Application Note

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Optimizing Separation of Oligonucleotides with Anion-Exchange Chromatography

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Abstract

Purification of synthetic oligonucleotides can represent a unique challenge. Impurities associated with the synthesis of the full-length product often includes truncations, incomplete thiolation, and base loss, which must be considered when developing a purification method. This application note describes common method parameters that can be optimized when purifying oligonucleotides ranging from 25 to 100 bases in length.

Introduction

Over the last several decades, there has been continued interest and focus on new therapeutic modalities for the regulation of protein expression at the RNA and DNA level.[1] Oligonucleotides represent an attractive approach for regulation of protein expression and gene editing.[2] These oligonucleotides can range in size from a few bases to hundreds of bases. Because of diversity in sequence, modifications, and length, there are several optimization variables that should be considered when developing a liquid chromatography purification method. Anion exchange is a widely used purification technique for oligonucleotides. This methodology can be preferred when there is a need to avoid volatile buffers such as those used in ion-pair reversed-phase (IP-RP) chromatography for more traditional purification buffers, such as Tris or PBS. This can become more attractive as the scale of purification increases, requiring larger buffer volumes.

In this study, the impact of mobile phase composition and column temperature on oligonucleotides separation using strong anion-exchange (SAX) chromatography has been investigated. The inclusion of organic solvents and higher temperatures played a significant role on the chromatographic performance of the oligonucleotides. However, high column temperature when paired with high pH may also cause oligonucleotides degradation, and therefore should be monitored.[3] To determine this, oligonucleotide samples were subjected to SAX fractionation with higher-pH mobile phase (pH 12) at 30 °C, and the collected fractions were analyzed by LC/MS.

Experimental

Method

For method optimization, a range of oligonucleotides were investigated, including Agilent RNA resolution standard (part number 5190-9028), unpurified DNA oligonucleotides (25, 50, 75, and 100 mer lengths) from Integrated DNA Technologies (IDT), and purified single-stranded guide RNA (sgRNA). Anion-exchange chromatographic separation was performed and optimized on an Agilent PL-SAX 1,000 Å, 2.1 × 50 mm, 5 µm column (part number PL1951-1502). LC/MS grade acetonitrile and Milli-Q purified water were used for the LC/UV and LC/MS analysis. Detailed steps for solvent buffer preparation are shown in Table 1.

Instrumentation

For LC-DAD

An Agilent 1290 Infinity II LC system was used in combination with an Agilent 1290 Infinity II diode array detector.

- Agilent 1290 Infinity II high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent InfinityLab sample thermostat (option 101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) with biocompatible heat exchanger
- Agilent 1290 Infinity II diode array detector (G7115A) with a bio-inert flow cell (option 28)

For LC/MS

- Agilent 1290 Infinity II high-speed pump (G7132A)
- Agilent 1290 Infinity II multisampler (G7167B) with Agilent InfinityLab sample thermostat (option 101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) with standard heat exchanger
- Agilent 1290 Infinity II diode array detector (G7115A) with a standard flow cell
- Agilent 6530 quadrupole time-of-flight LC/MS system (G6530B) with Agilent Jet Stream technology ESI source

Software

- **Acquisition:** Agilent MassHunter Acquisition software for instrument control (B.08.00) or later versions.
- **Data analysis:** Agilent MassHunter qualitative analysis software (B.07.00) or later versions.

Table 1. Mobile phase preparation for SAX chromatography.

