Ultra-High-Resolution Separation of Oligonucleotides on Pellicular Anion-Exchange UHPLC Columns

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Key Words
DNA, ssDNA, siRNA, RNAi, antisense, aptamers, nucleic acids, oligonucleotides, oligodeoxynucleotides, DNAPac PA200, DNAPac PA200 RS

Goal

To demonstrate selectivity and support resolution for separation of therapeutic and diagnostic oligonucleotides and their derivatives.

Introduction

DNA expression alterations have been implicated in initiation and progression of various diseases. Several research groups have developed approaches that rely on oligonucleotides (ONs) to diagnose and/or help control the progression of some of these otherwise "undruggable" diseases. Therapeutic modalities include *antisense* oligonucleotides (ASO),[1] aptamers,[2] and short interfering *RNA* (siRNA, RNAi),[3] among others, to modulate expression of genes involved in diseases. Other therapeutic options using oligonucleotides are also under development.[4-6]

Because nucleic acids are susceptible to *in vivo* degradation by endo- and exo-nucleases, several modifications to oligonucleotide structure are applied to improve their stability and biological persistence. Some of these modifications include 2'-O substitutions (e.g. 2'-O-methyl) on ribose, backbone modifications (e.g., phosphorothioate and phosphoro*di*thioates, phosphoramidate linkages, abasic inverted linkages, and insertion of methylene bridges between phosphorous and the 5' oxygen to elongate the linkage). Internal ribo-sugar modifications, ("locked" and "unlocked" nucleic acids)[7-16] may also be used. These ON modifications used for ASO, aptamer, and siRNA therapeutics complicate the methods required to characterize the identity and purity of preparations of these potential therapeutics.

Chromatographic analyses are performed for these therapeutic and diagnostic ON applications, as well as in manufacturing and research environments where oligonucleotide purity and impurity identification may assist developers satisfy their respective regulatory agencies. Oligonucleotide characteristics influencing chromatographic interactions include length, base composition, the presence of coupling or synthetic deprotection failures, and dyes (or "molecular beacons"). Each modification may be combined with the others in any given oligonucleotide, making thorough characterization potentially very difficult.

The most commonly used chromatographic methods for ON characterization are anion-exchange and/or ion-pair reversed-phase chromatography. These modes utilize ionic interactions between the ONs and the stationary phase (for anion exchange), and between the ONs and the ion-pair reagent (for IP-RPLC) rendering the ONs more hydrophobic and enhancing reversed-phase retention. Both approaches enjoy wide popularity as both deliver good resolution and analysis throughput for oligonucleotides.[17-20] Haupt and Pingoud[21] reported that anion-exchange-based ssNA separations were superior to both direct reversed-phase and ion-pairing reversed-phase approaches because more ssNAs were resolved per unit of time with the anion exchange process. Hence, this

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application note will focus on a *pellicular* anion-exchanger designed for high-resolution ON separations.

Here, the utility for ON characterization using the Thermo Scientific™ DNAPac™ PA200 RS column is described. This column employs a pellicular strong anion exchange surface coating applied to 4 µm non-porous polymer particles. The pellicular nature of the DNAPac PA200 RS phase produces very narrow peak width, and the polymer structure supports retention control by pH, as well as use of solvent modifiers (e.g., CH_3CN). Changes to oligonucleotide selectivity may also be produced by the use of alternate eluent salts (e.g., Cl^- or ClO_4^-). The DNAPac PA200 RS column provides increased throughput of normal-length single-stranded nucleic acids (e.g., 15–25-mers) on short (50 mm) columns, and industry-leading resolution on longer (150 or 250 mm columns). Curved eluent concentration gradients may also be applied to improve resolution of longer oligonucleotide samples.

Included here are example separations of:
1) oligodeoxythymidine samples with 12–60 bases,
2) RNAi samples of identical length and sequence with 2',5'-linkages that can be inavertently introduced into sequences where unprotected ribose residues are present during siRNA strand annealing,[22] and
3) disastereoisomers generated by introduction of phosphorothioate linkages into oligonucleotides.

Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm
- Fisher Scientific Tris base (BP152-500)
- Fisher Scientific 2-amino-2-methyl-1-propanol (AMP, AC104060010)
- Fisher Scientific Sodium chloride, 99.5% purity for analysis (S640-500)
- Fisher Scientific HCl (A466-500)
- Limited-volume PP autosampler vials with caps (Dionex P/N 045539)
- Deoxythymidine oligonucleotide samples (purchased from Midland Certified Reagent Co. Inc.)

