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Nota applicativa

Suitability of XBridge™ Premier GTx BEH™ SEC 450 Å 2.5 µm Column for Size-based Separations of Nucleic Acids

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Solo per uso a scopo di ricerca. Il sistema non è destinato all'utilizzo per procedure diagnostiche.

Abstract

Size-exclusion chromatography is routinely used for the analysis of molecular integrity. Here, we report the analysis of nucleic acids in both single stranded and double stranded form using XBridge Premier GTx BEH SEC Columns with 450 Å pore and 2.5 µm particles. Separations showing excellent resolution as well as sharp and symmetrical nucleic acid peaks are achieved with more than one type of mobile phase. With this column technology, it is possible to efficiently analyze ssRNA and dsDNA, where there is fractionation power up to 6000 and 3000 nucleotides/base pairs in length, respectively. These results provide examples of system suitability testing that confirm the applicability of the XBridge Premier GTx BEH SEC 450 Å 2.5 µm Columns for the analysis of nucleic acid drug substances, including small to medium sized therapeutic mRNA.

Introduction

Size exclusion chromatography (SEC) is extensively used for the analysis of proteins. More recently, SEC has been used to analyze antibody oligo conjugates and single guide RNA (sgRNA).[1,2,3] While the packing materials used in these studies have pore sizes (110–300 Å) sufficient to analyze 100–200 nucleotide (nt) nucleic acids, larger pore size packing materials are required to facilitate the resolution of longer nucleic acids such as messenger RNA (mRNA) where the lengths exceed 1000 nt or more. Here, we report the utility of XBridge Premier GTx BEH SEC 450 Å 2.5 µm Columns to resolve longer nucleic acids. The BEH-diol particles in these Columns are not only suitable for analysis of adeno-associated AAV capsids but also for RNA and dsDNA with a variety of mobile phase conditions.[4] The wide-pore BEH particles and high-performance surface technology used for column hardware construction ensure the quick start up of long nucleic acid SEC separations and reliable resolution. The pore size is optimal for excellent resolution of small to medium sized nucleic acid species. With this column technology, it is possible to efficiently analyze ssRNA and dsDNA, where there is fractionation power up to 6000 and 3000 nucleotides/base pairs in length, respectively.

Experimental

2x PBS preparation: Phosphate buffered saline salt mixture (Sigma (p/n: P-3583) from four packets were dissolved in 2 L of 18.2 MΩ water to make 20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4. The solution was filtered with a 1000 mL Nalgene™ Rapid-Flow™ Sterile Disposable PES filter with 0.1 µm pore size unit (p/n: 567–0010).

50 bp DNA ladder (NEB p/n: N3236L), 100 bp plus DNA ladder (Thermo Scientific Gene ruler p/n: SM1143): The frozen sample is thawed at at ambient conditions for 15 min, mixed by pipetting the fluid up and down ten times, and made 50 µL aliquot lots using 300 µL polypropylene screw neck vials using PE caps (p/n: 186004169 < https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186004169-polyethylene-septumless-screw-cap-for-12-x-32-mm-vials-100-pk.html>). The caps are tightened to prevent evaporation during storage in -20 °C freezer.

Single stranded RNA (ssRNA) ladders: High range RNA ladder (Thermo Ribo ruler p/n: SM1821), NEB ssRNA ladder (N0362S) were used. These vials stored at -80 °C, thawed at ambient temperature, diluted 20 µL of the RNA ladder with 80 µL of 1.25 mM EDTA, mixed with pipetting up and down before transferring into the injection vials.

100 mM Ammonium acetate pH 6.2: A stock solution of 200 mM Ammonium acetate was prepared and the pH adjusted to 6.2 (from initial pH 6.099) using 1 M sodium hydroxide. 100 mM ammonium acetate 6.2 was prepared by 1:1 dilution of 200 mM Ammonium acetate pH 6.2 solution with 18.2 MΩ water.

1XPBS, 75 mM sodium perchlorate, 20% acetonitrile: This mobile phase was prepared by mixing 900 mL of Milli-Q water with 200 mL of 10X PBS, 21 g of sodium Perchlorate Monohydrate and 400 mL of Acetonitrile. The mixture was then brought to a final 2 L volume with 18.2 MΩ water. It was stored at room temperature.

