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Separation of oligonucleotide phosphorothioate diastereoisomers by pellicular anion-exchange chromatography

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ABSTRACT

Synthetic oligonucleotides (ONs) are often prepared for development of therapeutic candidates. Among the modifications most often incorporated into therapeutic ONs are phosphorothioate (PT) linkages. The PT linkage introduces an additional chiral center at phosphorus to the chiral centers in D-ribose (and 2-deoxy-D-ribose) of the nucleic acid. Therefore, modified linkages can produce a diastereoisomer pair ([Rp] and [Sp]) at each PT linkage. These isomers are of identical length, sequence, charge and mass, and are not reliably separated by most chromatographic approaches (e.g., reversed phase chromatography) unless the ON is very short. Further these isomers are not distinguishable by single-stage mass spectrometry. During chromatography of a purified anti-NGF (nerve growth factor) aptamer containing 37 bases with 2 PT linkages by monolithic pellicular anion-exchange (pAE) column, we observed four components. The four components were postulated to be: (i) distinct folding conformations; (ii) fully and partially athioated aptamers; or (iii) PT diastereoisomers. Fractionation of the components, followed by de- and re- naturation failed to produce the original forms by refolding, eliminating option (i). Mass spectrometry of the fractionated, desalted samples revealed no significant mass differences, eliminating option (ii). Oxidative conversion of the PT to phosphodiester (PO) linkages in each of the purified components produced a single chromatographic peak, co-eluting with authentic PO aptamer, and having the PO aptamer mass. We conclude that the components resolved by pAE chromatography are diastereoisomers arising from the two PT linkages. Hence, pAE chromatography further enhances characterization of ON therapeutics harboring limited PT linkages and having up to 37 bases.

1. Introduction

For nearly thirty years pharmaceutical chemists have prepared oligonucleotides (ONs) for possible therapeutic use. In these ONs, structural modifications were introduced to improve resistance to nucleolytic degradation [1], optimize duplex stability [2,3] and improve aptamer affinity interactions [4]. Characterization of impurities in ON preparations is a requirement for regulatory filings during development of therapeutic ONs. The phosphorothioate (PT) linkage is likely the most widely employed ON backbone modification. The PT linkages introduce chirality at the modified phosphorus centers producing two isomers (Rp and Sp) [5]. Since the nucleic acid backbone harbors other chiral centers, the added PT chirality produces diastereoisomers. Introduction of an [Sp] (but not [Rp]) linkage at the 3′ end of a PT ON provides protection from rapid degradation by plasma 3′-exonucleases, and stereoselective nucleases cleaving either [Rp] or [Sp] isomers are reported [1,6–10]. An examination of PT linkages for antisense efficacy revealed that ONs with all [Rp] conformations enhanced RNAse H recruitment (a key component of the antisense process) more than those with the [Sp], or stereo-random conformations [11,12]. This reveals that the different PT-linkage isomers participate differentially in biochemical reactions.

RNAse-H recruitment is not a component of either RNAi or aptamer activity, but PT linkages may be combined with other modifications to increase nuclease resistance and improve biological half-life for aptamer and siRNA therapeutics. Where a limited number of phosphorothioate linkages are introduced [13], a method to resolve and separate the PT isomers creates opportunities to verify and perhaps even control the relative proportions of diastereoisomers in candidate ONs, and to more fully characterize the therapeutics during preclinical development. While ion-pair reversed-phase chromatography (IP-RPLC) is often coupled to single-stage ESI-MS (Electro-spray-ionization mass spectrometry) for resolving impurities, this will not differentiate the PT diastereoisomers, as they have identical mass. NMR analyses

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require significant amounts of material [8–10,12], and bulk solution methods such as optical rotation [11], may not reliably discriminate between Rp–Sp and Sp–Rp linkage pairs.

Early efforts to resolve and purify PT diastereoisomers by reversed-phase chromatography were limited to oligos of 2–5 bases with 1-3 phosphorothioate linkages [14–17]. However, Ion-pair RPLC-MS and CE methods did not resolve the diastereoisomers of 21-base oligonucleotides or their metabolites [18]. A 20-base PT ON and its metabolites were differentiated by ion-pair RPLC with ESI-MS, but this approach also failed to resolve the diastereoisomers [19]. Phosphorodithioate modifications also provide nuclease protection, but do not introduce phosphorus-chirality [20]. Yang et al. reported purification of phosphorodithioates by anion exchange chromatography on a porous phase, observing partial resolution of the diastereoisomers of a contaminating monophosphorothioate linkage in a 14-base ON [21]. Oligonucleotides harboring PT linkages between each base produce 2^n isomers where "n" is the number of linkages. Hence a 14-base fully 'thioated" ON would have 2^{13} (8192) different diastereoisomers. Clearly this exceeds potential resolution by HPLC techniques. However, Bergot and Egan observed that anion-exchange chromatography could resolve fully phosphorothioated from incompletely phosphorothioated synthetic PT oligonucleotides [22]. Similarly, Thayer et al. [23] observed peak broadening due to differential retention of the diastereoisomers of 12 and 15-base fully phosphorothioated DNA, and resolution of fully phosphorothioated DNA from those likely harboring residual phosphodiester (PO) linkages. Recently, a pellicular anion-exchanger was shown to resolve the diastereoisomers of 11 and 12-base oligos with a single PT linkage where the sulfur was conjugated to a nitroxide spin label [24]. While we found no reports on resolution of oligonucleotides greater than 14 bases harboring any number of PT linkages, resolution of the components of another isobaric modification, aberrant (2′,5′-) linkage isomers in a 21-base RNA, employed pellicular anion-exchange chromatography [25–27]. Similarly, Chen et al. documented the pellicular anion-exchange chromatographic resolution of PO from phosphoramidate linkage isomers [28]. These reports indicate that anion-exchange chromatography, especially on pellicular media, may be useful for separation or purification of the PT isomers in oligonucleotides having lengths consistent with both siRNA and aptamer applications. This report is the first demonstrating PT diastereoisomer resolution for an aptamer having potential therapeutic application. The ability to separate stereospecifically distinct forms now appears available. In light of this new capability, methods to synthesize oligonucleotides enriched in stereospecific forms would be useful for preparation of therapeutic ONs. Two reports on enrichment of chirally specific synthetic ONs appeared in 2003 [29,30]. Those observations may provide potential avenues for preparation of clinical quantities of therapeutic ONs enriched for one or the other stereospecific PT isomers.