  $dT_{12\text{-}18}$ D-1218
  $dT_{19\text{-}24}$ D-1924
  $dT_{25\text{-}30}$ D-2530
  $dT_{31\text{-}40}$ D-3140
  $dT_{41\text{-}60}$ D-4160
- Mixed-base oligonucleotide "Sense" siRNAs harboring phosphorothioate linkages at specific predefined positions in the sequence, and eGFP "Antisense" oligonucleotides harboring aberrant 2',5' phosphodiester linkages in specific predefined sequence positions, were obtained from Integrated DNA Technologies.
- Antisense ONs with 2',5'-linkages:.

  2-5_0: 5′-AUG AAC UUC AGG GUC AGC UUG -3′
  2-5_1: 5′-A*UG AAC UUC AGG GUC AGC UUG -3′
  2-5_5: 5′-AUG AA*C UUC AGG GUC AGC UUG -3′
  2-5_10: 5′-AUG AAC UUC A*GG GUC AGC UUG -3′
  2-5_15: 5′-AUG AAC UUC AGG GUC* AGC UUG -3′
  2-5_20: 5′-AUG AAC UUC AGG GUC AGC UU*G -3′

  * indicates position of 2',5'-linkage
- Phosphorothioate-containing eGFP "Sense" ONs

  eGFP Sense-2PS: 5′-AGC UGA$_s$ CCC UGA AG$_s$U UCA UdCdT -3′

  "s" indicates position of phosphorothioate linkage.

Buffer Preparation

- Tris buffer, 2.0 M concentrate (pH 8.0): Dissolve 242.28 ± 0.05 g Tris base in 742.7 ± 0.1 g DI water. Bring to pH 8.0 with 112.7 g concentrated HCl and verify pH using a calibrated pH meter. Filter the solution through 0.2 µm pore membrane filter (density [d]=1.097).
- AMP buffer, 0.2 M (pH 9.5): Dissolve 18.38 ± 0.05 g 97% AMP in 968.58 ± 0.05 g DI water. Bring to pH 9.5 by addition of 13.76 ± 0.05 g concentrated HCl. Verify or adjust pH using the calibrated pH meter. Filter the solution through 0.2 µm pore membrane filter.
- Sodium chloride, 5.0 M concentrate: Dissolve 584.4 ± 0.1 g sodium chloride in 1766.0 ± 0.1 g of DI water. Filter the solution through 0.2 µm pore membrane filter (d=1.175).
- Tris buffer, 40 mM (pH 8): Dilute 21.94 ± 0.05 g of Tris buffer, 2.0 M concentrate, pH 8.0 into 980.0 ± 0.05 g DI water. Mix well and transfer into chromatographic eluent A reservoir.
- 1.0 M NaCl, 0.04 M Tris (pH 8.0). Dilute 21.94 ± 0.05 g of Tris buffer, 2.0 M concentrate, pH 8.0 and 235.0 ± 0.05 g 5.0 M NaCl concentrate into 780 g DI water. Mix well and transfer into chromatographic eluent B reservoir.
- AMP buffer, 40 mM (pH 9.5): Dilute 219.4 ± 0.1 g of 0.2 M AMP buffer pH 9.5 into 800.0 ± 0.1 g DI water. Mix well and transfer into chromatographic eluent A reservoir.
- 1.25 M NaCl, 0.04 M AMP (pH 9.5). Dilute 219.4 ± 0.1 g of 0.2 M AMP buffer pH 9.5 and 235.0 ± 0.1 g 5.0 M NaCl concentrate into 600 ± 0.1 g DI water. Mix well and transfer into chromatographic eluent B reservoir.

Sample Preparation

Dilute each 25 unit (25 A_{260}) dry ON sample (dT_{12-18}, dT_{19-24}, dT_{25-30}, dT_{31-40}, dT_{41-60}) into 1.25 mL DI water to prepare a 20 A/mL stock. Transfer 8.0 µL of each stock into a sample vial and add 392 µL DI water. Cap the vial(s). Mix well and place vial(s) in autosampler rack. (Note: store the dissolved standards at -20 °C.) In this note, 15 µL injections were employed.

