Pellicular anion-exchange chromatography applied to RNAi assays for monitoring strand stoichiometry and RNA stability.

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Abstract

RNAi products require both single strand and duplex impurity assays. Duplex assays should allow titration of guide or passenger strands that may be in excess. These assays are chromatographic, and typically employ two steps: First, as is common to other single-stranded therapeutics, denaturing conditions must resolve both guide and passenger strand, and thus may reveal impurities formed during the annealing process. Next, native conditions, where the duplex is stable, are used to resolve the duplex and both single strands from one another. This allows analysis of possible duplex impurities, such as those arising from annealed impurities from the guide, passenger, or both strands. RNAi component strands, or those of other therapeutic modes, may harbor one or two phosphorothioate linkages. These introduce diastereoisomers that may impact the RNAi stability or efficacy. Resolution of the diastereoisomers allows assessment of the influence of each in the process, and where the separation can be accomplished at mg levels, can support bioanalytical identification of the contribution of each diastereomer. We describe here simple anion-exchange chromatographic approaches to accomplish these separations.

Introduction:

Among the continually increasing numbers of RNA therapeutic modes, four have been identified as clinically promising. These are Antisense oligonucleotides (ONs) [1], Ribozymes [2], Aptamers [3], and RNA interference (RNAi) [4,5] ONs. While several new non-coding RNA forms have been described recently, these four therapeutic modes show promise. The RNAi approach conscripts a subset of microRNA (miRNA) processing machinery, allowing exogenously supplied RNA of specific sequence to stimulate turnover of accordingly-specific mRNA [6]. Where the activity (or defect) in a specific protein is known to produce or support a disease state, turnover of the mRNA encoding that specific protein will suppress progression of that disease, or it symptoms. Numerous examples of gene-product "Knock-Down" have been described, ranging from control of Cholesterol levels [7] to suppression of Ebola Virus replication [8]. Understandably, this has stimulated intense interest in RNAi as a new therapeutic model.

We have developed pellicular anion exchange chromatography (pAXLC) as a productive approach to evaluate ON purity for nearly two decades, and recently coupled this technique to ESI-MS using an automated desalting protocol to identify the positions of aberrant 2′,5′-linkages in RNA [9], and to help verify that four components in an anti-NGF aptamer resolved by this technique were phosphorothioate diastereoisomers [10].

The RNAi duplexes are prepared from ONs produced on efficient nucleic acid synthesizers using increasingly inexpensive materials, often in parallel. Some groups can prepare over 1000 different ONs in a single day. ONs to be used as therapeutic candidates are usually modified to introduce resistance to plasma and tissue nucleases, and provide increased stability. Modifications to the sugar-phosphate backbone may include phosphorothioate (PS[11]), phosphoramidate (PN [12]) and phosphothioamidate linkages (PSN [13]), as well as 2′,5′-linkages [14] and 3′-3′ capping [1].

In addition, the ribose-sugar may be modified with 2′ fluoro [15], 2′-O-methyl , and numerous other 2′-alkyl modifications [16], or include a bridge between the 2′ oxygen and 4′ carbon resulting in a "locked" nucleic acid (LNA [17]). Other decorations on therapeutic ONs may include polyethylene-glycolylation (PEGylation [3]) and even addition of cholesterol [4].

Resolution of these components from their unlabeled parent compound present further analytical challenges. Characterization of therapeutic ONs includes evaluation of ON quantity, length, base composition, purity, mass, and the presence or absence of the above-mentioned decorations.

These analyses employ UV spectroscopy, Capillary- (or gel-) electrophoresis, and NMR. A good source of specific analytical information is found in reference [19]. Chromatographic methods include Reversed-phase (and ion-pair reversed-phase), size exclusion and anion-exchange chromatography. RPLC and AXLC are useful for base composition, length, and purity assessments. Size exclusion chromatography is typically used to determine the presence of excess single strand in duplex formulations, and also to evaluate duplex stability. However, both IP-RPLC and pAXLC are also useful and often faster for these assays. RPLC, IP-RPLC and AXLC have been directly coupled to ESI- (and MALDI-TOF) mass spectrometry, and these help provide mass information for full-length products, their impurities and for ON

metabolites [9,10,23]. This approach has also been most useful for identifying the chemical source of synthetic impurities. AXLC has also helped identify both 2′,5′-linkage isomers that may arise during release from the synthesizer [22],or during annealing of the single-stranded components of duplex RNAi therapeutics. Where only one or two PS linkages are employed, pAXLC can resolve their diastereoisomers [10,23]. In addition, where a fully phosphorothioated ON is prepared, linkages lacking the sulfur atom (residual phosphodiesters) can be resolved by pAXLC [24], and sometimes by IP-RPLC.