2. Materials and methods

2.1. Chromatographic equipment

Dionex ICS3000 and UltiMate-Titanium chromatography systems were controlled by Chromeleon Chromatography Management Software (version 6.8). Silica reversed phase cartridges used for desalting were from Dionex (Acclaim PA2 C18, 3 μm, 4.6 mm × 50 mm). Purification of the normal and PT-linked aptamer RNA was performed on a 5 mm × 150 mm DNASwift SAX-1S monolith, and analysis of the purified components employed a 2 mm × 250 mm DNAPac PA200 (both from Dionex). The chromatographic conditions were as described in the figure legends. Gradient elution of the samples employed an eluent system modified from that previously described [29]. In this case phosphate was removed from the eluent buffer mix, limiting the useful pH range to 7.0–12.3.

2.2. Chemicals

Sodium chloride (Cat. # S-7653, 99.5%), Trizma base (Tris, Cat. # T-1503, ≥99.9%), sodium phosphate (Cat. # S-9638, 99.1%), and diisopropylamine (DIPA, Cat. # 471224, ≥99.5%), were obtained from Sigma. Methane sulfonic acid (MSA, Cat. # 6428, ≥99%), 2-amino-2-methyl-1-propanol (AMP, Cat. # 08580, >97%), phosphoric acid (Cat. # 79617, 85%), ammonium formate (Cat. # 09739 ≥ 97%), and sodium perchlorate ($NaClO_4$, Cat. # 71853, >98%) were from Fluka. Methanol (Cat. # 230-4, High purity solvent) and acetonitrile (Cat. # 015-4, High Purity Solvent) were from Honeywell (Burdick and Jackson). Deionized water was prepared using a Millipore Milli-Q Plus deionizing system.

Aptamer sample: A 37 base anti-NGF aptamer with two PT linkages and its phosphodiester (PO) analog were prepared via solid phase synthesis and purified by IE HPLC using NaBr eluent in 20 mM NaOH mobile phases in a Lab-Packed column with Tosoh Super-Q 5PW resin at Archemix Corporation.

2.3. Methods

2.3.1. Oligoribonucleotide diastereoisomer purification, assay, and desalting

The 37-base anti-NGF aptamer with two PT linkages and multiple RNA protecting groups was provided by Archemix. This aptamer sequence is proprietary, with a base composition of A:9, C:10, G:8, U:8 with two dT linkages. The aptamer was chromatographed on a Dionex DNASwift SAX-1S (5 mm × 150 mm) monolith at 1.0 mL/min in a Tris–MSA eluent, at pH 7 with two different eluent salts. The perchlorate gradient was 79–119 mM $NaClO_4$ in 15 mL (6 column volumes) at 15 °C in Fig. 3 (top panel), and the chloride gradient was 400–600 mM NaCl at pH 7 and 45 °C in Fig. 3 (bottom panel). Analysis of purified fractions was performed on a 2 mm × 250 mm DNAPac PA200 at 30 °C and pH 7 using linear or curved gradients [31] of 300–525 mM NaCl at 300 μL/min. Using a thermostated Well-Plate Sampling (WPS) fraction collecting autosampler (Dionex), we configured a system for automatic purification and desalting (Fig. 1.) This system employed two eluents for DNASwift and DNAPac chromatography (employing Tris,

