Application Note

Biopharma/Pharma

Agilent

Trusted Answers

Choosing Appropriate Pore Size Columns for Size Exclusion Chromatography of Oligonucleotides

Authors

Andy Coffey, Anne Blackwell, and Charu Kumar
Agilent Technologies, Inc.

Abstract

The use of analytical size exclusion chromatography (SEC) for the separation of oligonucleotides from other components based on hydrodynamic radius is complicated by the range of sizes that can be present, as well as fundamental differences in the conformation between DNA and RNA. This makes selecting the correct pore size SEC column more challenging.

Using oligonucleotide ladders, often developed for electrophoresis calibration, makes understanding the pore structure of SEC columns easier, but it remains essential to perform thorough method optimization to overcome challenges with some molecules. This application note provides guidance in choosing the appropriate pore size columns for oligonucleotide separations.

Introduction

SEC has become the method of choice for aggregate analysis of biotherapeutic molecules. Unlike proteins, which generally have a compact structure, oligonucleotides are much larger. They may be single- or double-stranded, and they may have a complex structure containing loops and hairpins. These structures may also vary depending on the conditions used for analysis.

This study used SEC of oligonucleotide standards available in a wide range of sizes. These sizes corresponded to differing dimensions in solution that covered the entire resolving range of the columns used in the study. This allowed a direct comparison of the chromatographic properties of each column, such as exclusion limit (rather than pore size) based on the number of nucleotides.

Although oligonucleotides are highly hydrophilic, they can exhibit secondary interactions through hydrogen bonding or from ionic interactions. Also, variations that introduce hydrophobic modifications such as labels can also cause secondary interactions. It is therefore advisable to screen different mobile phase conditions to ensure robust methodology.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Instrumentation

Data acquisition was performed on an Agilent 1260 Infinity II bio-inert LC system using Agilent OpenLab CDS software.

Sample preparation

DNA Ladder standards (part numbers S7025 and D0428) were purchased from Merck Sigma-Aldrich, dissolved in mobile phase, and stored frozen until needed.

RiboRuler HR was purchased from Thermo Fisher and diluted 1:10 in mobile phase before use.

Mobile phase preparation

Three different mobile phase solutions were investigated. These were prepared by dissolving the necessary compounds in Milli-Q water, then filtering through a 0.22 µm membrane filter. The eluents were:

- 150 mM sodium phosphate, pH 7.0
- 2X PBS (approximately 20 mM phosphate + 280 mM NaCl), pH 7.4
- 50 mM potassium phosphate, 200 mM potassium chloride, pH 7.0

Table 1. HPLC method conditions.

Parameter	Value
Column	See Table 2
Mobile Phases	– 150 mM sodium phosphate, pH 7.0; or – 2X PBS (approximately 20 mM phosphate + 280 mM NaCl), pH 7.4; or – 50 mM potassium phosphate, 200 mM potassium chloride, pH 7.0
Flow Rate	0.35 or 0.175 mL/min
Column Temperature	30 °C
Injection Volume	5 µL
Total Run Time	30 minutes per injection
Detection	UV, 260 nm

Table 2. Columns tested.

Column	Description
A	Agilent AdvanceBio SEC 500 Å, 4.6 × 300 mm, 2.7 µm
B	Agilent AdvanceBio SEC 1000 Å, 4.6 × 300 mm, 2.7 µm
C	Waters GTxResolve Premier SEC 1,000 Å, 4.6 × 300 mm, 3 µm

Table 3. DNA standards used.

50 bp DNA Ladder	1 kb DNA Ladder
50 bp	500 bp
100 bp	1,000 bp
150 bp	1,500 bp
200 bp	2,000 bp
250 bp	2,500 bp
300 bp	3,000 bp
350 bp	4,000 bp
400 bp	5,000 bp
450 bp	6,000 bp
500 bp	8,000 bp
600 bp	10,000 bp
700 bp	
800 bp	
900 bp	
1,000 bp	
2,000 bp	
3,000 bp	

Results and discussion

The first experiment was to determine if using a slower flow rate would improve chromatographic performance as very large molecules diffuse more slowly. Figure 1 shows the separation of a 50 bp DNA ladder performed at 0.175 and 0.35 mL/min. The latter is the flow rate normally used with 4.6 mm id SEC columns.

