Application Note

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# From Narrow to Ultrawide Pores: Characterization of mAbs, AAVs, mRNA, and pDNA

## Using size-exclusion chromatography


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## Abstract

Biopharmaceutical modalities such as monoclonal antibodies (mAbs), transgene carrying adeno-associated virus (AAV), and messenger ribonucleic acid (mRNA) easily differ over one order of magnitude in molecular weight and coincidently also vary in size. This has spurred the development of novel wide-pore size-exclusion chromatography (SEC) columns for the evaluation of size variants such as monomers, aggregates (dimers, multimers), and fragments altogether in one chromatographic column volume. Here, the entire family of the Agilent AdvanceBio SEC 2.7 µm columns, with pore sizes ranging from 130 Å to 1000 Å, has been evaluated for various classes of biopharmaceuticals, including mAbs, AAVs, mRNA, and plasmid deoxyribonucleic acid (pDNA).

## Introduction

The pharmaceutical industry has undergone a profound transformation over the past four decades, driven by successive waves of innovation in therapeutic and prophylactic modalities. The introduction of recombinant proteins and monoclonal antibodies (mAbs) in the 1980s marked a paradigm shift from small molecules to complex biologics, enabling highly specific targeting of disease pathways. More recently, the emergence of nucleic acid-based medicines, including messenger ribonucleic acid (mRNA)-loaded lipid nanoparticles (LNPs) and deoxynucleic acid (DNA)-carrying adeno-associated viruses (AAVs), has further expanded the landscape by allowing in vivo protein synthesis and gene modulation.[1] The molecular weight of these products, including their aggregates and potential degradation products (fragments) is spanning over several orders of magnitude—approximately 150 kDa (~ 5 nm) for mAbs, 300 kDa to 3 MDa (several tens of nm) for mRNA, 3.8 MDa (~ 25 nm) for AAVs and a few to several tens of MDa for plasmid DNA (pDNA) (up to several hundreds of nm depending on topology). Studying size heterogeneity of these modalities is essential to evaluate their quality, safety, and efficacy. Aggregates and fragments have shown to reduce therapeutic effectiveness, trigger adverse immunogenic responses, and impact product stability and shelf life.

Size-exclusion chromatography (SEC) is the method-of-choice to provide robust size-based information under native conditions. In recent years, a new generation of SEC columns has been introduced with (1) sub-3 µm particle size, (2) optimized stationary-phase chemistry and column materials to limit nonspecific interaction and bleeding, (3) increased pore size beyond 300 Å, and (4) improved mechanical stability of the packed bed to extend the maximum pressure tolerability of the column to 400 bar and above. This improves the separation performance and allows various detection approaches to be explored like ultraviolet (UV), fluorescence, multi-angle light scattering (MALS) and mass spectrometry (MS).[2] Importantly, pore size expansion broadens the applicability of SEC and allows larger modalities to be studied.

This application note evaluates the Agilent AdvanceBio SEC columns with pore sizes 130 Å to 1000 Å for the characterization of mAbs, AAVs, mRNA, and pDNA. Several relevant applications are highlighted to demonstrate the usability of an optimal pore size for a given biopharmaceutical sample.

## Experimental

### Materials

Type I water was produced using an Arium Pro ultrapure lab water system (Sartorius). Sodium chloride (≥ 99.0%, ReagentPlus), sodium phosphate dibasic (≥ 99.0%, ACS reagent), sodium phosphate monobasic monohydrate (≥ 99.0%, ACS reagent), and urea were purchased from Merck. Ethylenediaminetetraacetate (EDTA) was bought from Panreac AppliChem. Pierce bovine serum albumin (BSA) standard (2 mg/mL), DEPC-treated water, and nuclease-free Tris-HCl buffer (pH 7.5) were acquired from Thermo Fisher Scientific and gel filtration standard was procured from Bio-Rad. Humanized monoclonal antibody trastuzumab (21 mg/mL) was obtained from Roche. AAV serotype 5 samples ($2 \times 10^{13}$ vg/mL for AAV5-CMV-GFP and $2 \times 10^{13}$ vg/mL for AAV5-empty) were sourced from Virovek. Firefly luciferase (FLuc) mRNA (1 µg/µL) and Cas9 mRNA (1 µg/µL) were ordered from TriLink BioTechnologies. IVT mRNA (4.5 kb, 1 µg/µL) was produced in house from linearized pDNA (7.5 kbp). Single guide RNA (sgRNA) was synthesized at Integrated DNA Technologies. The single stranded RNA (ssRNA) ladder (0.5–9 kb), restriction enzyme SpeI, NEBuffer r.1.1, RNase T1 and RNase 4 were sourced from New England Biolabs.