A great advantage of Anion-exchange liquid chromatography (AXLC) is that it allows control of selectivity by several readily modifiable chromatographic parameters. These parameters include pH, salt type (NaCl vs NaClO4 vs LiBr etc), use of solvent modifiers and temperature [25]. These factors can often be used as orthogonal mechanisms to support separation of virtually identical ONs. We have described resolution of ONs differing: by one base in length; by exchange of a single base for another [25]; by replacement of a single 3′,5′-linkage for a 2′,5′-linkage [9,21], and recently reported separation of the diastereoisomers of ONs having one or two phosphorothioate linkages [10]. Note that in the last two examples, the ON harbors identical length, mass, charge, and sequence. While AXLC on traditional porous media can probably accomplish these separations, their porous nature results in diffusive mass transfer between the mobile and stationary phases. This can severely limit resolution of ONs. This can be overcome by employing very low linear velocities (flow rates). However, this renders the separations quite time-consuming. Since developers of therapeutic ONs are required to evaluate numerous candidates, and their impurities, traditional AXLC media may not adequately support the necessary analytical throughput. Pellicular anion-exchangers employ a thin coating (of latex or nanobead) on a non-porous substrate resin to support convective mass transport. This mode results in essentially immediate partitioning of the ONs between the mobile and stationary phases, greatly improving peak shape, and consequently resolution and throughput. In this report we describe the use of temperature to control selectivity for separations of an RNAi duplex from its guide and passenger strand components. Because these separations can reveal impurities from each component they represent stability-indicating methods.

Figure 1: Effect of temperature on DNASwift Peak Width and resolution.

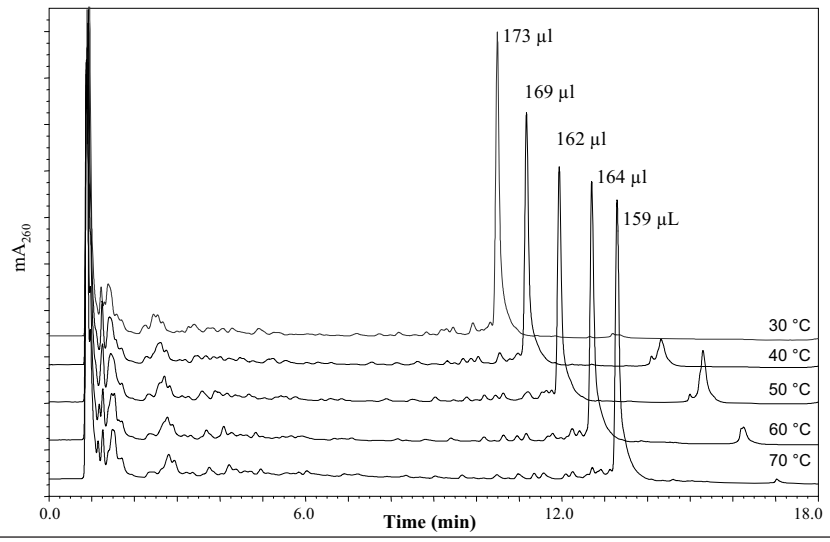

Figure 1. Effect of temperature on retention and peak width of a 20-base ODN. Column: DNASwift SAX-1S. System: Dionex DX600 inert quaternary gradient LC system. Gradient conditions: 100 – 800 mM NaCl in 15 min at 1.77 mL/min, pH 8 and at the indicated temperatures. Sample sequence: 5′ GGG ATG CAG ATC ACT TTC CG 3′.
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Figure 2: Effect of temperature on duplex, guide and passenger strands.