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ UltiMate™ Dionex™ 3000 RSLC Analytical LC system consisting of:
	Solvent degas system (SRD 3600, P/N 5035.9230)
	DGP-3600RS dual ternary gradient pump (P/N 5040.0066)
	TCC-3000RS thermal compartment (P/N 5730.0000)
	WPS-3000 TBRS autosampler (P/N 5841.0020)
	DAD3000RS absorbance detector (P/N 5082.0020)
	Semi-micro flow cell, PEEK, 2.5 µL, 7 mm path length (P/N 6082.0500)
	Nylon 0.2 µm filter unit (Fisher Scientific 0974026A)
	pH Meter Accumet XL150 (Fisher Scientific 13-636-XL150)
	Thermo Scientific™ Orion™ ROSS™ combination pH electrode (Fisher Scientific 13-641-729)

Separation conditions for Figures 1 and 3:

Column	DNAPac PA200 RS (4 µm), 4.6 × 150 mm (P/N 082509 Figures 1 & 3)
	DNAPac PA200 (8 µm) 4.0 × 250 mm (P/N 063000 Figure 3 only)
Mobile phase A	40 mM Tris, pH 8
Mobile phase B	40 mM Tris, pH 8, 1.0 M NaCl
Flow rate	1.0 mL/min
Temperature	30 °C
Diode-array detector	260 nm

Separation conditions for Figure 2:

Column	DNAPac PA200 RS (4 µm) 4.6 × 250 mm (P/N 082510)
Mobile phase A	40 mM AMP, pH 9.5
Mobile phase B	40 mM AMP, pH 9.5, 1.25 M NaCl
Flow rate	1.0 mL/min
Temperature	30 °C
Diode-array detector	260 nm

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

At pH values between 6.5 and 8, standard ONs harbor one negative charge for each phosphodiester bond. Hence, a terminally nonphosphorylated ON will harbor a charge equal to one less than the number of bases. At constant pH, a linear gradient of salt will elute oligonucleotides primarily in order of increasing charge (and therefore increasing length). Since the relative charge *difference* between ONs will decrease with increasing ON length, the separation (resolution) between ONs will also decrease with length.

Thermo Scientific HPLC instruments support the use of *curved* gradients that start at one gradient slope, and continually lower gradient slopes over time.[18] Since ONs of increasing length are less well resolved with linear gradients, such curved (concave) gradients that reduce slope over time provide a means to improve the resolution of increasingly longer ONs.[20]

Figure 1 employs a concave gradient that modifies only a single command in the gradient program (designation of curve 3 instead of 5, see Table 1). In this example, 84 separate components were resolved in less than 10 minutes. In this example, a collection of deoxythymidine oligonucleotides including components of 12 to 60 nucleotides long were mixed to demonstrate the range of ON lengths resolved by this column. The peaks labeled 1–7, 10, and 13 represent "failure" sequences where incomplete coupling between dT additions result in shorter than expected ONs. Since the smallest length prepared was 12 nt long, these first peaks represent ONs of four to eleven bases, and thus 3–10 negative charges. In addition to these numbered peaks, 26 other components were resolved that represent other forms of ON synthetic failures, such as incomplete group (e.g. trityl- or cyanoethyl-) deprotection. Further analysis by coupling to MS and HRAM-MS instruments supports and extends ON characterization.[18-19]