### Sample preparation

Lyophilized trastuzumab was dissolved in water for injection to 21 mg/mL and stressed by incubation at 40 °C for 4 weeks, followed by dilution to 2 mg/mL prior to injection. The 7.5 kbp pDNA was linearized by adding restriction enzyme SpeI (incubation at 37 °C) according to manufacturer's protocol. pDNA was injected at 1 µg/µL. To prepare the RNase T1 digest, 100 µg of FLuc mRNA was digested with 5000 U of RNase T1 in a 100 mM Tris-HCl pH 7.5 buffer with 40 mM EDTA for 30 minutes at 37 °C. Prior to RNase 4 digestion, 100 µg FLuc mRNA was denatured by addition of 3 M urea, followed by incubation at 90 °C for 10 minutes. After the sample was cooled down to room temperature, digestion was completed by addition of 500 U of RNase 4 in 1× NEBuffer r1.1 and incubation for 1 hour at 37 °C. Both the RNase T1 and RNase 4 digest were concentrated to 2 µg/µL using a refrigerated CentriVap Concentrator (Labconco). The ssRNA ladder was injected without dilution. sgRNA was dissolved in DEPC water to 1 µg/µL and combined with Cas9 mRNA in a 1:1 (wt:wt) ratio.

### Instrumentation and method

Details of instrument configuration can be found in Table 1. Method parameters are summarized in Table 2. Data were acquired and processed in the Agilent OpenLab CDS (version 2.7).

**Table 1.** Details of the instrument configuration used.

| Parameter | Value |
|---|---|
| Pump | Agilent 1260 Infinity II Bio-Inert Pump (G5654A) |
| Autosampler | Agilent 1260 Infinity II Bio-Inert Multisampler (G5668A) |
| Column Compartment | Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) |
| Detector (DAD) | Agilent 1260 Infinity II Diode Array Detector (G7115A) |
| UV Flow Cell | Agilent Bio-Inert Flow Cell, 13 µL, 10 mm path length (G5615–60022) |
| Detector (FLD) | Agilent 1260 Infinity II Fluorescence Detector (G7121B) |
| FLD Flow Cell | Agilent FLD Flow Cell, standard 8 µL (G1321-60005) |

**Table 2.** LC method parameters.

| Parameter | Value |
|---|---|
| Column | – 4.6 × 300 mm, Agilent AdvanceBio SEC 2.7 µm, 130 Å/500 Å/1000 Å (p/n PL1580-5350, PL1580-5301, PL1580-5325, PL1580-5302)<br>– 4.6 × 150 mm, Agilent AdvanceBio SEC 2.7 µm, 1000 Å (p/n PL1580-3302) |
| Flow Rate | 0.35 mL/min |
| Mobile Phase | 50 mM phosphate buffer, 400 mM NaCl, pH 7.2 |
| Column Temperature | 25 °C |
| Autosampler Temperature | 4 °C |
| Injection | 1 µL/5 µL (for mAb) |
| Detection DAD | 260/4 nm, 5 Hz<br>280/4 nm, 5 Hz |
| Detection FLD | Ex 280 nm, Em 348 nm, 4.63 Hz |
| Run Time | 24 min |