	100x Stock Buffer	Mobile Phase A	Mobile Phase B
10 mM Tris, pH 8.0	1 M Tris, pH 8.0, adjusted with HCl	10 mL of stock buffer was mixed with 990 mL of water	10 mL stock buffer was mixed with 116.88 g of NaCl (2 M), and water was added up to 1 L
10 mM Tris, pH 8.0 + 10% ACN	1 M Tris, pH 8.0, adjusted with HCl	10 mL of stock buffer was mixed with 100 mL of ACN and 890 mL of water	10 mL stock buffer was mixed with 100 mL ACN, 116.88 g of NaCl (2 M), and water was added up to 1 L
10 mM NaOH, pH 12	N/A	2.5 mL of 4 M NaOH was mixed with 997.5 mL of water	2.5 mL 4 M NaOH was mixed with 116.88 g of NaCl (2 M), and water was added up to 1 L

General method settings

Method conditions and settings are outlined in Tables 2 to 5.

Results and discussion

Optimizing SAX method for oligonucleotides analysis

In the initial investigation of using SAX for oligonucleotides separation, 10 mM Tris (pH 8.0) was used as the buffer with 0.5 M of NaCl in solvent B. However, it was found that 0.5 M NaCl as an eluent was not strong enough to elute most of the oligonucleotides (data not shown). As such, 2 M of NaCl was used as the salt eluent in solvent B in the following anion-exchange separations (Table 1). In Figure 1, a comparison is made for the LC/UV analysis of RNA resolution standard, 25, 50, 75, and 100 mer DNA oligonucleotides using different mobile phase compositions with the column compartment temperature set at 30 °C. The gradient for each of the solvent compositions was scaled to match the retention times obtained across the runs to allow for comparison of the chromatographic performance.

Table 2. LC-DAD conditions.

	Value
Column	Agilent PL-SAX 1000 Å, 5 µm, 2.1 × 50 mm column (p/n PL1951-1502)
Solvent A and B	See Results and discussion section
Gradient	See Results and discussion section
Flow Rate	0.5 mL/min
Column Temperature	See Results and discussion section
Detection (DAD)	270/4 nm (references 360/20 nm) Peak width >0.025 min (10 Hz)
Injection Volume	1 µL
Injector Temperature	10 °C

Table 3. LC/MS conditions.

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide, 2.1 × 50 mm, 2.7 µm column (p/n 659750-702)
Solvent A	10 mM hexylamine, 50 mM HFIP, 5% methanol, 10 µM EDTA in water
Solvent B	Methanol
Gradient	See Results and discussion section
Flow Rate	0.4 mL/min
Column Temperature	70 °C
Detection (DAD)	260/4 nm (references 360/20 nm) Peak width >0.1 min (2.5 Hz)
Injection volume	1 µL
Injector temperature	10 °C

Table 4. LC/Q-TOF settings.

Source Settings	Agilent Jet Stream Technology
Ionization Mode	Negative ionization
Drying Gas Temperature	275 °C
Drying Gas Flow	12 L/min
Nebulizer Pressure	45 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Nozzle Voltage	1,000 V
Capillary Voltage	3,500 V
Acquisition Settings	
Fragmentor Voltage	100 V
Mass Range	800 to 3,200 *m/z*
Acquisition Model	Extended dynamic range mode (2 GHz)
Scan Rate	1 spectra/s
Acquisition	Centroid and profile

Table 5. Deconvolution settings.

Agilent MassHunter BioConfirm Software (B.07.00) Settings	
Deconvolution Algorithm	Maximum entropy
Subtract Baseline	7
Adduct	Proton loss
Mass Range	20,000 to 40,000
Mass Step	0.1
Use Limited *m/z* Range	N/A

The introduction of 10% acetonitrile to 10 mM Tris (pH 8.0) or raising the buffer's pH to 12 with 10 mM NaOH showed significantly better performance compared to 10 mM Tris (pH 8.0) buffer alone. Specifically, higher separation efficiencies and sharper peak shapes were observed for the oligonucleotides shown in Figure 1. Noticeably, more peaks were observed eluting before the main peak for the 75 mer and 100 mer DNA when using 10 mM NaOH, pH 12 buffer (Figure 1, right panel). This suggested that although 10 mM NaOH (pH 12) buffer can yield higher resolution and sharper peaks than 10 mM Tris (pH 8.0) buffer for the smaller oligonucleotides (i.e., RNA resolution standard, 25 mer, and 50 mer DNA), it is important to determine whether higher pH mobile phases provide better impurity separation without inducing purification-related degradation. Therefore, the identity of those peaks was investigated through SAX fraction collection and LC/MS analysis, as detailed in the following sections.