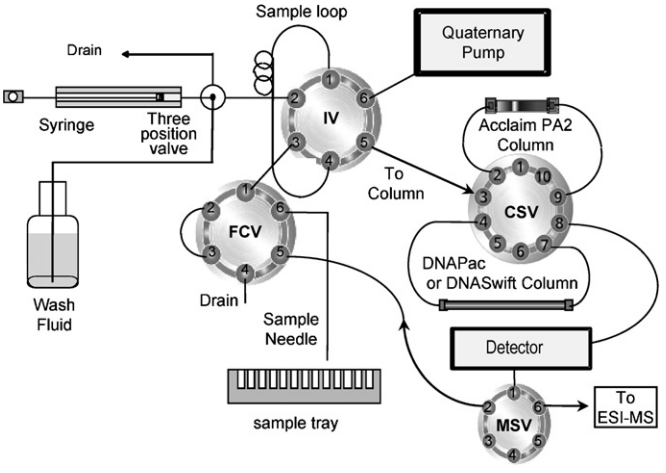

Fig. 1. Automated purification and desalting with a well plate autosampler: Depicted is the layout of an inert 4-eluent HPLC system with a fraction-collecting autosampler for purification, desalting and analysis of oligonucleotides. See Section 2.3.1 for details of automated desalting after purification. Valve labels: IV, injection valve; CSV, column selection valve; FCV, fraction collection valve; MSV, mass spectrometer valve.

AMP and/or DIPA buffered solutions), with and without 1.25 M NaCl. Eluting peaks were collected into 96-well plates. After purification the system was programmed to switch to the two other eluent reservoirs of the quaternary gradient system (these containing 20 mM ammonium formate in deionized water, with or without 40% MeOH), and to change the column selection valve for automated desalting on a 4.6 mm × 50 mm (3 μm dp) Acclaim PA-II reversed-phase column (Dionex). Samples were injected from the well-plates onto the Acclaim column and salt (eluting in the column void) was monitored by a Dionex ED50 conductivity detector. A step to 40% MeOH eluted the purified, (now desalted) oligonucleotides and collected them into new vials. These were dried by centrifugal evaporation and stored at −20 °C until resuspended in deionized H_2O for later analyses.

2.3.2. ESI-MS of oligonucleotide

ESI-MS was performed on an Oligo HTCS LC-MS system purchased from Novatia LLC (Princeton, NJ). The Oligo HTCS LC-MS system consisted of a CTC HTS-PAL autosampler (LEAP Technologies, Carrboro, NC), Paradigm MS4 HPLC with UV detector (Michrom BioResources, Auburn, CA), and Thermo LCQ Fleet MS system (Thermo Electron, San Jose, CA). All devices were controlled using the Xcalibur software data system and the ProMass utility (Thermo Electron) was used for deconvolution [32]. The ESI conditions were as specified [32] (their reference 5). The Novatia LLC desalting method was modified by reducing the eluent flow rate to 300 μL/min, and delaying the oligonucleotide elution step by 0.5 min for removal of excess salt co-collected with the aptamer components during anion-exchange chromatography.

2.3.3. Desulfurization of the RNA aptamer

Desulfurization of the RNA aptamer employed the method of Fearon et al. [33]. Briefly, RNA fractions separated on the DNASwift monolith were quantified using peak area during rechromatography on a DNAPac PA200 anion-exchange column. Samples of each fraction containing the component mixtures (19–40 μg in 30 μL) were added to 22.5 μL of 1 M N-methyl-imidazole in Tetrahydrofuran (NMI/THF), mixed with 12 μL of 0.5 M iodine in NMI/THF: deionized H_2O (15:85, v/v), and centrifuged to unite the sample. To each sample, 4.5 μL of 1 M (N,N-dimethylamino)-pyridine in THF was added, the sample mixed, centrifuged to reunite the sample, and incubated at 22 °C for 2.5 h. The desulfurization reaction was quenched by addition of 8 μL sodium thiosulfate (1 M in deionized H_2O). Each sample was directly desalted on gravity-packed 5 mm × 50 mm Superfine DNA grade Sephadex G-25 (Pharmacia, now GE/Healthcare) columns equilibrated with 40 mM Tris buffer at pH 7. The collected fractions were evaporated to dryness, resuspended and further desalted by reversed-phase cartridge chromatography [27], again dried in the Speed-Vac and stored at −20 °C until resuspended for further analyses. The dried samples were resuspended in 100 μL deionized H_2O prior to DNAPac PA200 chromatographic or ESI-MS analysis.

3. Results

3.1. Separation of aptamer components

During preclinical research, a 37-base anti-NGF aptamer was prepared and a large-scale purification performed. While a single major peak was observed during reversed-phase chromatographic analyses, chromatography of the purified sample on a DNAPac PA200 revealed the presence of four components that were differentially resolved at pH values from 7 to 11.5 (Fig. 2). The number of resolved components decreased as the pH increased from 10 to 11.5, consistent with denaturation of double-stranded conformations expected in an aptamer. However, at least two components

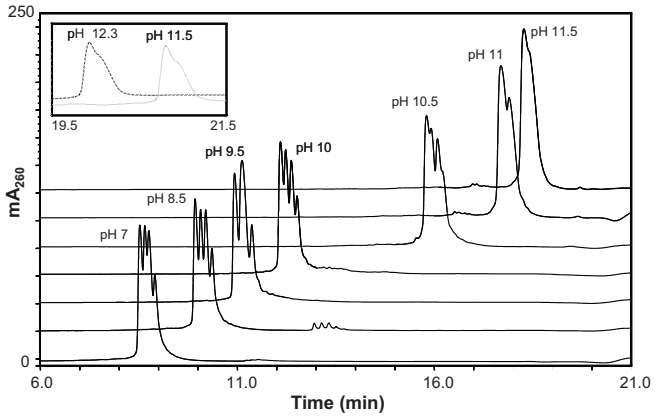

Fig. 2. Chromatography of the anti-NGF oligonucleotide, a 37-base aptamer with PT linkages at two specific positions in the sequence, at pH values from 7 to 11.5 using a DNAPac PA200 2 mm × 250 mm column, $NaClO_4$ eluents, and a curved gradient [31]. Gradient: 65–170 mM $NaClO_4$ in 15 min (curve 4), 300 μL/min, 30 °C and pH as indicated. Inset: chromatography of the aptamer in NaCl using 250–950 mM NaCl in 23.3 min at pH 12.3, 300 μL/min at 30 °C.