## Results and discussion

The selection of the right pore size for a given application is key in SEC. Figure 1 shows the UV 260 nm (for nucleic acids) and 280 nm (for proteins) chromatograms of different classes of biopharmaceuticals obtained on SEC columns with particle pore sizes varying from 130 Å to 1000 Å. In general, from a qualitative perspective, the optimal pore size for the sample at hand is the one where the main peak appears in the middle of the separation window between exclusion limit (i.e., the upper size at which molecules are unable to enter the pores of the particles and elute in the interparticle space) and the permeation limit (i.e., where molecules are small enough to fully enter the pores). This leaves plenty of separation space to study aggregates and fragments. One can notice that 300 Å pore size is optimal to separate proteins like BSA and mAbs while larger modalities (AAVs, mRNA, and pDNA) are excluded from the pores. Interestingly, these constructs elute at or before the exclusion limit of the column and still get separated in the external void fraction of the column due to hydrodynamic chromatography (HDC), which relies on the interstitial pore volume. Large analytes (with respect to the interparticle flow-through pores) will get distributed along local velocity streamlines and get separated to some extent. AAVs and mRNA, on the other hand, benefit from, respectively, 500 Å and 1000 Å pore sizes that allow to resolve AAV aggregates and double-stranded RNA (dsRNA) impurities from the main capsid or mRNA peak. An ~100 nm pDNA requires larger pore sizes for true SEC separation, though some structural information can be gleaned by using a smaller pore SEC column and exploiting HDC or slalom separation mechanisms. The latter is typically performed at

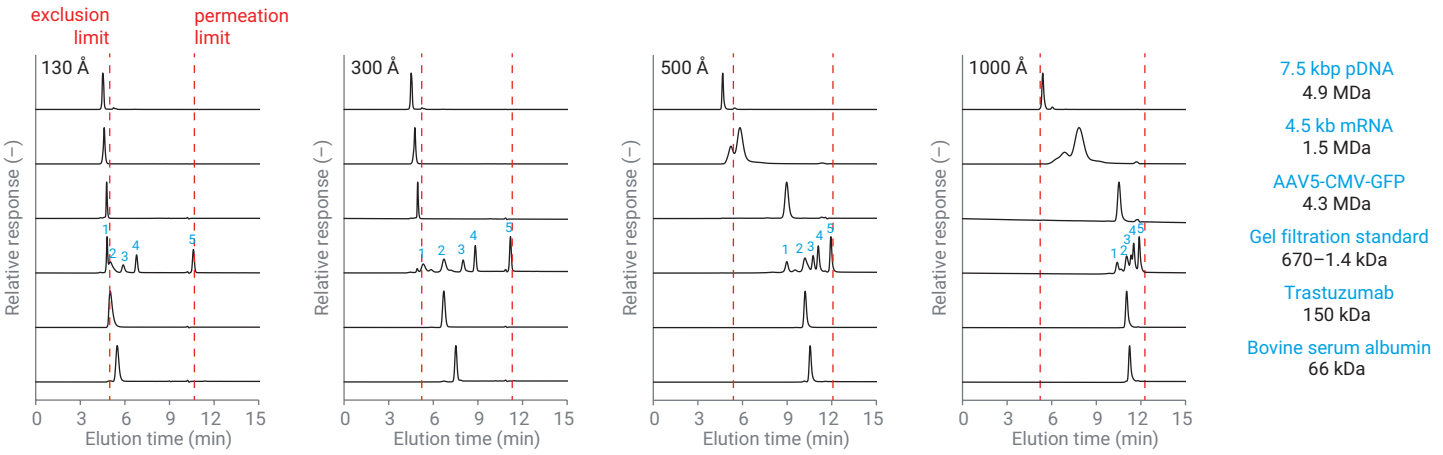


**Figure 1.** Overview of the separation space for various biopharmaceutical modalities on the Agilent AdvanceBio SEC columns with pore sizes ranging from 130 Å to 1000 Å. For clarity, the compounds of the gel filtration standard are annotated: thyroglobulin (1), γ-globulin (2), ovalbumin (3), myoglobin (4), and vitamin B12 (5). Depending on the size of the analyte, an appropriate pore size should be selected, or a combination of pore sizes can be used when a large dynamic size range is expected. The exclusion and permeation limits were empirically determined based on the elution profiles of the constructs, linking to an average external porosity of 0.37 and a total porosity of 0.82.