The effect of temperature on sgRNA was also investigated in this study, as RNA is prone to form secondary structures that could yield broad peak shapes leading to poor chromatographic results. In this study, a 105 mer purified sgRNA was analyzed with 10 mM Tris (pH 8.0) buffer and compared to the same buffer containing 10% ACN with the column compartment temperature set at 30, 60, and 80 °C (Figure 2). From these results, it was clear that the use of 10% ACN in 10 mM Tris (pH 8.0) buffer reduced secondary structures, yielding better chromatographic results. Moreover, systematic increase in column compartment temperature also led to significant improvements in the sgRNA's peak shape. In this study, the use of 10% ACN in 10 mM Tris (pH 8.0) as the mobile phase solvent for SAX at 80 °C yielded the best chromatographic result for the 105 mer sgRNA (Figure 2).

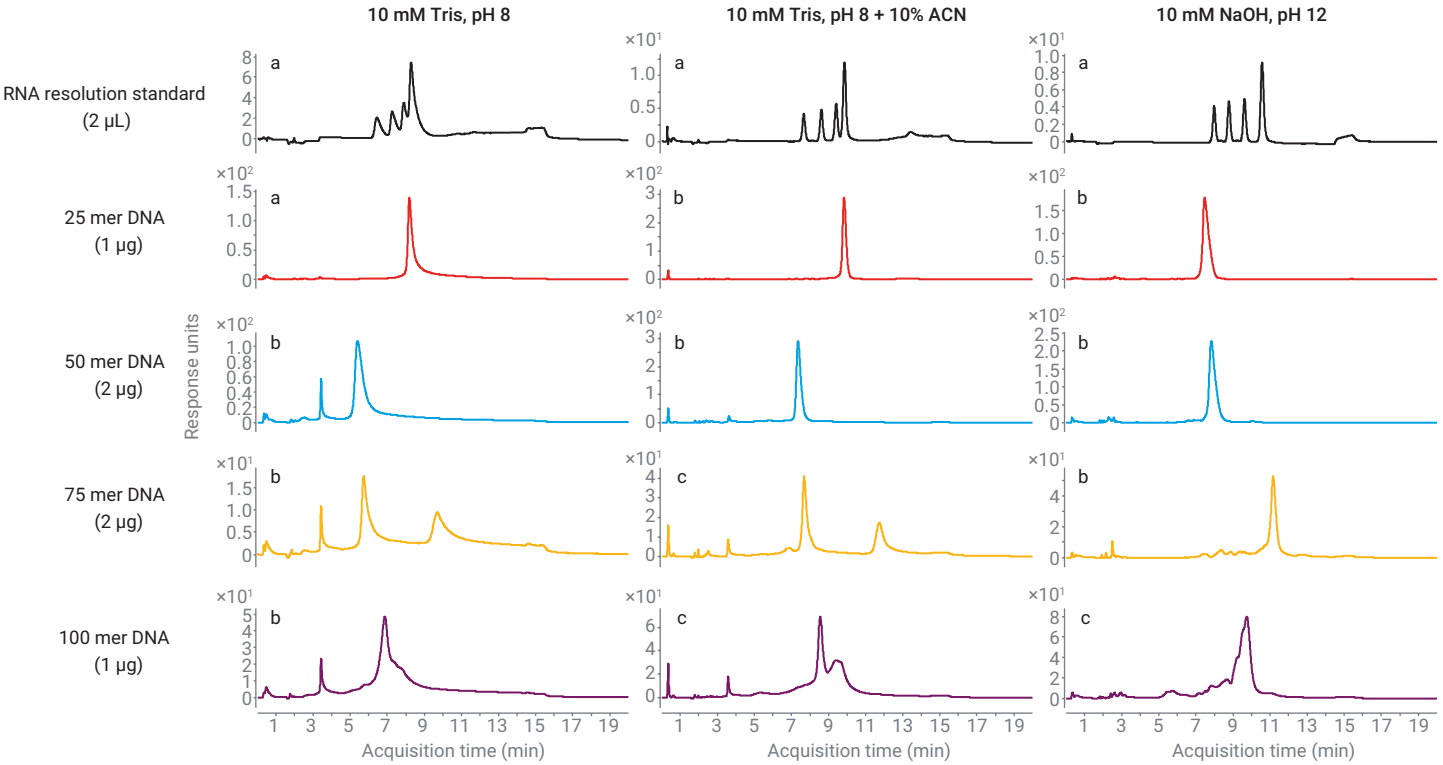

Figure 1. Optimizing mobile phase for oligonucleotide separation. LC gradient and temperature: (a) 10 to 30% B in 10 minutes, 30 °C; (b) 20 to 40% B in 10 minutes, 30 °C; (c) 25 to 45% B in 10 minutes, 30 °C.