The main difference was the peak height – at the lower flow rate, the peaks spent longer in the detector flow cell, resulting in a greater response. Other factors such as peak width and resolution appeared to be very similar.

However, when testing larger oligonucleotides using the 1 kb DNA ladder (Figure 2), the peak response was similar, confirming that larger molecules have slower diffusion times. Consequently, the resolution of the largest molecules was improved, and subsequent experiments were performed using the lower flow rate. Figure 3 shows the separation of the 1 kb DNA ladder using the Agilent AdvanceBio SEC 1000 Å, 2.7 µm column with three different mobile phase solutions as described in Table 1. The total buffer/salt concentration was either 150 or 300 mM, ensuring that nonspecific secondary interactions were minimized. The molecules eluted at almost the same retention time (RT) with very similar peak shape and resolution. The 2X PBS mobile phase conditions were used for creating the calibration data plots shown in Figure 4. Individual retention times are listed in Table 4 and were determined by integration of chromatograms shown in Figure 5.

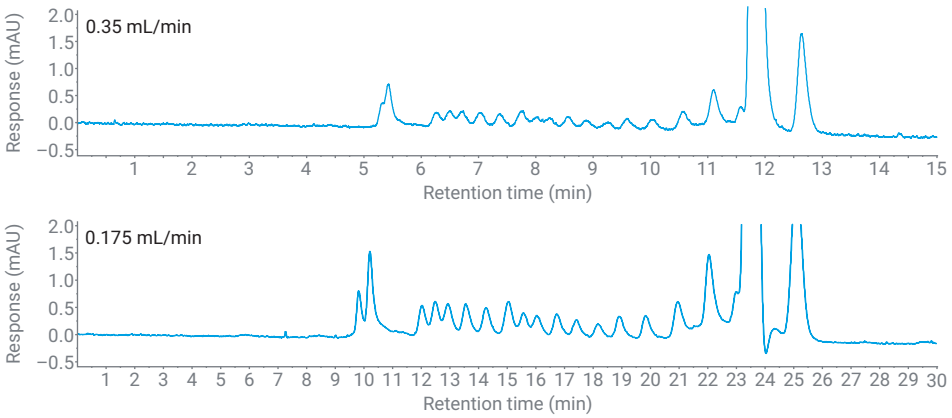

Figure 1. Separation of a 50 bp DNA ladder on an Agilent AdvanceBio SEC 1000 Å (column B), mobile phase 2X PBS.

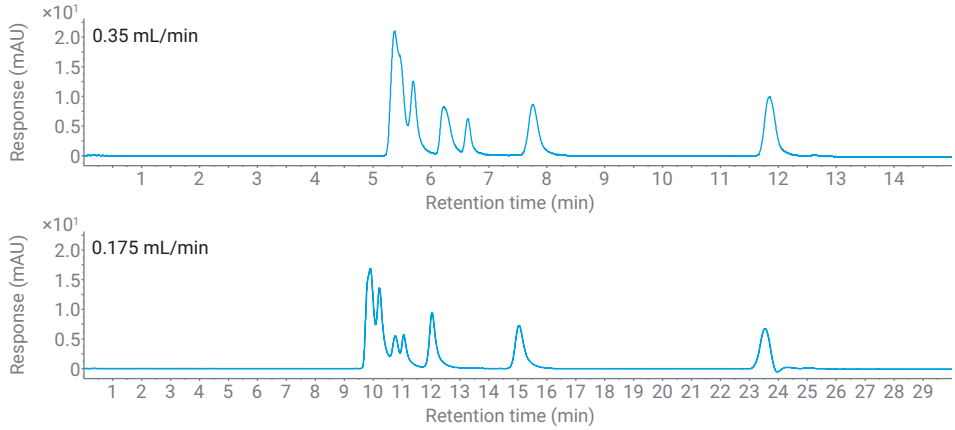

Figure 2. Separation of a 1 kb DNA ladder on an Agilent AdvanceBio SEC 1000 Å (column B), mobile phase 2X PBS.