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class Bio with Quaternary Solvent Manager with 100 µL mixer, FTN-SM with 15 µL MP35N Needle p/n: 700005421, CH-30A heater with an Active Preheater 18.5" p/n: 205001755 and post-column tubing to TUV: 0.005" ID x 22.5" LG MP35N Welded Tube p/n: 700008914] [Equivalent to an ACQUITY Premier QSM FTN instrument configured with a High pH Kit]
Detection:	ACQUITY TUV Detector (Titanium Flow Cell, 5 mm, 1500 nL)
Wavelength:	260 nm
Detection:	ACQUITY RI Detector
Data Acquisition:	Empower™ Pro 3 Feature Release 3
Vials:	Max Recovery Vials and Caps (Waters p/n: 186000327C) and Waters™ 300 µL polypropylene screw neck vial (Waters p/n: 186004112)

Column:	XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column, 7.8 x 300 mm (Waters p/n: 186010586); XBridge Protein BEH SEC Column (steel hardware), 450 Å, 3.5 µm, 7.8 mm X 300 mm (Waters p/n: 176003599)
Column temperature:	30 °C and 50 °C
Sample temperature:	6 °C
Sample manager washes:	18.2 MΩ water
Seal wash:	10% HPLC grade Methanol / 90% 18.2 MΩ water (v/v)
Injection volume:	20 µL for DNA ladders, 10 µL for RNA ladder
Flow rate:	0.288 mL/min or 0.25 mL/min
Mobile phase A:	2X PBS: Phosphate Buffered Saline (20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4) *(or)* 1XPBS, 75 mM sodium perchlorate, 20% acetonitrile
Mobile phase B:	100 mM Ammonium Acetate pH 6.2
Mobile phase C:	N/A
Mobile phase D:	1X PBS Sigma p/n: P3813 (10 mM Phosphate, 138 mM NaCl, 2.7 mM KCl pH 7.4)
Sample(s):	NEB 50 bp DNA Ladder (N3236L); Thermo Scientific GeneRuler 100 bp Plus DNA Ladder

	(SM1143); Thermo Scientific RiboRuler High Range RNA Ladder (SM1821)
Gradient:	Isocratic
Syringe draw rate:	30 µL/min
Needle placement:	1.0 mm
Data channels:	System Pressure and TUV
TUV sampling rate:	5 Hz (Recommended)
Filter time constant:	none
Data mode:	absorbance
Autozero on inject start:	yes
Autozero on wavelength:	Maintain Baseline

Results and Discussion

Nucleic acids of smaller lengths are preferentially analyzed by ion-pairing reversed phase liquid chromatography (IP-RP-LC) for qualitative and quantitative characterization.[5] However, this analytical method can exhibit resolution limitations for longer nucleic acids (>1000 nt). Moreover, ion-pairing based mobile phases can have a partial denaturation effect on double stranded DNA leading to ambiguity in peak assignment and data interpretation. Further, any type of aggregates formed with nucleic acids cannot be discerned by the IP-RP-LC methods. SEC, on the other hand, often conducted under native conditions, allows detection of any aggregates formed by longer RNAs, and it also keeps the complementary strands of DNA together to allow size-based separations.

Figure 1 depicts separations of dsDNA ladders as obtained with an XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column with more than one mobile phase composition. The chromatography peak profiles match well with the expected size-based separation observed on agarose-gel electrophoresis systems where the early eluting DNA species match with the slowest-migrating gel bands. Similarly, the late eluting peaks from SEC correspond well with the fastest migrating gel bands. Bands of intermediate size exhibited the appropriate elution order between these pairs. As predicted, the longer size nucleic acids (>1500 bp) in the 100 bp ladder eluted earlier than longest nucleic acid in the of 50 bp ladder (a 1350 bp species). Peak areas per species matched well to band profiles observed by electrophoresis. Both PBS and ammonium acetate mobile phases yielded similar separations with sharp and symmetrical peaks observed for all the components of the nucleic acid ladder.