Collecting SAX fractionated 100 mer sample at high pH

Crude 100 mer DNA, 6 µg, was subjected to SAX fractionation with 10 mM NaOH as the mobile phase buffer at pH 12 to determine the identities of the early eluting peaks. Six fractions were collected starting from 7 to 9 minutes, with 20 seconds of eluent collected per fraction (Figure 3A). The crude and fractionated samples were subjected to LC/UV analysis using IP-RP with 100 mM TEAA, pH 8.5 as the mobile phase buffer (Figure 3B to E). The LC/UV analysis of the fractionated samples showed that fraction 4 yielded the purest sample, while fractions 3 and 5 yielded some impurities, as demonstrated by the fronting and tailing of the peak shapes, respectively.

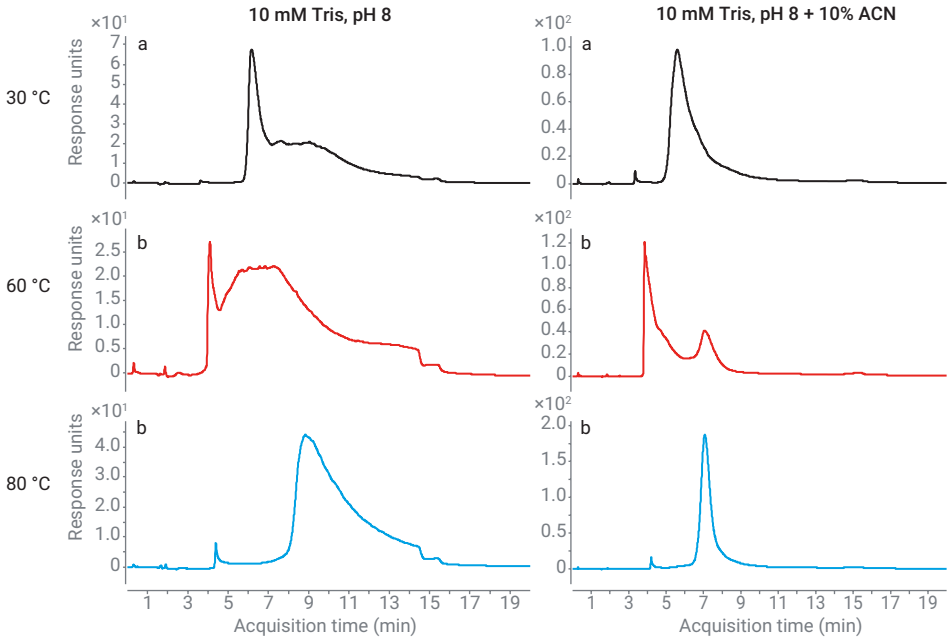

Figure 2. Effect of temperature on gRNA separation. Sample: sgRNA (purified). LC gradient and temperature: (a) 20 to 40% B in 10 minutes; (b) 30 to 50% B in 10 minutes.

