ABSTRACT

The need for multiple approaches to determine API yields and identify contaminants generated during therapeutic oligonucleotide (ON) development stems from regulatory guidelines. Among the possible contaminants unique to RNA are linkage isomers that are difficult to identify by MS and LC-MS techniques. Using non-porous bead-based and higher capacity monolithic anion exchange phases for ON analysis and purification, we demonstrate an alternative method for evaluation of these isomers, and suggest an independent linkage-confirming technique. Non-porous bead and monolithic columns support resolution of ONs with control of selectivity, and improved sample throughput in R&D or quality assessment environments. The monolithic phases are scalable to laboratory scale purification. We will discuss specific approaches for evaluation of contaminants unique to synthetic RNA, as well as options for scalable purification.

Oligonucleotide Linkage vs Retention: DNAPac PA200 4 × 250mm Column

RNA synthetic routes may produce occasional 2’–5’ phosphodiester bonds where biologically ‘normal’ RNA would have 3’–5’ linkages. During the TIDES 2005 conference the opinion was expressed that the presence of these aberrant linkages are likely to have biological consequences, and that they represent a difficult analytical challenge because:

1. They do not introduce a change in mass that might be detected by mass spectrometry
2. They are not expected to introduce a change in hydrophobicity that would produce a selectivity change in reversed-phase chromatography, and
3. They are unlikely to produce changes in ionic character of RNA that would allow differentiation by ion-exchange chromatography.

Initial studies employed poly-A or poly-U 21mers with 0, 2, 4, or 8 consecutive 2’–5’ linkages at the 5’ end of the oligonucleotide. Figure 1 shows five consecutive 2’–5’ linkages.

In Figure 2 a curved NaCl gradient applied to the DNAPac PA200 column readily resolves the cartridge-purified rA21mer RNAs in order of decreasing number of 2’–5’ linkages.
In Figure 3, the curved NaCl gradient applied to the DNAPac PA200 column readily resolves the machine-grade rU$_{21}$ mer RNAs in order of decreasing number of 2'–5' linkages. Since elution before the normal full length oligomer is characteristic of failure sequences often contaminating purified oligonucleotides, an independent assay is necessary to confirm the presence of the aberrant linkages.

The exonucleases in Figure 4 excise terminal monophosphates from oligonucleotides. Phosphodiesterase-I (PDAse-I) processes from the 3' end, producing 5' monophosphates, and Phosphodiesterase-II (PDAse-II) processes from the 5' end, producing 3' monophosphates.

If one or both of these exonucleases fails to process through 2'–5' linkages, it should produce oligonucleotide fragments indicating the presence of those aberrant linkages.

In Figure 5, the PDAse-II control shows no interfering peaks and the undigested rA$_{21}$ mer elutes at ~19 minutes. Exposure of the rA$_{21}$ mer to either PDAse reveals complete digestion by both enzymes in 18 hr.

In Figure 6, the PDAse-I control shows no interfering peaks and the undigested rA$_{21}$ mers with 0-, 2, 4, and 8 aberrant linkages elute between 15.1 and 18.5 minutes. Exposure of these compounds to PDAse-I reveals complete digestion of all oligonucleotides in 18 hr.
In Figure 7 the undigested rA_mers with 2 aberrant linkages elutes from the DNAPac PA200 column at 18.2 minutes, and exposure of this oligo to PDAse-I results in complete digestion.

Exposure of this compound to PDAse-II for 18 hours reveals a major digestion product at ~ 3.57 minutes, and minor products at 4.4, 5.2, and 6.8 minutes. Exposure for more time (24 hr), and an additional 17 hours after addition of more enzyme did not change this pattern.

Next (Figure 9) we repeated the assay with the pyrimidine base (U) to compare these results with the purine base (A). The results obtained with the rA_mer are fully repeated with the rU_mer. Hence, the PDAse-II product appears to work equally well for purines and pyrimidines.

RNA with inadvertent 2’–5’ linkages (Figure 10 A) are unlikely to have them placed consecutively, or at multiple locations like the test oligo-nucleotides previously described.