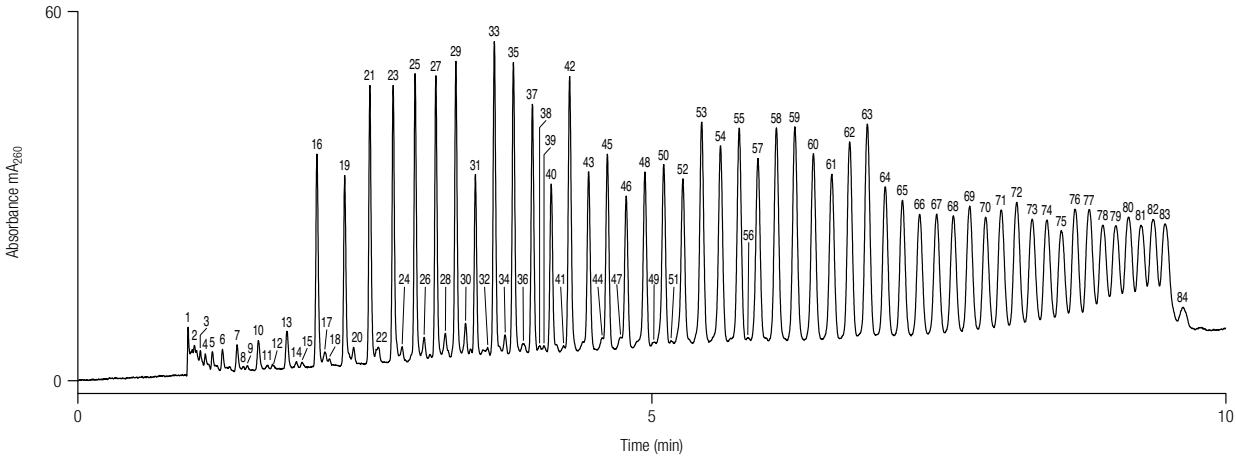

Figure 1. Separation of deoxythymidine oligodeoxynucleotides through a 4.6 x 150 mm DNAPac PA200 RS (4 µm) column using a 410–650 mm NaCl gradient in 8.4 minutes at 1 mL/min. This DNAPac PA200 RS 4.6 x 150 mm chromatogram of $dT_{12\text{-}61}$ resolves 49 full-length synthetic oligonucleotides (ONs), with eight failure sequences eluting early (between 1.0 and 2.0 minutes). The chromatogram also reveals separation of 26 ONs that are incompletely deprotected or otherwise modified, and which therefore do not co-elute with, but elute between, the fully deprotected deoxythymidine ONs in this 8.4 minute gradient.

Table 1. Gradient for Figure 1.

Time (s)	%A	%B	Curve
-10.0	59.0	41.0	5
0.0	59.0	41.0	5
8.4	35.0	65.0	3
8.5	20.0	80.0	5

Chromatography of homo-oligonucleotides may demonstrate resolution of numerous ON components, but does not represent the expected results when mixed-base oligonucleotides are used. In order to further demonstrate the resolution capability of the DNAPac PA200 RS column, a mixed-base 21-mer comprising the antisense strand of an eGFP siRNA ON pair was employed. In this case, aberrant 2',5'-linkages were introduced at specified positions along the strand. These aberrant linkages may be introduced at positions in siRNA sequences where an unprotected 2' hydroxyl is available for phosphoryl-migration during the annealing at elevated temperature of sense and antisense siRNA strands. The ONs with aberrant linkages at different positions were mixed together at different concentrations (to aid identification) and chromatographed as shown in Figure 2. The peaks are labeled with the position of the aberrant linkages (2',5'-) and the peak labeled "0" indicates the elution position of the ON with all normal 3',5'-linkages. All six ONs (representing both identical length and *sequence*) were resolved within ten minutes. Since the introduction of 2',5'-linkages does not alter the ON *mass*, the column must resolve these sequences based on some other intrinsic property of these ONs. Previous work by Schlech et. al.[23] and Giannaris and Damha[24] indicates that differences in solution conformation are introduced by the 2',5'-linkage.

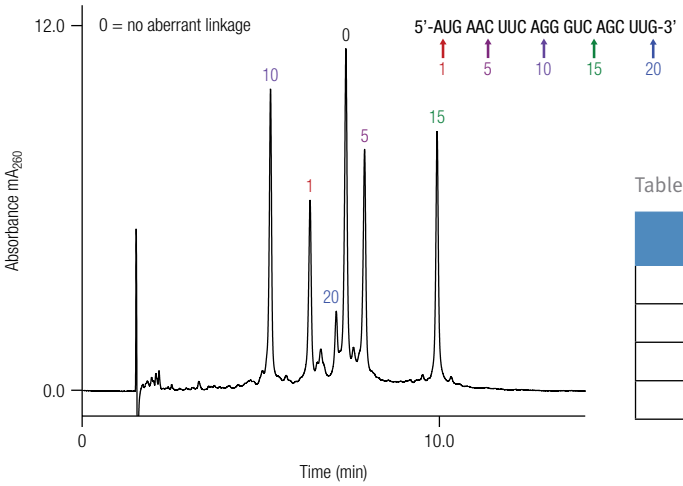

Table 2. Gradient for Figure 2.

Time (s)	%A	%B	Curve
-10.0	51.0	49.0	5
0.0	51.0	49.0	5
19.5	30.0	70.0	5
20.5	20.0	80.0	5

Figure 2. Resolution of a 21-base eGFP antisense strand with all normal 3',5' linkages and five different ONs of the same sequence, but with 2',5'-linkages inserted at positions 1, 5, 10, 15, and 20, on a DNAPac PA200 RS 4.6 x 250 mm column. The aberrant linkage positions are depicted above the chromatographic peaks. The DNAPac PA200 RS column resolved the oligonucleotides harboring aberrant (2',5'-) linkages at each of these positions in the sequence.

Another common backbone modification is the phosphorothioate (PS) linkage where one of the two nonbridging oxygens in the phosphodiester is replaced with a sulfur atom. This results in a pair of diastereoisomers (Rp and Sp) that are differentially susceptible to different exonucleases (suggesting conformational differences). Introduction of a single PS linkage in an ON leads to diasteromer pairs that can be resolved on the DNAPac PA200 (8 µm) column.[25]

In order to evaluate separation of the Rp and Sp diastereomers, the eGFP sense strand was prepared with PS linkages in positions 6 and 14 (counting in 5'-3' direction) and the ON was chromatographed using the DNAPac PA200(8 µm) and RS (4 µm) columns. Figure 3 compares the resolution obtained using these two columns, and peak labels indicate resolution of the diastereomer ON peak pairs.