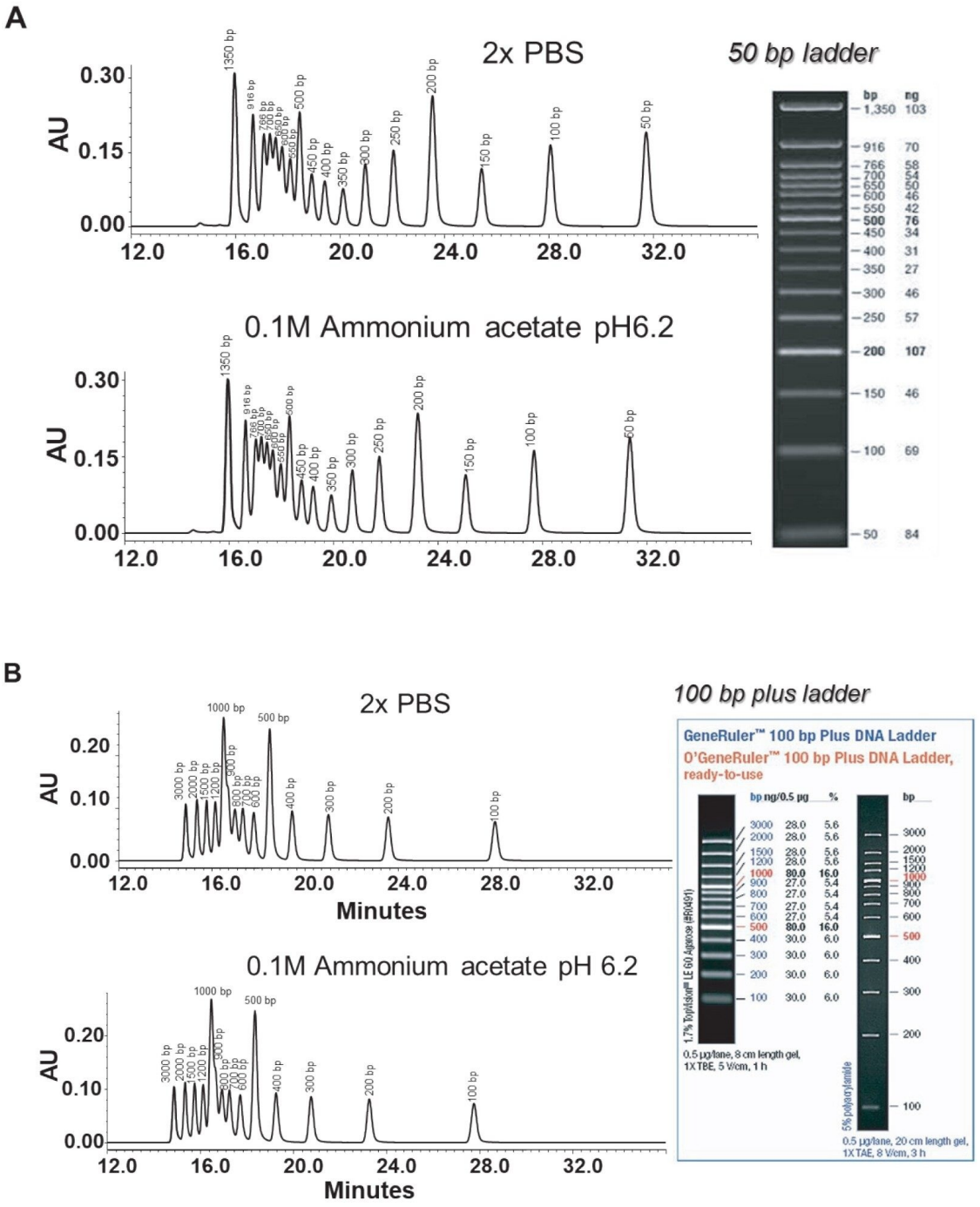

Figure 1.SEC-based separation of DNA ladders using an XBridge Premier GTx BEH SEC 450 Å 2.5 µ m Column. Components of 50 bp (A) or 100 bp plus DNA ladders (20 µL) (B) were resolved with a

7.8x300 mm column using either 2X PBS (top) or 0.1 M ammonium acetate, pH 6.2 (bottom) mobile phase at 50 °C and 0.288 mL/min flow rate. Note the peak profile correspondence between the SEC peaks and agarose gel (right side) electrophoresis bands. UV absorbance levels at 260 nm also aligned well with the amounts of individual components of the 50 bp (1350, 500, and 200 bp) and 100 bp (1000 and 500 bp) ladders. Gel electrophoresis results are displayed as reported on certificate information provided with each standard.

Investigations were also made into the utility of these SEC Columns for single stranded RNA separations. Here, a comparison was made between the use of a steel hardware, 3.5 µm 450 Å SEC Column versus the XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column. Figure 2A shows the chromatography peak profile of a single stranded RNA ladder spanning 200 nt to 6000 nt. Eight (8) RNA peak features (6000, 4000, 3000, 2000, 1500, 1000, 500, and 200 nt) were observed. However, resolution on the 4000 and 3000 nt ladder species was seen to be a challenge. Interestingly, one smaller peak was noticed at 20.5 (or 19.8) min just before 6000 nt RNA peak and another at 30 (or 28.6) min. Although the exact origin of these peaks is unknown, there is potential for the peak at 20.5 min to be a high molecular weight aggregate species because of its early elution compared to the longest nucleic acid expected in the sample. This is information that can be uniquely observed from SEC analysis. In this work, a specially formulated mobile phase was applied. It was made of PBS buffer with the addition of 20% acetonitrile co-solvent and perchlorate additive.