high flow rates, stretching the large DNA/RNA molecules that are excluded from the pores to an extended nonequilibrium configuration because of the high shear force. As a result, large nucleic acids susceptible to slalom effects will elute later, leading to a reversed-elution behaviour as in HDC/SEC. Note that although the molecular weight (MW) of pDNA and AAVs are similar (4.9 versus 4.3 MDa), their elution times differ due to the size and folding architecture of the AAV capsid (~25 nm), which is drastically smaller than that of the 7.5 kbp supercoiled pDNA (> 100 nm). The same holds true for 4.5 kb mRNA (1.5 MDa) which has an extended and flexible conformation and hence, increased hydrodynamic radius compared to AAVs.

## Characterization of size variants of mAbs using 300 Å pore size SEC column

SEC columns packed with 300 Å pore size particles are preferred to study mAb size heterogeneity. Stress conditions during manufacturing, storage, shipment, etc., can result in the formation of high (HMW) and low molecular weight (LMW) species that can affect drug safety and efficacy. The SEC–UV (280 nm) chromatogram of trastuzumab, a humanized anti-HER2 mAb, is presented in Figure 2. Incubation of the mAb at 37 °C for several weeks reduces the potency due to isomerization of heavy chain aspartate 102, which is resolved by cation exchange chromatography (CEX).[3] This stress also induces clipping above the hinge, resulting in LMW species: a full mAb deprived of one Fab arm (i.e., ~100 kDa mAb-Fab fragment) and a ~50 kDa Fab fragment, both of which are visualized by SEC. Temperature stress furthermore appears to reduce the amount of noncovalent dimer.[4] HMW and LMW species can be detected ≤ 1%. A column with a slightly smaller particle and pore size (i.e. AdvanceBio SEC 1.9 µm, 200 Å) is better suited to resolve the ~100 kDa mAb-Fab fragment from the mAb monomer.