The top panel shows the separation on the 8 µm column in 4.0 × 250 mm format, and the bottom panel the 4 µm column in 4.6 × 150 mm format. Even though the 4 µm column is only 60% the length of the 8 µm column, it produced 28–42% better resolution.

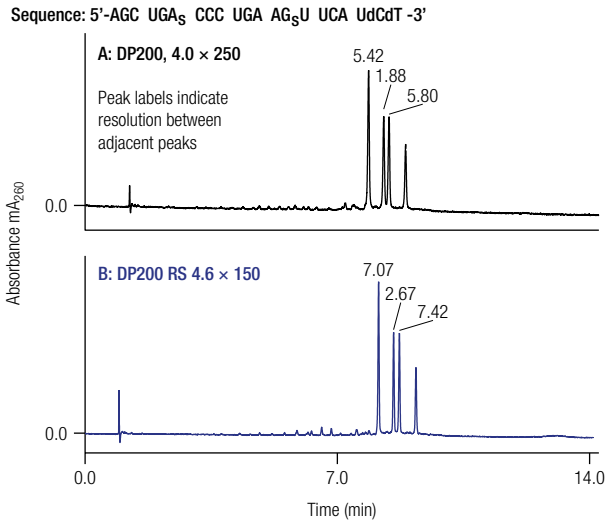

Table 3. Gradients for Figure 3.

Time (s)	%A	%B	Curve
-10.0	70.0	30.0	5
0.0	70.0	30.0	5
12.8	37.5	62.5	5
13.3	20.0	80.0	5

Time (s)	%A	%B	Curve
-10.0	65.0	35.0	5
0.0	65.0	35.0	5
10.0	32.5	67.5	5
10.4	20.0	80.0	5

Figure 3. Resolution of phosphorothioate diastereoisomers on a DNAPac PA200 RS 4.6 x 250 mm column. These chromatograms compare the resolution of two diastereoisomer pairs of a 21-base eGFP sense strand. The oligonucleotide harbors phosphorothioate linkages at positions 6 and 14 as shown in the sequence above the chromatogram. Peak labels indicate resolution between each pair of eluting ONs. The top panel shows the resolution using a 4.0 x 250 mm DNAPac PA200 (8 µm) column. The bottom panel shows the results on the same sequence using the 4.6 x 150 mm DNAPac PA200 RS (4 µm) column. The DNAPac PA200 RS (4 µm) column resolves the diastereoisomers with improved peak widths in essentially identical times, providing 28–42% increases in resolution between the diastereoisomer pairs.

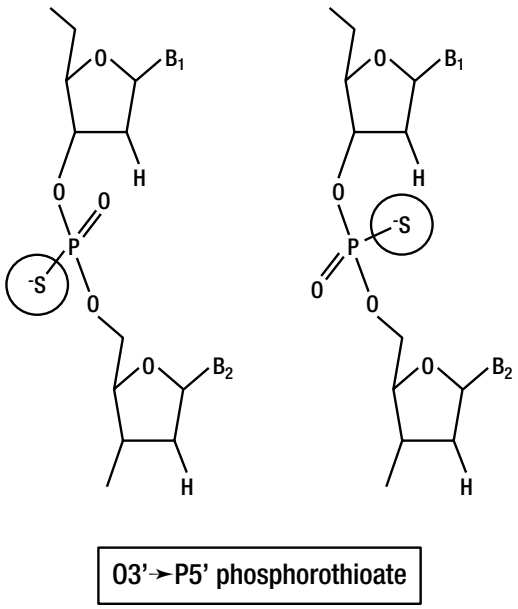

Figure 4. Diastereoisomer phosphorothioate linkages.

Conclusion

The DNAPac PA200 RS column provides industry-leading resolution of ONs used in therapeutic and diagnostic applications, including:

- Separation of oligonucleotides 60 nucleotides long with an average separation speed of between 8 and 9 bases per minute in a 10 minute chromatogram.
- Separation of siRNA oligonucleotide strands of identical length, sequence, and mass that differ only by the presence of aberrant 2', 5' -linkages along the oligonucleotide, including resolution of such ONs where the position of the aberrant linkage occurs in different positions.
- Separation of the Rp and Sp diastereoisomers in ONs harboring one or two phosphorothioate linkages.

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