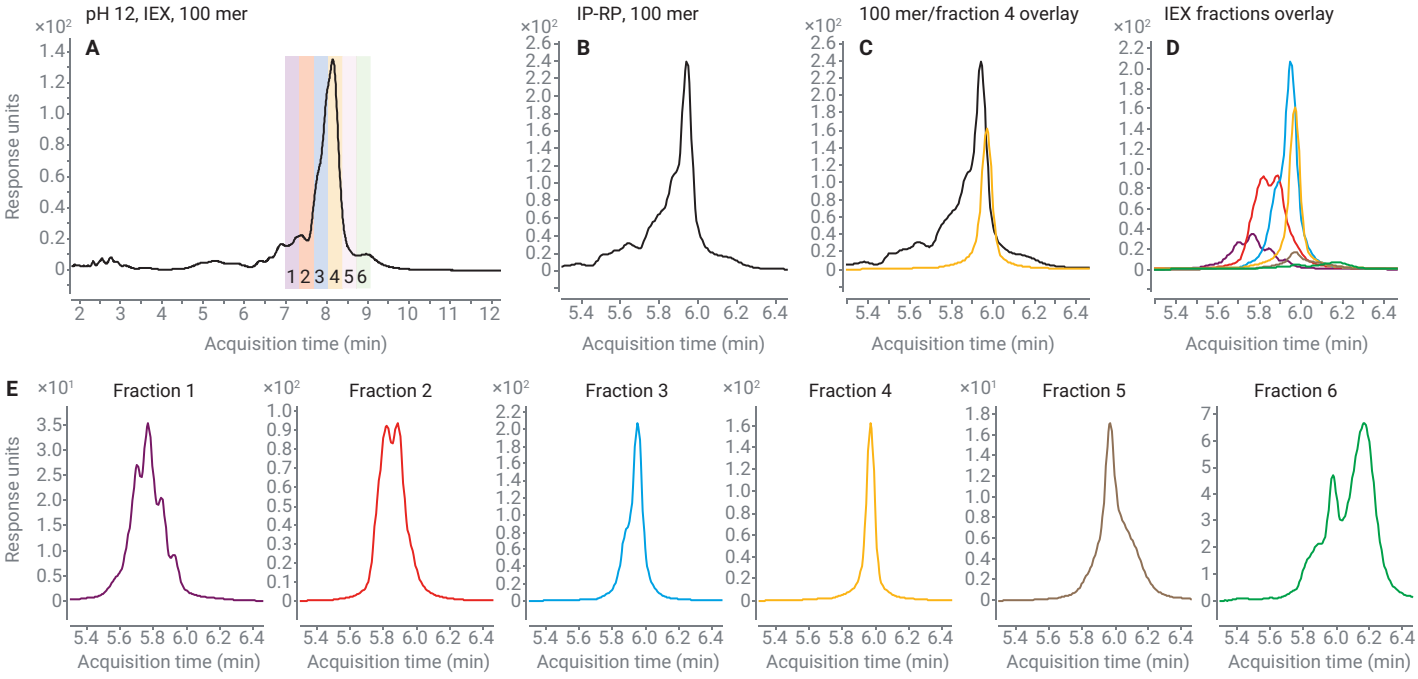

Figure 3. LC/UV analysis of SAX fractionated 100 mer DNA.