were discernable (as a peak doublet) at the highest pH examined (11.5) where hydrogen bonding is usually absent for short double stranded segments. These components persisted even during DNAPac chromatography at pH 12.3 (Fig. 2 inset), above the pH where Watson–Crick and G-tetrad ladder hydrogen bonds are broken [34].

3.2. Folding conformation analysis

In order to determine if these aptamer components represented unusually stable folding conformations [35], a larger amount (24 μg) of the the 37-base aptamer was chromatographed on a DNASwift hybrid monolith (monolithic substrate coated with a cationic polymer latex) at 15 °C to prepare larger quantities of the different components. Five fractions were collected from this sample (Fig. 3, top panel). These fractions were diluted 1:3 in DI H_2O to reduce the salt concentration, and reanalyzed on the DNAPac PA200 to verify that different components were present in each fraction (Fig. 4 bottom five traces). Each fraction revealed a primary component, consistent with its original elution position during DNASwift elution. If the different components represented different folding conformations, exposure of the samples to very high (denaturing) temperatures followed by a programmed transition to cold temperatures would allow them to refold, provided that they represent conformation variants. The remaining volumes of the fractions collected in Fig. 3 (top panel) were desalted and recollected using the system depicted in Fig. 1. These independent fractions were heated to 96 °C in a PCR processor (Techne) for 10 min, cooled to 5 °C at −20 °C/min, and reanalyzed on a DNAPac PA200 column as described earlier. The top five traces in Fig. 4 reveal that the desalted, heat-denatured, then cooled ONs in fractions 2–6 did not exhibit elution profile changes expected for conformation variants (i.e., they did not produce the initially observed four component elution pattern).

3.3. Incomplete phosphorothioation assay

The presence of four components in the purified anti-NGF aptamer could also be interpreted as indicative of aptamer copies having incomplete phosphorothioation. Specifically, these could have PT linkages at neither of the two positions, at both positions, and at one of each of the two positions intended, thus producing four components. While this is unlikely given the synthetic process, it is readily evaluated by comparing the mass values of the different

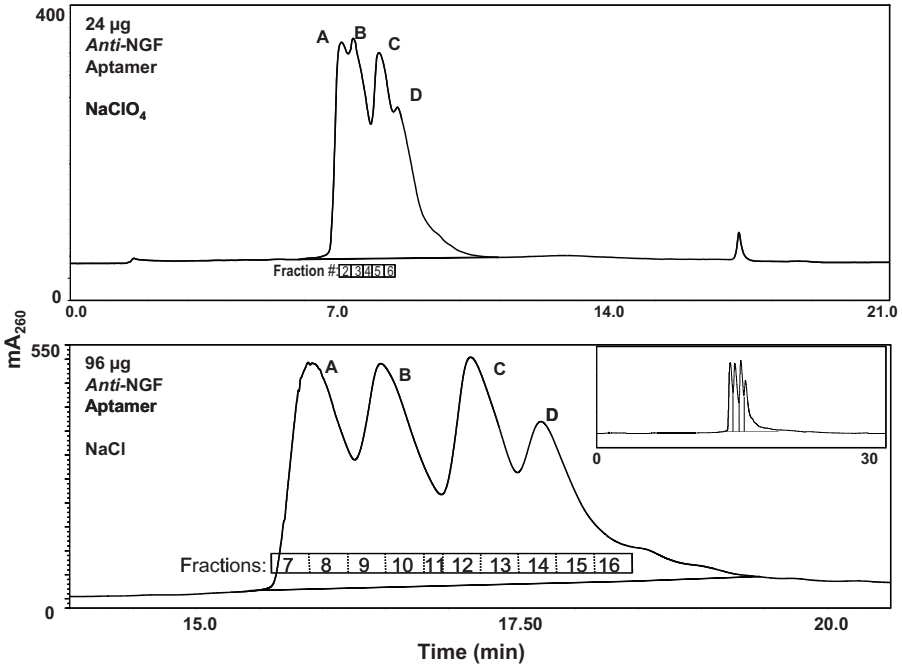

Fig. 3. Purification of 24- and 96-μg of the anti-NGF aptamer on a DNASwift SAX-1S monolithic anion-exchanger. Top panel: 24 μg sample; five fractions were collected as indicated across the peaks eluting during the linear gradient: 79–119 mM $NaClO_4$ at 1.0 mL/min, pH 7 and 15 °C. Bottom panel: 96 μg sample; ten fractions were collected as indicated across the peaks eluting during the linear gradient: 400–600 mM NaCl at 1.0 mL/min, pH 7 and 45 °C. Inset: full chromatogram.

components. The mass of sulfur differs from that of oxygen by 16 u. Hence, the aptamer with neither PT linkage would differ in mass from that harboring both PT linkages by 32 u, and from that with only one PT linkage by 16 u. The fractions collected in Fig. 3 (bottom panel) were, desalted and subjected to ESI-MS analysis (Table 1). While we do not suggest that deconvoluted ESI-MS is quantitative for this assay, we would expect one of the four components to harbor a mass of the fully phosphorothioated oligo (12474), two of the four to exhibit a mass 16 u lower (12,458), and the remaining component to present a mass 32 u below the aptamer target mass (12,442).