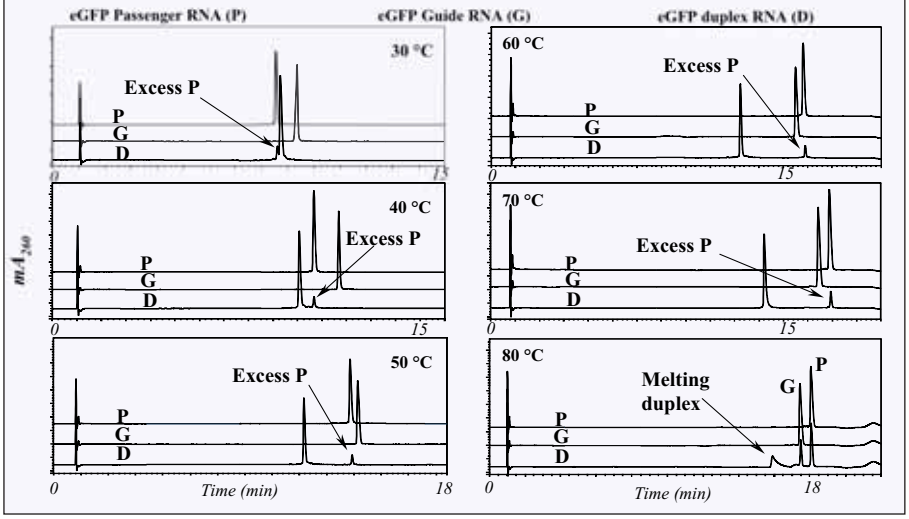

Figure 2. Effect of temperature on resolution of sense, antisense and duplex RNA.
The sense sequence is 5 -AGCUGACCCUGAAGUUCAUdCdT-3 ,and the antisense sequence is that shown in table 4. Column: DNAPac PA-200. System: Dionex UltiMate 3000 Titanium inert quaternary gradient LC system. Gradient conditions: 325 – 750 mM NaCl in 17.2 min at 300 µL/min, pH 7 and at the indicated temperatures.
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Table 1: Oligonucleotide Sequences:

DESIGNATION	SEQUENCE			LENGTH
AR25	5′	GGG ATG CAG ATC ACT TTC Cg	3′	20-mer
eGFP Passenger (no PS)	5′	AUG AAC UUC AGG GUC AGC UdTdG	3′	21-mer
eGFP Guide (no PS)	5′	AGC UGA CCC UGA AGU UCA UdCdT	3′	21-mer
eGFP Guide (1PS 6)	5′	AGC UGAs CCC UGA AGU UCA UdCdT	3′	21-mer
eGFP Guide (1PS 14)	5′	AGC UGA CCC UGA AGsU UCA UdCdT	3′	21-mer
eGFP Guide (1PS 6,14)	5′	AGC UGAs CCC UGA AGsU UCA UdCdT	3′	21-mer

Anti-NGF Aptamer: This sequence is proprietary; Base composition is A:9, C:10, G:8, U:8 with two dT linkages.