However, they are likely to have only one inadvertent 2’–5’ linkage, or if more than one, placed at non-adjacent positions, as suggested in Figure 10 B.

Undigested rA_mers with no aberrant linkages elutes at 19.4 minutes, and exposure of this oligo to PDAse-II results in complete digestion (Figure 8). Exposure of this compound with 2, 4, or 8 aberrant linkages to PDAse-II for 41 hours reveals major digestion products at ~ 3.56 (rA_2(L2)), 4.86 (rA_4(L4)), and 7.38 (rA_8(L8)) minutes. The DNAPac PA200 elution position of each of these fully digested products is consistent with oligos of length equal to the number of consecutive aberrant linkages.
In Figure 11 the new suite of mixed-base 21-mers with aberrant linkages were chromatographed on the DNA Pac PA200 at pH 7. At this pH samples Dio-5, Dio-6, and Dio-9 are completely resolved from all other samples, and are shown as red in the table. The pairs unresolved at this pH are shown as black.

In Figure 12 the fully resolved components are not shown, and the rest of the new suite of mixed-base 21-mers with aberrant linkages were chromatographed on the DNA Pac PA200 at pH 9. Components resolved at pH 9 are now depicted in red in the table.

In Figure 13 the fully resolved components are not shown, and the rest of the new suite of mixed-base 21-mers with aberrant linkages were chromatographed on the DNA Pac PA200 at pH 10. Components resolved at pH 10 are now depicted in red in the table.

**Figure 11. Retention of Mixed-Base 21-mers with 2′–5′ Linkages: pH 7**
DNA Pac PA200, 66–132 mM NaClO/10 min, 1 mL/min, 30 °C

**Figure 12. Retention of Mixed-Base 21-mers with 2′–5′ Linkages: pH 9**
DNA Pac PA200, 76–142 mM NaClO/10 min, 1 mL/min, 30 °C

**Figure 13. Retention of Mixed-Base 21-mers with 2′–5′ Linkages: pH 10**
DNA Pac PA200, 99–165 mM NaClO/10 min, 1 mL/min, 30 °C
Chromatography of PDAse-II treated mixed-base RNA 21 mers containing one or two aberrant 2′–5′ linkages at different positions in the RNA, result in specific, differentially retained, degradation products. The chromatographic elution position is indicative of the length of the digested fragment.

**CONCLUSIONS: I**

**Observations on RNA with aberrant 2′–5′ linkages**

- Phosphodiesterase-I processes through RNA with 2′–5′ linkages.
- Phosphodiesterase-II appears to stop at 2′–5′ linkages.
- This Phosphodiesterase-II product appears to *skip over* 2′–5′ linkages to produce partial digests from 5′-end located 2′–5′ linkage oligos.
- Exhaustive (overnight) Phosphodiesterase-II treatment does not continue to process through 2′–5′ linkages, even with extra enzyme and time.
- Exposure to pH 11 for 20 minutes or less does not reveal detectable on-column RNA degradation.

**DNAPac PA200**

- Resolves rA, rU 2′–5′ linkage isomers.
- Retains mixed-base RNA in a pH-dependent manner (as with DNA).
- Resolves mixed-base 21-mer linkage isomers, but may require different pH values to resolve all pairs.
- Retains PDAse-II degradation products of each of the 11 mixed-base 2′–5′ linkage isomers to unique positions.
- Is recommended for analytical application at low sample concentrations.

Since RNA is thought to degrade quickly at pH > 9, chromatography at high pH is usually discouraged. Here we observe that changes in flow (to increase the residence time of RNA on-column) during DNAPac PA200 chromatography at pH 11 does not change the relative amounts of the full-length sequence or impurities, indicating that exposure to pH 11 for 20 min or less does not result in RNA degradation on the DNAPac column.

In Figure 14 the fully resolved components are not shown, and the rest of the new suite of mixed-base 21-mers with aberrant linkages were chromatographed on the DNAPac PA200 at pH 11. All components are now resolved at some pH and are now depicted in red in the table. Dio-2 and Dio-3 are not fully resolved, but can be differentiated at pH 11.