The ESI analyses shown in Table 1 indicated ~90% of each sample's mass was that of the aptamer with two PT linkages, even though the proportions of the largest chromatographically observed component (A, B, C or D) vary from at least 57 (B, fraction 23) to 96 (A, fraction 20) percent. Component C comprises 70% of fraction 27, and component D comprises 59% of fraction 30. We observe no correlation of any chromatographic component, with a comparable percentage of different mass, in any of the fractions from the larger sample.

3.4. Conversion of PT to PO linkages

During anion-exchange chromatography, oligonucleotides harboring phosphorothioate linkages elute significantly later than the same oligos with only PO linkages [22–23]. Indeed, an ON harboring a single residual PO linkage in a 27-base "fully 'thioated" sequence eluted earlier than the same ON without the residual PO linkage [22].

Evidence that pAE may resolve these diastereoisomers is provided by Yang et al. [21] where an 80-min gradient permitted partial resolution of [Rp] and [Sp] diastereoisomers on a 14-base singly phosphorothioated DNA using a fully porous AE column, and by Grant et al. [24] where a 47 min gradient produced full resolution of the diastereoisomers on a 12-base (nitroxide-labeled) DNA using a pellicular AE column. If the four aptamer components resolved here on the pellicular anion-exchangers represent the four diastereoisomers, each will have the same mass, and each will be retained to a greater degree than the same sequence containing only PO linkages. Fig. 5 compares the retention of the anti-NGF aptamer with that of an identical aptamer lacking phosphoroth-

Table 1
ESI-MS analysis of DNASwift-purified putative diastereoisomeric aptamer components.

Aptamer fraction	Chromatographic assay[a] (Form)				ESI-MS results (mass)[b]			
	% as form A	% as form B	% as form C	% as form D	% Target Mass (12474)	% as Target −16	% as Target −32	Ion-Intensity
7	96	2	2	0	90	0	0	3.2E+5
8	89	11	0	0	91	0	0	4.7E+5
9	52	48	0	0	91	0	0	5.1E+5
10	42	57	1	0	90	0	0	5.9E+5
11	24	26	48	2	91	0	0	4.9E+5
12	16	20	61	3	90	0	0	3.9E+5
13	14	16	70	0	92	1.2	0	3.2E+5
14	7	11	41	41	91	0.4	0	3.3E+5
15	5	7	39	49	91	0.4	0	3.3E+5
16	0	4	37	59	92	0	0	2.7E+5

[a] Chromatographic assay: the percentages of each form are indicated as relative peak areas on a 2 mm × 250 mm DNAPac PA200 (conditions as in Fig. 4).
[b] ESI-MS results (mass): the % Target Mass indicates the presence of the aptamer with two PT linkages. Percentages as, −16 and −32 (u) indicate the percentages as PO linkages at one or both PT linkage sites.

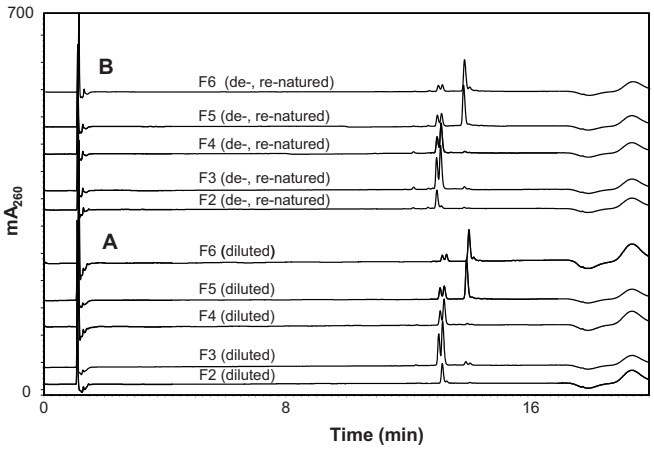

Fig. 4. Analysis of the fractions collected in Fig. 3 (Top panel) on the DNAPac PA200. Gradient: 300–525 mM NaCl in 15 min with 40 mM Tris pH 7 at 300 μL/min and 25 °C. (A) Samples 2–6 diluted 1:3 with deionized water (v:v). (B) Samples 2–6 after desalting, denaturing at 96 °C for 10 min and reannealing using a gradient of −20 °C/min to 5 °C prior to DNAPac analysis.