Figure 3: Resolution of phosphorothioate diastereoisomers by pAXLC

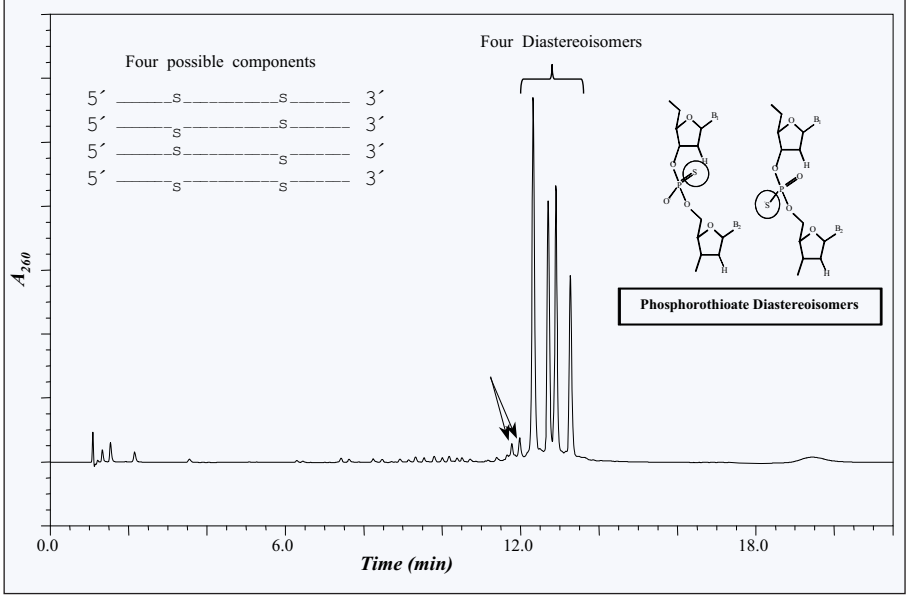

Figure 3. Separation of isobaric Phosphorothioate diastereoisomers on the DNASwift SAX-1X. Chromatography of a commercial sample of the indicated sequence with phosphorothioate linkages only at positions 6 and 14 resolves three peaks in DNA form and resolves all four in the RNA sample. Arrows indicate early eluting impurities likely to be phosphorothioate oxidation products. System: Dionex DX600 inert quaternary gradient LC system. Gradient conditions: 300-600 mM NaCl in 16.7 min at 1.5 mL/min, 30 °C and pH 7. Sample: eGFP (sense strand): 5 AGC UGAs CCC UGA AGsU UCA UdCdT 3 . "Reprinted from Journal of Chromatography-B, 878, J.R. Thayer, K.J. Flook, A. Woodruff, S. Rao and C.A., Pohl. New monolith technology for automated anion-exchange purification of nucleic acids. Pages 933-941, Copyright (2010) with permission from Elsevier."

Experimental:

Chemicals

Sodium chloride (99.5%), Trizma base (≥99.9%), sodium phosphate (99.1%), and diisopropylamine (DIPA, ≥99.5%), were Sigma brand and obtained from Sigma (St. Louis, Mo, USA). Methane sulfonic acid (MSA, ≥99%), 2-amino-2-methyl-1-propanol (AMP, >97%), phosphoric acid (85%), ammonium formate (≥97%), and sodium perchlorate (NaClO4, >98%) were Fluka brand, also obtained from Sigma as above. Methanol (High purity solvent) and acetonitrile (High Purity Solvent) were from Honeywell (Burdick and Jackson, Muskegon MI, USA). Deionized water was prepared using a Millipore Milli-Q Plus deionizing system.

Figure 4: Differential elution of PS-RNAs by pAXLC

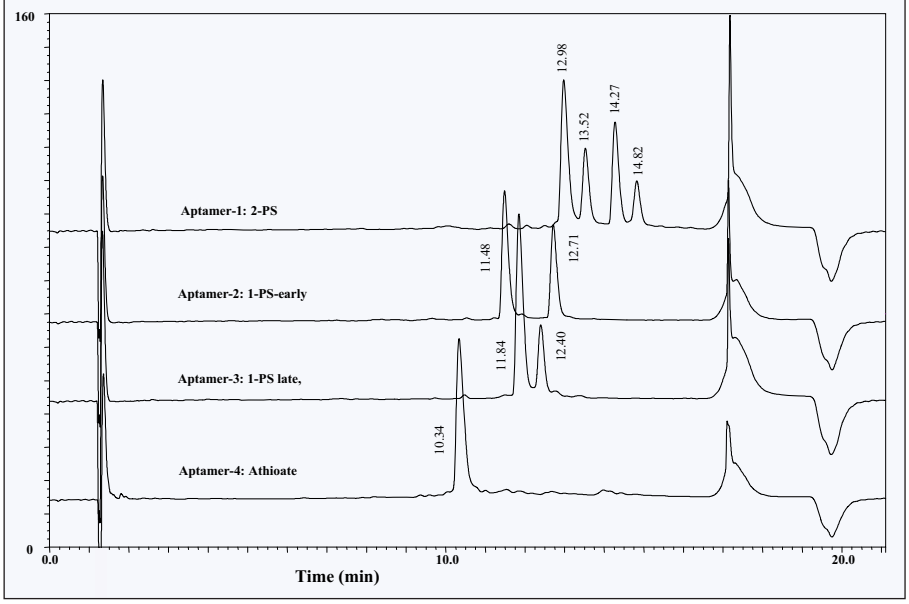

Figure 4. Separation of Phosphorothioate diastereoisomers in a 37-base anti-NGF aptamer by pAXLC. Column: DNAPac PA200 (4x250 mm). Gradient conditions: 400-513 mM NaCl in 40 mM NaCl in 14.6 minutes at 1.0 mL/min, pH 7 and 41 °C.

Oligonucleotides

The oligonucleotide used in this report are listed in Table 1. The AR25 DNA was obtained from Integrated DNA Technologies (Coralville, IL USA). The eGFP oligonucleotides (guide and passenger strands, with and without PS linkages) were obtained from both BioSearch Technologies (Novato, CA USA) and Integrated DNA Technologies (Coralville, IL USA).