Since RNA is thought to degrade quickly at pH > 9, chromatography at high pH is usually discouraged. Here we observe that changes in flow (to increase the residence time of RNA on-column) during DNAPac PA200 chromatography at pH 11 does not change the relative amounts of the full-length sequence or impurities, indicating that exposure to pH 11 for 20 min or less does not result in RNA degradation on the DNAPac column.
EFFECT OF LINKAGE ISOMERISM ON THE PROSWIFT WAX-1S 4.6 × 50 mm

Monolithic WAX retention

The ProSwift WAX Monolith

- Weak Anion exchange monolithic polymer phase.
- Solid polymer rod with an uninterrupted interconnected network of channels of controlled size range producing a morphology that supports fast analyte mass transfer.
- Features high speed, low back-pressure separations with high resolution.
- The WAX monolith also exhibits 10–15 times the capacity of the pellicular DNAPac phase on a volume basis.
- These features support increased throughput and productivity.

We used a 25 mer mixed-base oligo with and without, the protecting trityl- group to compare the dynamic loading capacity of the ProSwift Monolith to that of the DNAPac PA100, using comparably sized columns. Based on these comparisons the ProSwift WAX monolith has much greater dynamic capacity (Figure 17).

Comparing the peak width (at half-height) of samples injected onto both columns, we observe a significantly better sample handling capacity on the ProSwift monolith. Based on these comparisons the ProSwift WAX monolith has 10–15 times the capacity of the DNAPac column, on a bed volume basis (Figure 18).

The ProSwift WAX-1S column fully resolves very similar oligonucleotides (here homopolymers of dA) up to 40 or more bases long, from their failure sequences.

Figure 17. Oligonucleotide Dynamic Capacity Comparison:

Aldehyde Reductase Primer (25 base) ± Trityl group

DNAPac PA100 (4 × 50 mm)
150–600 mM NaCl in 18 min
Flow: 1.00 ml/min
WV L 260 nm

ProSwift WAX-1X (4.6 × 50 mm)
300–925 mM NaCl in 18 min
WV L 260 nm

Figure 18. Dynamic Loading Capacity of Anion-Exchange Columns

DNAPac PA100 (4 × 50 mm) and ProSwift WAX (4.6 × 50 mm)

Figure 19. Elution of PdA Oligonucleotides
12- to 60-mers: ProSwift WAX
400–625 mM NaCl in 35 min, 20 mM Tris pH 8, 38 °C, Curve 4, 1 mL/min
In Figure 20, a repeat of the rA$_{21}$ mer DNAPac study on the ProSwift WAX monolith, produced the same result. The undigested oligos elute in order of decreasing number of aberrant linkages. Also the elution positions of the digestion products are consistent with the number of consecutive aberrant linkages.

In Figure 21 a repeat of the rU$_{21}$ mer DNAPac study on the ProSwift WAX monolith also produced the same result. The elution positions of the digestion products are consistent with the number of consecutive aberrant linkages.

In Figure 22 the new suite of mixed-base 21-mers with aberrant linkages was chromatographed on the ProSwift WAX-1S at pH 8. At this pH, samples Dio-5, Dio-6, Dio-9 and Dio-10 are completely resolved from all other samples, and are shown as red in the table. The pairs unresolved at this pH are shown as black in the table.
In Figure 23 the same samples are chromatographed on the ProSwift WAX-1S at pH 9. All components not resolved at pH 8 are resolved at pH 9 so they are depicted in red in the table. Where the DNAPac PA200 required several pH values to resolve all of these components, the ProSwift monolith resolved them at only 2 pH values.

**PROSWIFT WAX CONCLUSIONS**

ProSwift WAX:

- Exhibits >10 fold more capacity per bed volume, than the DNAPac PA100 column.
- Can resolve oligonucleotides of 40 or more bases.
- Retains mixed-base RNA in a pH-dependent manner (as with DNA).
- Resolves mixed-base 21-mer linkage isomers, but may require different pH values to resolve all pairs.
- Retains PDase-II degradation products of each of the mixed-base 2’→5’ linkage isomers to unique positions.
- Is recommended for high resolution purification and analysis.
- Will be available in larger sizes with a linear increase in sample handling (up to ~5 mg on a 10 × 100 mm format).