Given the challenge to resolve the 3000 and 4000 nt species, an XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column was applied. The separation achieved with this column is provided in Figure 2B. The effect of using a smaller particle, higher efficiency column is immediately seen in the improved resolution of the 3000/4000 nt critical pair. The peak-to-valley ratio obtained with the 3.5 µm column was 1.3, while 1.6 was achieved with the GTx BEH SEC 2.5 µm Column. Moreover, the new separation appears to have partially resolved some form of heterogeneity within the 500 nt species. The hypothetical resolution improvement for switching from a 3.5 µm to 2.5 µm packing is approximately 20%. It is possible that the separation was also improved by the use of MaxPeak High Performance Surfaces and a reduction in secondary interactions between the nucleic acid analytes and the column hardware.

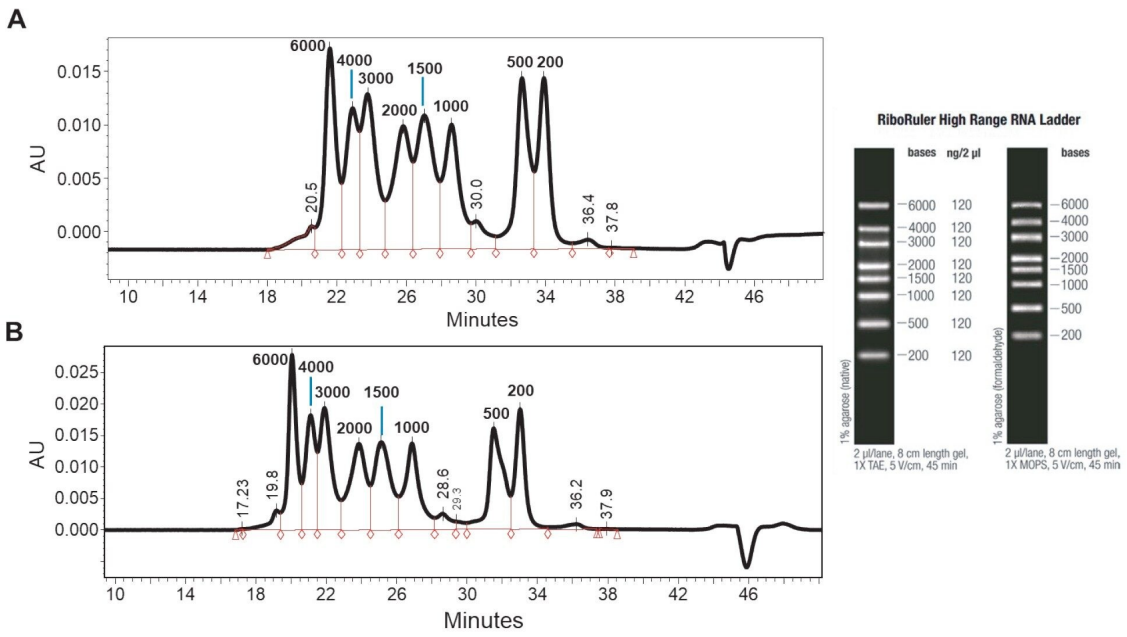

Figure 2. SEC-based separation of single stranded RNA components of a high range RNA ladder using an XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column. A. Separation of the ssRNA ladder on a 7.8 x 300 mm steel hardware, 3.5 µm 450 Å particle column using perchlorate and ACN - containing 1X PBS buffer. B. Separation as achieved with an XBridge Premier GTx BEH SEC 450 Å 2.5 µm 7.8 x 300 mm Column. Chromatographic peaks are annotated based on the expected size distribution of RNA molecules. Gel electrophoresis results are displayed as reported on certificate information provided with the standard.

Conclusion

Both small to medium sized DNA and RNA can be resolved with an XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column using one or more types of mobile phase compositions. Resolution is optimal for <3000 bp double stranded DNA and <6000 nt RNA. SEC provides an attractive method for drug substance integrity and purity measurements and is one of the few techniques available for aggregate analyses. While it is not the highest resolution separation technique, it is generally simple to implement. With the efficiency of a 2.5 µm packing material, low adsorption hardware, and optimization of column dimensions up to traditional 7.8 x 300 mm

configuration, GTx BEH SEC 450 Å 2.5 µm Columns make it possible to achieve robust resolution and requisite system suitability for new RNA and DNA assays.

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