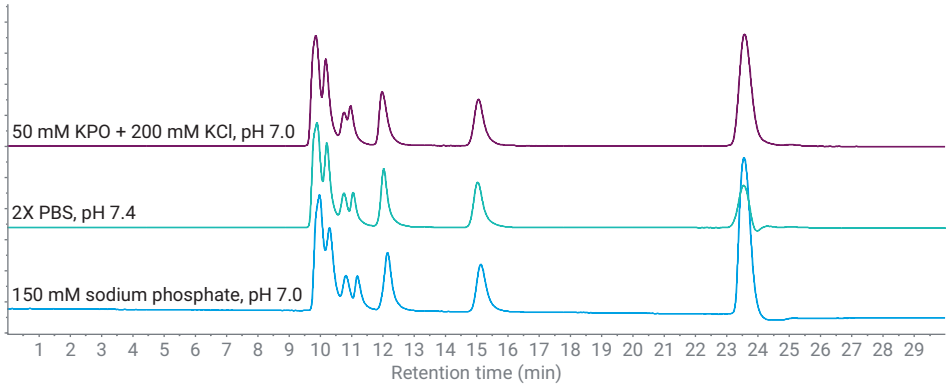

Figure 3. Separation of a 1 kb DNA ladder on an Agilent AdvanceBio SEC 1000 Å (column B) showing the effect of mobile phase.

Table 4. Retention times of DNA ladder peaks.

dsDNA (bp)	RT (min)		
	Column A	Column B	Column C
3,000	9.10	9.81	11.24
2,000		10.21	
1,000		12.02	
900	9.81	12.48	12.54
800	9.97	12.92	12.75
700	10.27	13.56	13.17
600	10.67	14.26	13.62
500	11.19	15.05	14.16
450	11.57	15.57	14.50
400	11.98	16.02	14.85
350	12.59	16.72	15.38
300	13.26	17.44	15.91
250	14.11	18.18	16.53
200	15.10	18.90	17.19
150	16.42	19.84	18.04
100	18.25	20.94	19.17
50	20.17	22.05	20.30

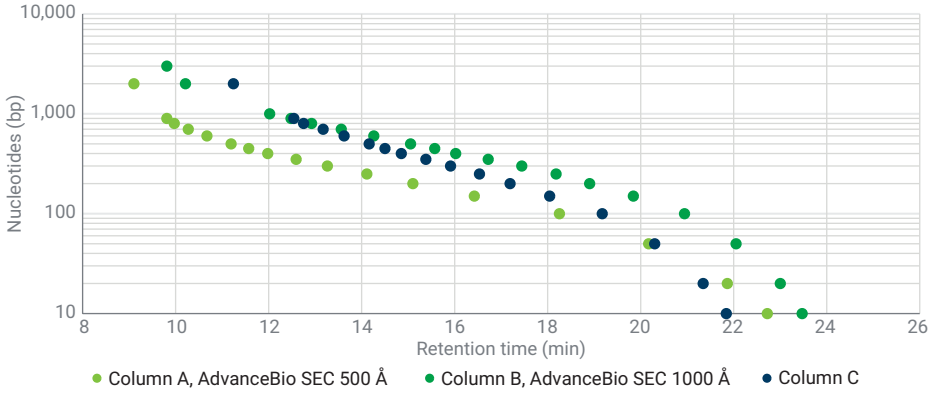

Figure 4. Oligonucleotide calibration curve comparison.