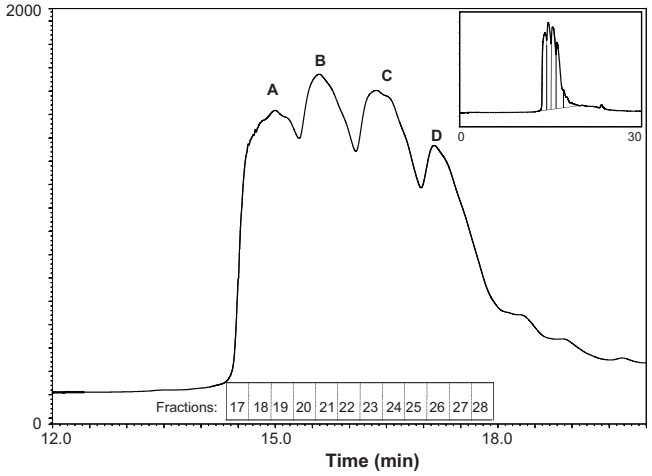

Fig. 6. Purification of 0.5 mg of anti-NGF aptamer on a 5 mm × 150 mm DNASwift SAX-1S column. Conditions as in Fig. 3 (B): twelve fractions were collected as shown between 12.3 and 18 min. Inset: full chromatogram.

ioate linkages. The all PO aptamer elutes significantly earlier than any of the PT aptamer components, and produces only a single peak instead of four. This is consistent with the four components observed during pellicular anion-exchange chromatography being diastereoisomers.

Fearon et al. reported a method to remove sulfur atoms from oligonucleotides containing phosphorothioate linkages [33], providing a mechanism to test the possibility that pellicular anion exchange chromatography resolves PT diastereoisomers. For this experiment, we collected the components from a 500 μg (40 nmol) sample of the 37-base 2 PT anti-NGF aptamer, as shown in Fig. 6.

The collected fractions were desalted and analyzed on the DNAPac PA200 to characterize the relative amounts of the components in each fraction. Fractions with similar component ratios were pooled and the constituent forms of each fraction (or pool) were verified chromatographically by comparison with the unfractionated sample on the DNAPac PA200 (Fig. 7).

The collected fractions (and pools) were desulfurized as described in Section 2.3.3, and reanalyzed by DNAPac PA200 chromatography. As control samples, the authentic all PO aptamer and the original anti-NGF PT aptamer were also chromatographed. With the exception of minor amounts of incompletely oxidized oligonucleotide, elution of all desulfurized components in each fraction aligned with the elution position of the authentic aptamer lacking phosphorothioate linkages (Fig. 8).

To verify that the samples were appropriately desulfurized, we measured the mass of the desalted products by ESI-MS. Table 2 summarizes the ESI-MS data for each sample. In this table, a blank sample oxidized and desalted along with the PT aptamer samples serves as a control. As expected, this blank produced very low signal intensities and a primary mass unrelated to either (PO or 2PT) aptamer. Conversely, the synthetic all PO samples (with and without chromatographic purification and desalting) serve as positive

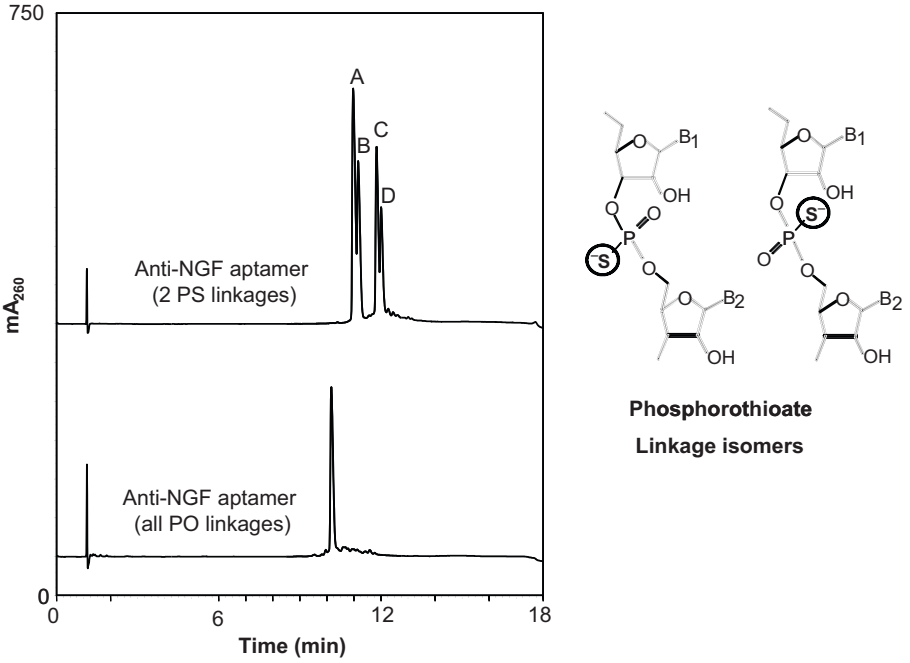

Fig. 5. Analysis of anti-NGF aptamer containing 2 PT linkages and an all PO version of the same aptamer on a 2 mm × 250 mm DNAPac PA200. The isomeric PT linkage structures are also depicted. Conditions were as shown in Fig. 4.

