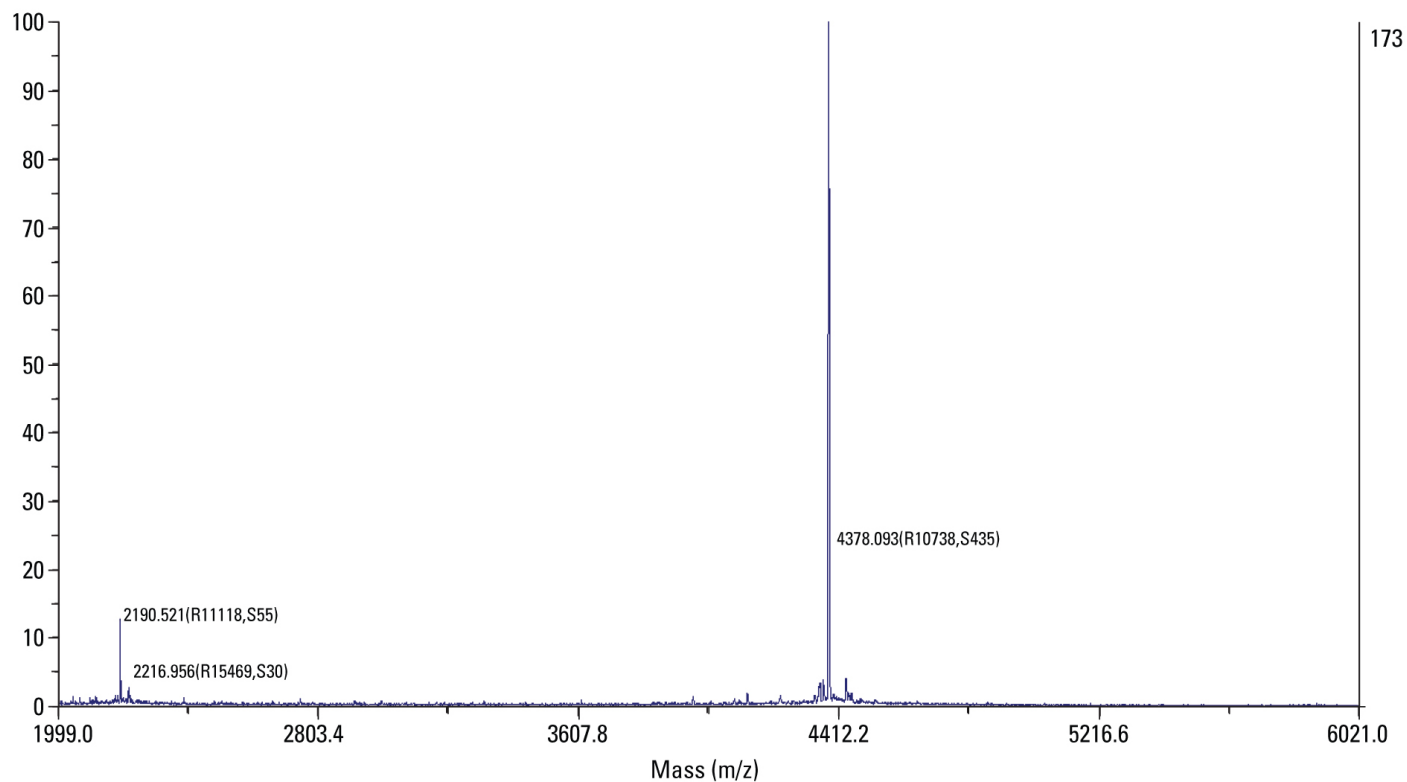




Figure 6: MALDI TOF MS of Purified PNA-Peptide based Oligonucleotide Biotherapeutic



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 column¹.
- 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 oligonucleotides².



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)