Methods

The ONs were chromatographed on Dionex DNAPac PA200 pellicular packed bed, or DNASwift SAX-1S (5mm×150mm) hybrid (pellicular) monolith. Chromatographic conditions are provided in the figure legends. The chromatographic systems included either ternary or quaternary gradient pumps (DGP 3600 or GP50), WPS TBFC fraction-collecting autosamplers, UVD340U Photodiode array detectors and TCC100 column ovens. Samples were injected from well-plates or 300µL vials and where appropriate, the pH was monitored using a Dionex ED50 electrochemical detector.

Results and Discussion:

We evaluated the effect of temperature using a 20-base ON (AR25) as indicated in Figure 1. Here the sample was eluted with a common gradient (100 – 800 mM NaCl in 15 minutes) applied to the DNASwift hybrid monolith at temperatures from 30 to 70 °C. Each peak is labeled with the peak width (at half-height) expressed in µL of volume. Retention increases with temperature, but the peak volume shows a decreasing trend. These combined effects increase resolution, especially near the full length product.

We next examined the effect of temperature independently on guide, passenger and annealed duplex for the eGFP ONs (Figure 2). Here we observed that the different single-strands, and the duplex all exhibit different retention dependencies with temperature. Hence, not all ONs respond to changes in temperature to the same degree. This may allow tuning of selectivity to resolve critical pairs of therapeutic ONs. In this case, retention of the passenger strand increased most, while retention of the duplex increased least, with temperature. Thus, the order of elution is seen to change between both 30 to 40, and 50 to 60 °C. The presence of a small excess of passenger strand is noted at 30-70 °C, and the relative amount of this excess is stable below 70°C. At 70 °C, the peak area of this excess passenger peak increases slightly. This may indicate 70 °C to be near the T_m for the duplex. The trace at 80 °C confirms this supposition as the duplex is shown to be

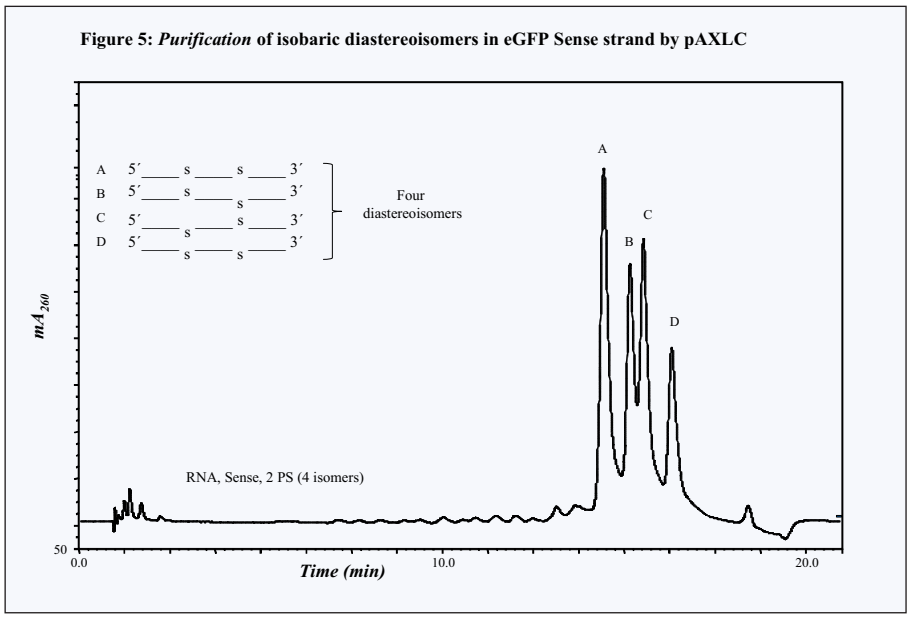

Figure 5. Purification of isobaric diastereoisomers by pAXLC. Column: DNASwift SAX-1S, 5x150mm. Gradient conditions: 300 – 600 mM NaCl in 16.7 min, in 40mM Tris, at 1.5 mL/min, pH 7 and 30 °C.

melting, releasing both passenger and guide strands as free oligonucleotides.

In each frame of Figure 2, small peaks representing fractional percentages of the ss and duplexed RNA elute before these major peaks. These represent minor impurities present in the individual strands before annealing. Similar impurities also elute after the annealed duplex, indicating some duplex components are not perfect duplexes. These imperfect duplexes may contain both "n+x" and "n-x" impurities, resulting in duplexes both larger and smaller that the expected complementary duplex.