LC/MS analysis of SAX fractionated sample

While the LC/UV analysis seemingly yielded the pure 100 mer DNA oligo in fraction 4 (Figure 3), we wanted to confirm the identity of the peak and potential impurities in other fractions by LC/MS. The collected fractions were dried down by SpeedVac, and resuspended with water for IP-RP analysis by LC/MS. In Figure 4, LC/MS analysis of the crude 100 mer DNA sample showed that the starting sample was predominantly the 100 mer DNA, with the detection of some depurinated products in the sample. Interestingly, fraction 4 showed much greater sodium adducts, where the additional sodium ions may be from the NaCl used for SAX fractionation to elute the oligo off the PL-SAX column. When examining fraction 3, similar adduct formation and depurinated products were observed. More importantly, n-5 truncated products were detected in fraction 3, demonstrating that the earlier-eluting peaks observed for 100 mer DNA were truncated products when analyzed with the 10 mM NaOH, pH 12 buffer (refer to Figure 1 or Figure 3A).

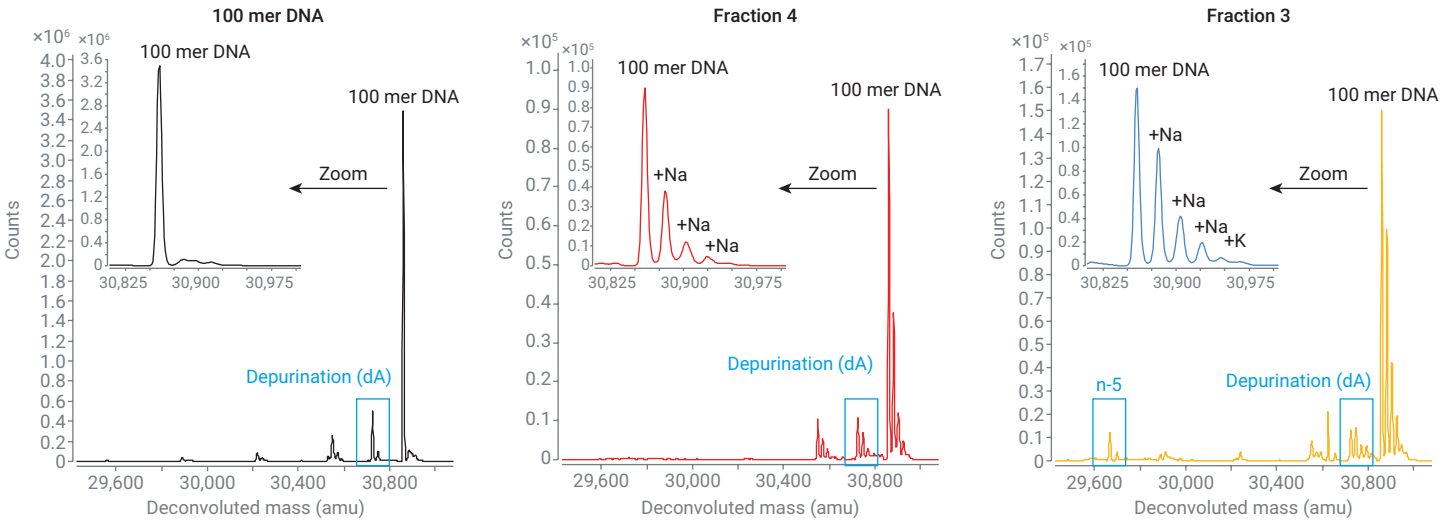

Figure 4. LC/MS analysis of ion-exchanged 100 mer DNA.

Conclusion

This application note shows that mobile phase composition plays an important role in chromatographic separation of oligonucleotides. Importantly, the use of organic solvents and higher temperatures could significantly improve the separation efficiency. While higher-pH mobile phase buffers may also improve peak shapes of shorter oligonucleotides, longer oligonucleotides can suffer from pH-mediated degradation of the product, and should be monitored to determine the maximum temperature and pH combination to use before purification related degradation is induced. These methods demonstrated a systematic approach to optimize oligo separation with SAX. This study also showed a workflow to perform SAX fractionation of oligonucleotides and confirm sample purity using IP-RP LC/UV and LC/MS methods.

References

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