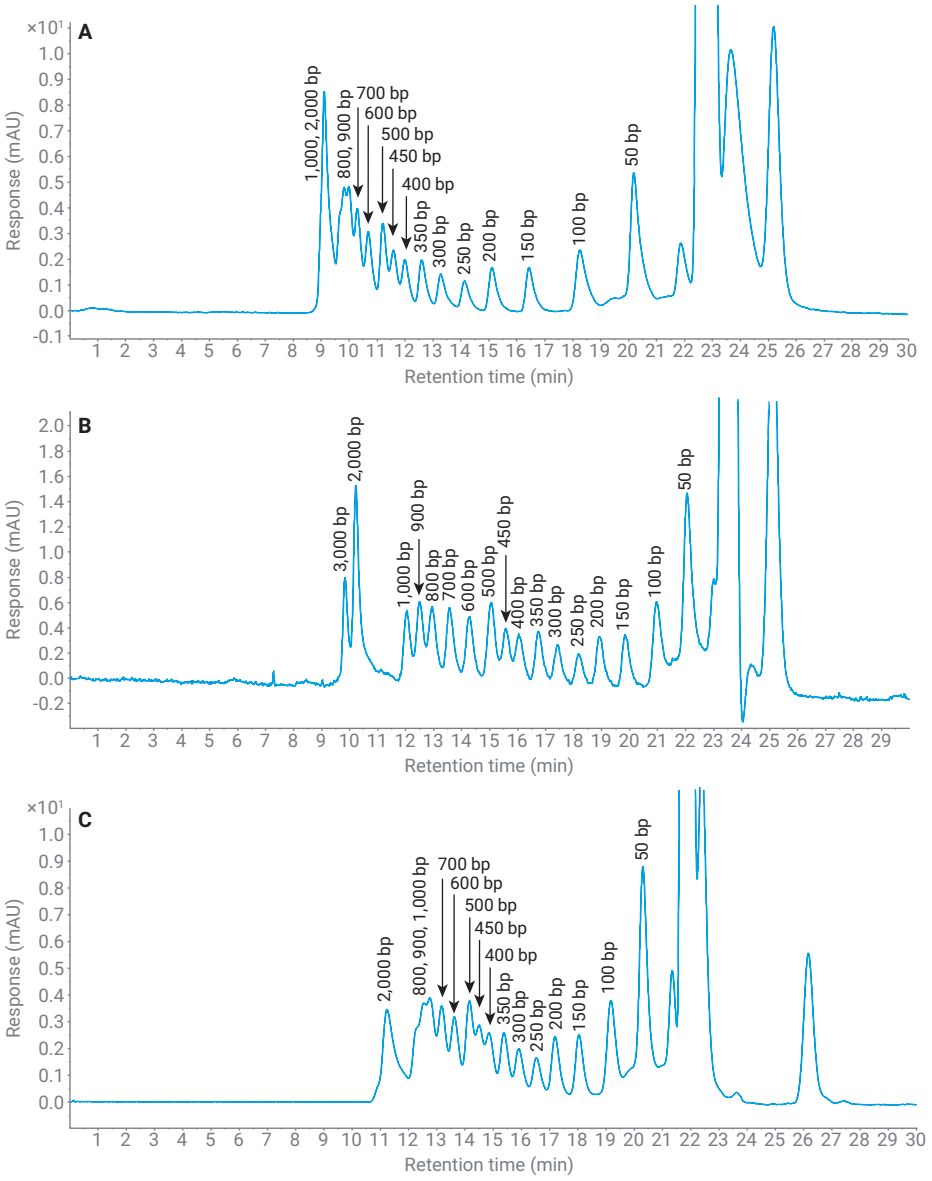

Figure 5 . Chromatograms of a 50 bp DNA ladder using (A) column A, Agilent AdvanceBio SEC 500 Å; (B) column B, AdvanceBio SEC 1000 Å; and (C) column C, with mobile phase 2X PBS, pH 7.4.

Figure 6 shows chromatograms of the 1 kb DNA ladder standard run on both 1,000 Å pore size columns, illustrating the difference in pore volume (from exclusion point to total permeation point).