The control of ON retention by temperature may allow resolution of ONs when they harbor quite different sequence or base composition, as in the case of RNAi duplex components. However, we also reported [5] that pAXLC sometimes resolves ON diastereoisomers arising from insertion of 1 or 2 PS linkages in the sequence. In this case we found that temperature influences resolution even of the paired diastereoisomers. This suggests that temperature changes can help manipulate selectivity even for ONs having an identical sequence. An example of the optimized separation of the diastereoisomers of the eGFP guide strand is shown in figure 3. Here, the four possible diastereomers formed by two PS linkages (at positions 6 and 14) were nicely resolved at 40 °C. The insert depicts the chiral difference at the phosphorus atom in the linkage.

We noticed that there were small, but relatively prominent impurities in this sample. In some cases, incomplete conversion of phosphodiester (PO) to PS linkages results in "residual" PO linkages, and these were shown to elute prior to the fully phosphorothioated ONs (see arrows). Since these impurities elute just prior to the PS diastereomers, and as PS linkages are known to be oxidizable, we hypothesized that these impurities might arise from oxidation of one or more of the diastereomers. If so, pAXLC can examine the stability of the linkages, offering the ability to assess another aspect of ON stability. To complement the eGFP data, , we employed four forms of an anti-NGF aptamer: without any PS linkages, with a single PS linkage at the 2nd or 15th position, and another ON with PS linkages at both positions. After examining the effect of temperature on these separations, we observed the best overall conditions (at pH 7 as used here) to require 41 °C. These separations are shown in Figure 4.

When the PS linkage was at position 15, this temperature placed the diastereomer pair, between the positions of the diastereomer pair having the linkage at position 2. In this case a small impurity peak was observed in both ONs harboring a single PS linkage, eluting at the position of the athioated anti-NGF aptamer. Similarly, the chromatogram of the aptamer with both PS linkages revealed impurity peaks at the positions of each of the singly 'thioated diastereoisomers. Hence, pAXLC under optimized temperature conditions allows stability indication for phosphorothioate oxidation. These analyses were performed with a column packed with non-porous pellicular beads. Figure 5 shows separation of the eGFP components on the latex-coated hybrid monolith. While the peak efficiency did not match that of the packed bed column, the hybrid monolith harbors a much higher loading capacity, and thus accomplishes the separation of much greater quantities. This hybrid monolith therefore can support collection of sufficient amounts of diastereoisomers for bioanalysis of the different chiral forms.

Conclusions:

We present here several example pAXLC separations demonstrating the utility of temperature control for improving the separation of important therapeutic oligonucleotide forms. Different ONs exhibit different rates of retention change with changes in temperature, and duplexed RNAs seem to change retention less than ssRNAs. These characteristics allow facile separation and analysis of both duplexed RNA and its component complementary ssRNA forms. While one may expect that different sequences, such as complementary RNAs, may show different selectivities with temperature, we show here that temperature control also facilitates resolution of ONs with identical sequence, when these ONs harbor different diastereoisomeric (chiral) centers.

These assays are stability-indicating methods because they reveal impurities that may accumulate in both ss- and duplexed RNA formulations. The examples given here show the presence of impurities with different lengths from both the complementary ssRNA components, and in the duplex forms. In addition the oxidation of PS linkages is shown to reveal the presence of specific and distinct diastereomeric products.

The high resolution separations of these RNAs by pAXLC using packed bed columns can be scaled up using a hybrid monolith to support bioanalytical assays allowing examination of the biochemical impact of the different chiral isomers. While not shown here, we have also prepared duplexes using the different purified diastereoisomers to prepare duplexes, allowing assessment of duplex stability (T_m, unpublished observations by JRT).

Finally, pAXLC (and AXLC) can be automatically coupled to ESI/MS for identification of some impurities, when they have different mass values. Indeed, coupling pAXLC to ESI/MS does permit resolution of the different diastereoisomeric forms, and thus supports forced degradation studies where PS linkages are present.

As demonstrated here, and in numerous other reports, AXLC, and in particular pAXLC is a powerful technique for analysis of several common attributes of both RNA and DNA ONs [19,23,25]. This method allows discrimination of PS diastereoisomers, aberrant linkage (2′,5′-

Linkage) isomers, can discriminate between PO, PS and PN linkages [9,10,12,18], can be coupled to ESI/MS for impurity identification [9,19,23], and is orthogonal to Reversed-Phase and Ion-Pair Reversed phase methods, as has been discussed in this symposium.

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