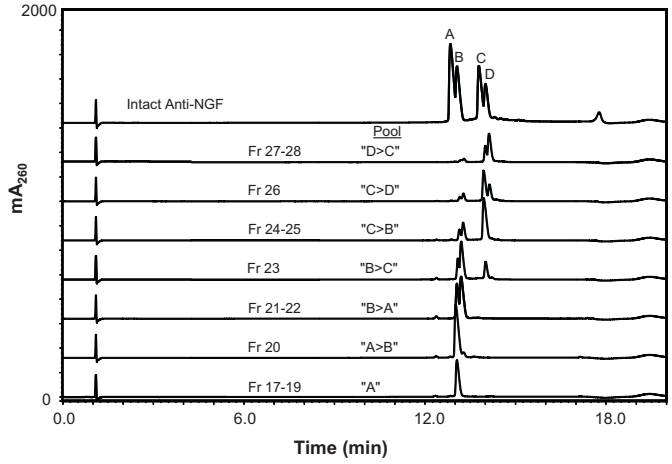

Fig. 7. Analysis of pooled fractions collected during DNASwift purification of 0.5 mg of anti-NGF aptamer before desulfurization on a 2 mm × 250 mm DNAPac PA200. Samples harboring similar proportions of the four components were pooled and reexamined. Samples are labeled according to their relative proportions of the two most abundant components (e.g., "A > B" indicates a higher proportion of component "A" than "B"). Gradient conditions were as in Fig. 4.

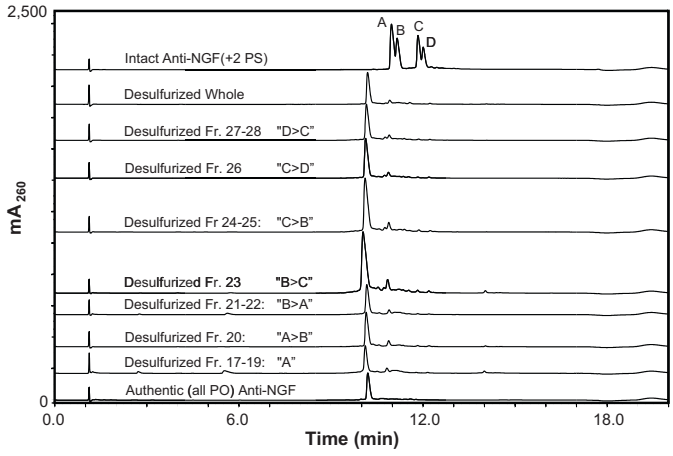

Fig. 8. Analysis of pooled fractions collected during DNASwift purification of 0.5 mg of anti-NGF aptamer after desulfurization on a 2 mm × 250 mm DNAPac PA200. Samples are as in Fig. 7, but after oxidative conversion of PT to PO linkages, and chromatographic conditions are as in Fig. 4.

controls. These samples both produce the PO aptamer mass at similar intensities and relative abundances. An unoxidized PT aptamer, providing the fully phosphorothioated mass serves as a negative control.

Table 2
ESI-MS determination of anti-NGF fractions after oxidation of PT to PO linkages.

Sample	Fraction(s)	Mass	Intensity	Abundance (%)
Desalt/ox/blank	–	9925.0	9.9E+02	22
All PO aptamer	–	12443.2	8.1E+04	76
Desalted all PO aptamer	–	12443.2	8.5E+04	77
Oxidized PS aptamer mixture	–	12443.9	4.6E+04	71
Oxidized PS aptamer "A"	17–19	12444.5	2.9E+04	55
Oxidized PS aptamer "A/B"	20	12443.6	3.1E+04	77
Oxidized PS aptamer "B/A"	21–22	12443.5	4.5E+04	67
Oxidized PS aptamer "B/C"	23	12443.5	4.6E+04	66
Oxidized PS aptamer "C/B"	24–25	12443.6	4.8E+04	69
Oxidized PS aptamer "C/D"	26	12443.4	5.0E+04	71
Oxidized PS aptamer "D/C"	27–28	12443.8	3.9E+04	68
Unoxidized PS aptamer	–	12475.0	2.4E+04	80

Fraction numbers indicate those of Fig. 6, pooled as described for Figs. 7 and 8.

4. Discussion

Characterization of components partially purified from oligonucleotide preparations can be difficult and time consuming. In our case, the instrumental setup depicted in Fig. 1 allowed automated purification, collection and desalting of ON samples, greatly simplifying the preparation of samples for the tests we performed.

Our first hypothesis for the identity of the components observed in the purified sample was that they represented conformation variants. Since aptamers are known to have specific and profound three-dimensional structures, this hypothesis cannot be trivially dismissed. One report describes an enzymatically transcribed 58-base aptamer sample that consists of two forms (apparently derived from a single parent sequence) that was incapable of conversion from one form to another [35].

In our case, the four components observed during analytical chromatography of the 37-mer are shown not to be conformation variants. First, at least two components persist during chromatography at pH 12.3, where nucleic acid hydrogen-bonds break (this technique was not attempted in [35]). Second, the separated components, when isolated, desalted and subjected to thermal denaturation and programmed cooling, did not produce the originally observed four chromatographic variants, but produced profiles essentially identical to those observed prior to denaturation.