In contrast, Figure 7 shows the chromatogram from an RNA ladder. RNA is most commonly single-stranded and the size is expressed in terms of number of nucleotides (nt) instead of base pairs (bp) used when describing DNA. One might expect a single strand of RNA containing 2,000 nucleotides to have the same size as a double-stranded DNA (dsDNA) molecule containing 2,000 base pairs. However, the double helix nature of the dsDNA appears to restrict the conformation that the molecule is able to adopt. Single-stranded RNA appears to be much more flexible and able to adopt a smaller size in solution than DNA, and therefore elutes later.

This behavior has been noted previously.[1]

It is also important to take into consideration the likely need to either increase the denaturing conditions (for instance by increasing the temperature, or to include a chaotrope in the mobile phase) to improve peak shape for RNA molecule separations.

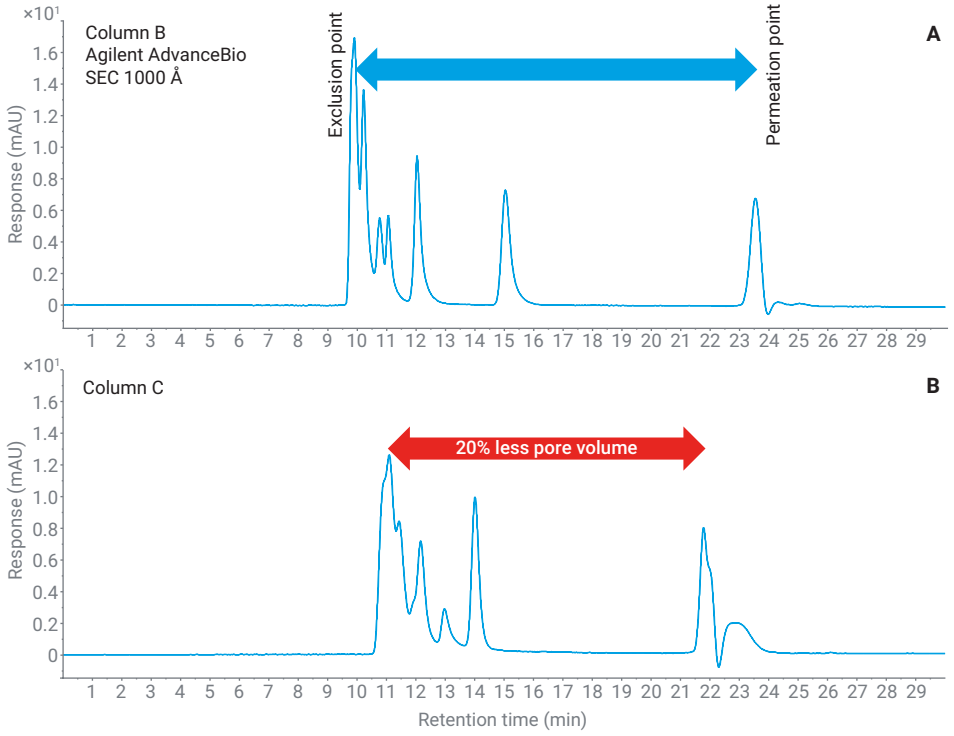

Figure 6. Chromatograms of a 1 kb DNA Ladder for 1000 Å columns B and C (mobile phase 2X PBS, pH 7.4) showing the difference in pore volume.

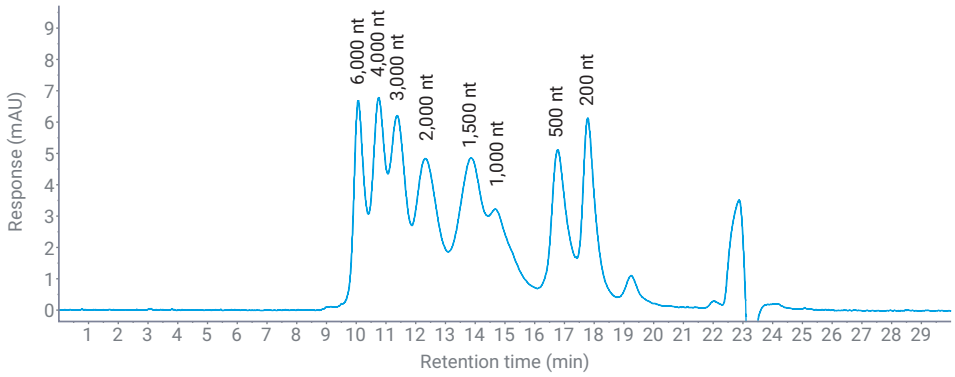

Figure 7. RiboRuler HR RNA Ladder on column A, Agilent AdvanceBio SEC 500 Å.

Conclusion

The columns used in this study are the latest Agilent AdvanceBio SEC columns in 500 and 1000 Å pore sizes, which provide exceptional pore volume for maximizing the useful range of oligonucleotides that can be separated.

It is difficult to compare the resolving range of globular proteins to the resolving range of oligonucleotides, but a useful indication comes from the pore size of the column. For example, 500 Å pore size columns are suitable for DNA up to approximately 500 to 1,000 bp while 1000 Å pore size columns are suitable for DNA up to approximately 2,000 to 3,000 bp (Figure 8).

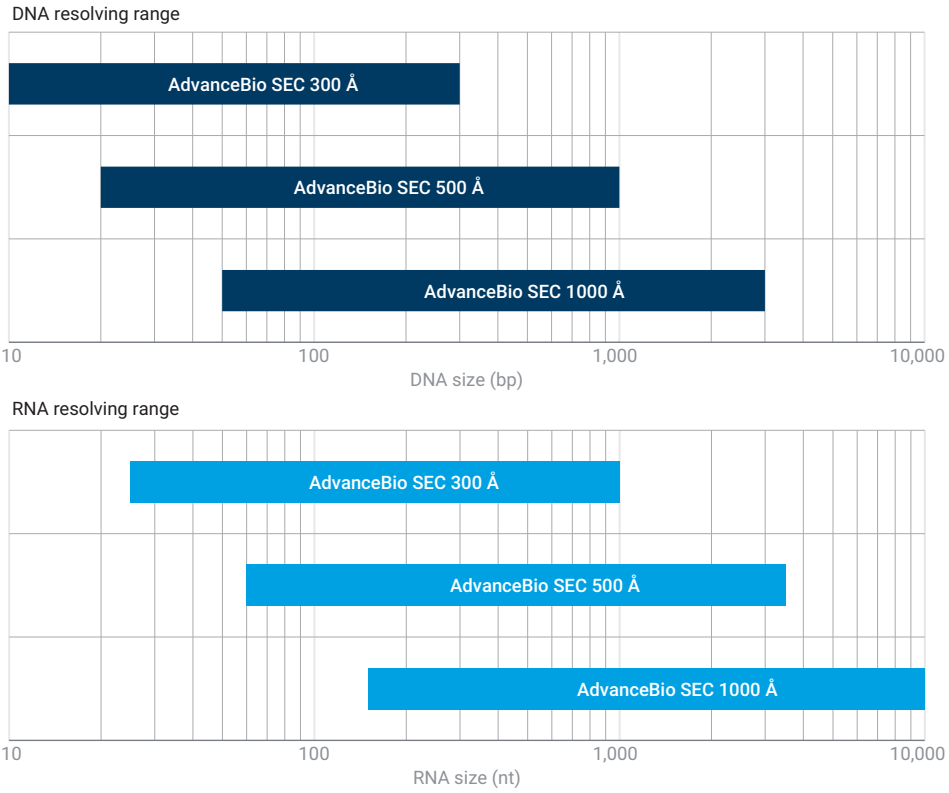

Figure 8. Expected DNA and RNA resolving ranges of molecules for different pore size columns using Agilent AdvanceBio columns.

Reference

1. Schneider, S.; Rieck, F. SEC-MALS for mRNA Characterization with the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector, *Agilent Technologies application note*, publication number 5994-7745EN, **2024**.

www.agilent.com

DE-004830

Printed in the USA, March 12, 2025
5994-8198EN

Agilent
Trusted Answers