Our second hypothesis, that the four components represented incompletely phosphorothioated forms, was also determined to be incorrect. Table 1 shows that each observed chromatographic component correlates to the mass expected for the full-length, fully phosphorothioated aptamer. Further, in no case was a signal with mass 32 u less than the aptamer with two PT linkages observed. This eliminates the possibility that the four components resolved here by pellicular anion-exchange chromatography were due to differential phosphorothioation.

We note that switching from perchlorate to chloride salt for our eluent system appears to improve both the apparent column capacity for the aptamer, and resolution of the four components that were first observed on the DNAPac column. Using $NaClO_4$, our attempts to purify the components from a small aliquot produced a minimal resolution of the four components. When we purified the components from a 96 μg aliquot with NaCl eluent we employed a longer gradient, but achieved resolution of the components greatly in excess of that expected based on the $NaClO_4$ eluent. While the differing gradient times renders this an indirect comparison, the use of NaCl as eluent salt enabled us to purify greater quantities (500 μg) as shown in Fig. 6, and discussed next.

Our third hypothesis was that the four components represent diastereomers introduced by the chiral nature of PT linkages (see structures in Fig. 5). This was examined by conversion of all PT linkages in each fractionated sample to PO linkages, and examining the products using two independent assays.

The first assay was chromatographic retention: each of the desulfurized samples eluted at the position of the authentic all PO aptamer (Fig. 8). In this figure, retention time variations are observable, but these represent a retention time range less than 10 s. Since the desulfurization process resulted in salt concentrations >1 M, minor inconsistencies in the two stages of desalting these samples could readily account for the very small change in retention on the low-capacity (DNAPac) analytical column.

The second assay was analysis of the desulfurized oligonucleotide component mass. Before desulfurization, each of the four components exhibited the mass of the aptamer with two PT linkages. After desulfurization, each of the four components exhibited the mass of the aptamer with no PT linkages. The only explanation for this observation is that the components are PT diastereoisomers unless the components represent aptamers with the PT linkages

at different positions in the sequence. Solid phase oligonucleotide synthesis procedures may allow this possibility, but would result in an extremely small proportion of incorrectly positioned PT linkages. Since the four components are present at $22.5 \pm 5.4\%$, the probability that they are incorrectly positioned PT linkages is vanishingly low.

We also attempted to perform differential enzyme digestions on the purified stereospecific components in order to identify their form (S_p vs R_p). Unfortunately, the anti-NGF aptamer harbors other protecting groups that severely inhibited the activity of the enzymes (Snake venom phosphodiesterase and Calf spleen phosphodiesterase [1]) so the diastereoisomer determination could not be completed.

5. Conclusions

We have demonstrated a method for resolving identical length (37-base) diastereoisomeric oligonucleotides harboring two internal phosphorothioate linkages, each with [Rp] and [Sp] isomers. That the separated forms are not alternate folding conformations was confirmed because heat denaturation and cooling did not generate the set of separable components initially observed for the unpurified sample. Further, chromatography at pH 12.3, where tetrad-ladder and Watson–Crick hydrogen bonds break, still produced peak doublets. Hence, the components are not hydrogen-bond dependent folding conformations.

These four separable components are not failure sequences (generated during synthesis as $n \pm x$ or incompletely thioated variants). PO ONs elute significantly earlier than ONs harboring PT linkages [22,23]; and an all PO form of this aptamer eluted earlier than any of the components observed in the aptamer sample harboring two PT linkages. That these components are diastereoisomers is supported by desalting and ESI-MS analysis of the fractions isolated from the original aptamer; all fractionated samples harbored different amounts of each component, but exhibited only mass values indicating the sequence with both phosphorothioate linkages.

Further, oxidative conversion of each component from the PT to PO forms resulted in oligonucleotides eluting only at the position of the authentic aptamer synthesized with only PO linkages. Finally, each of the four components separated by the pellicular anion exchangers yielded, upon desulfurization, components having a single mass; that of the all PO aptamer. These results all confirm the hypothesis that each component represents a full length 37-mer with 2 PT linkages. Hence, pellicular anion-exchange chromatography differentially retains the diastereomeric components of oligonucleotides harboring phosphorothioate linkages. When there are few such linkages, purification of the diastereoisomers on high capacity pellicular AE monoliths is possible. This report represents the first observation of PT diastereoisomer resolution for ONs larger than 14 bases, and does so for a 37-base modified aptamer harboring two such linkages. After beginning this study, we observed resolution of PT diastereoisomers in a 21-base eGFP siRNA sense strand harboring PT linkages at positions 6 and 14 in the sequence [36]. These observations, and prior reports for shorter ONs [21,24], indicate that phosphorothioate diastereoisomer resolution is a general attribute of pellicular anion-exchangers.

Pellicular anion exchangers provide a means to characterize phosphorothioate diastereoisomers of longer ONs, such as aptamers and the components of siRNA preparations, provided the oligonucleotides harbor only one to a few such linkages. This capability in a high-capacity monolithic anion exchanger extends the characterization of lab-scale preparations of aptamers, siRNA and other oligonucleotides where relatively few PT linkages are present.

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