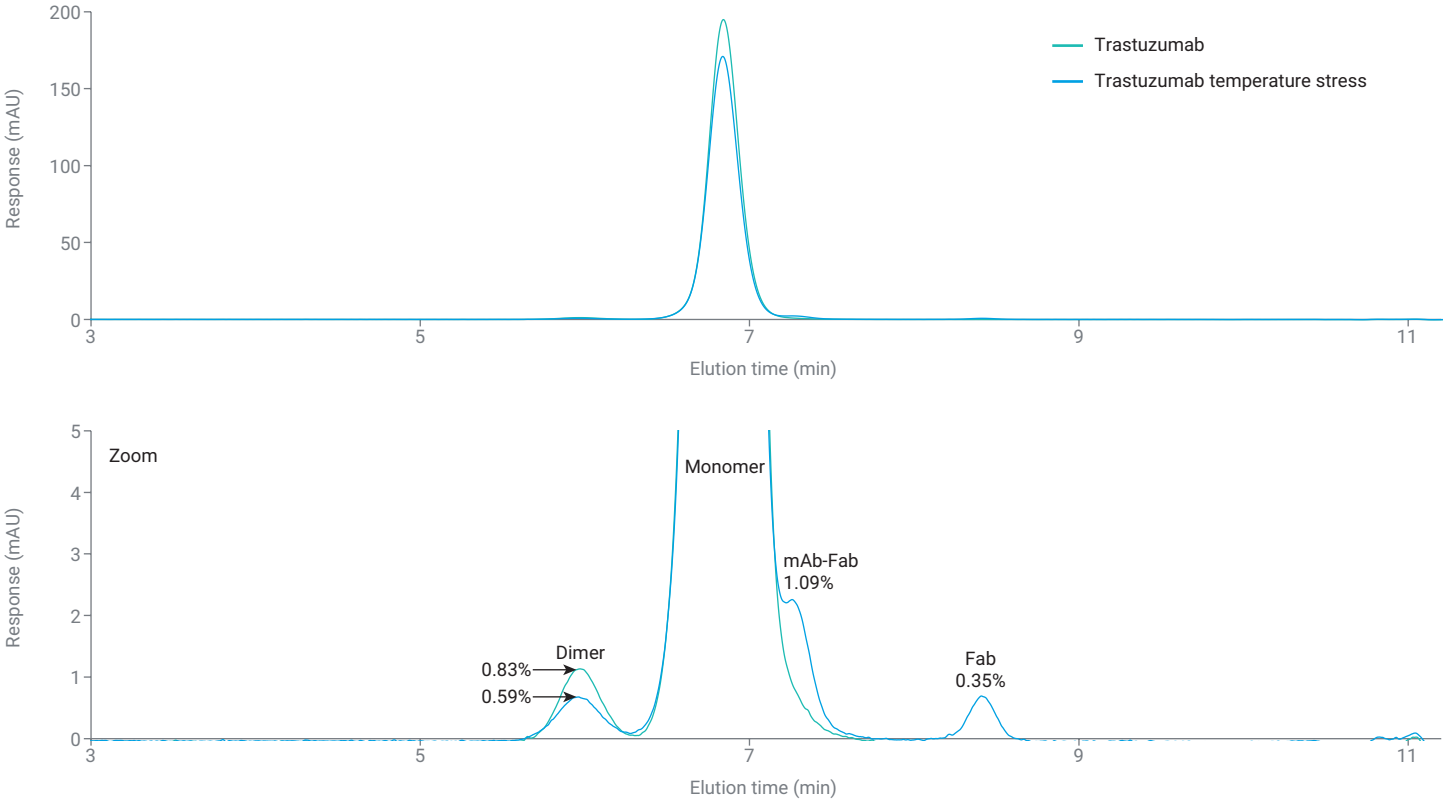


**Figure 2.** UV 280 nm chromatograms of nonstressed (cyan) and temperature-stressed trastuzumab (blue) on an Agilent AdvanceBio 300 Å SEC column showing the separation of HMW and LMW variants.

## Evaluation of AAVs using 500 Å pore size SEC column

An SEC column with 500 Å pore size particles, combined with fluorescence detection (FLD), allows for a sensitive method for the determination of aggregates in AAV samples (Figure 3), as was also demonstrated in an Agilent Technologies application note (p/n 5994-8897EN).[5] Several aggregates are present in the AAV5-empty sample, but interestingly its main aggregate species seems to be dissimilar to that of the AAV5-CMV-GFP sample. Based on the peak heights of the recorded UV chromatograms at 260 nm and 280 nm, an $A_{260/280}$ ratio can be calculated, which should be around 1.2 for a genome-filled AAV peak. This is indeed the case for both the aggregate and the AAV main peak of AAV5-CMV-GFP, and this value drops to 0.7 for both peaks for the AAV5-empty sample indicating more "pure" protein capsids (absence of DNA).[5]

## Study of mRNA size heterogeneity using serially coupled AdvanceBio SEC 1000 Å and 300 Å columns

mRNA is at least one order of magnitude larger in molecular size compared to mAbs, as was described for a 1.9 kb mRNA with a measured hydrodynamic diameter of 43 nm.[6] Hence, particles with larger pore size are required for the SEC analysis of mRNA. As shown in Figure 4, and reported earlier[7], 1000 Å pore size particles are optimal to analyze mRNA in the size range 0.5–9 kb and separate dsRNA from the 1.9 kb FLuc mRNA main peak. The dsRNA impurity, preceding the mRNA monomer, melts upon heating the sample at 80 °C for 30 minutes, illustrating its noncovalent nature. The analysis of FLuc mRNA RNase T1 and RNase 4 digests shows a huge peak of coeluting shorter oligonucleotide fragments near the permeation limit of the column. The peak eluting before the shorter digestion products corresponds to the approximately 120 b long poly(A)-tail, which is resistant to digestion.

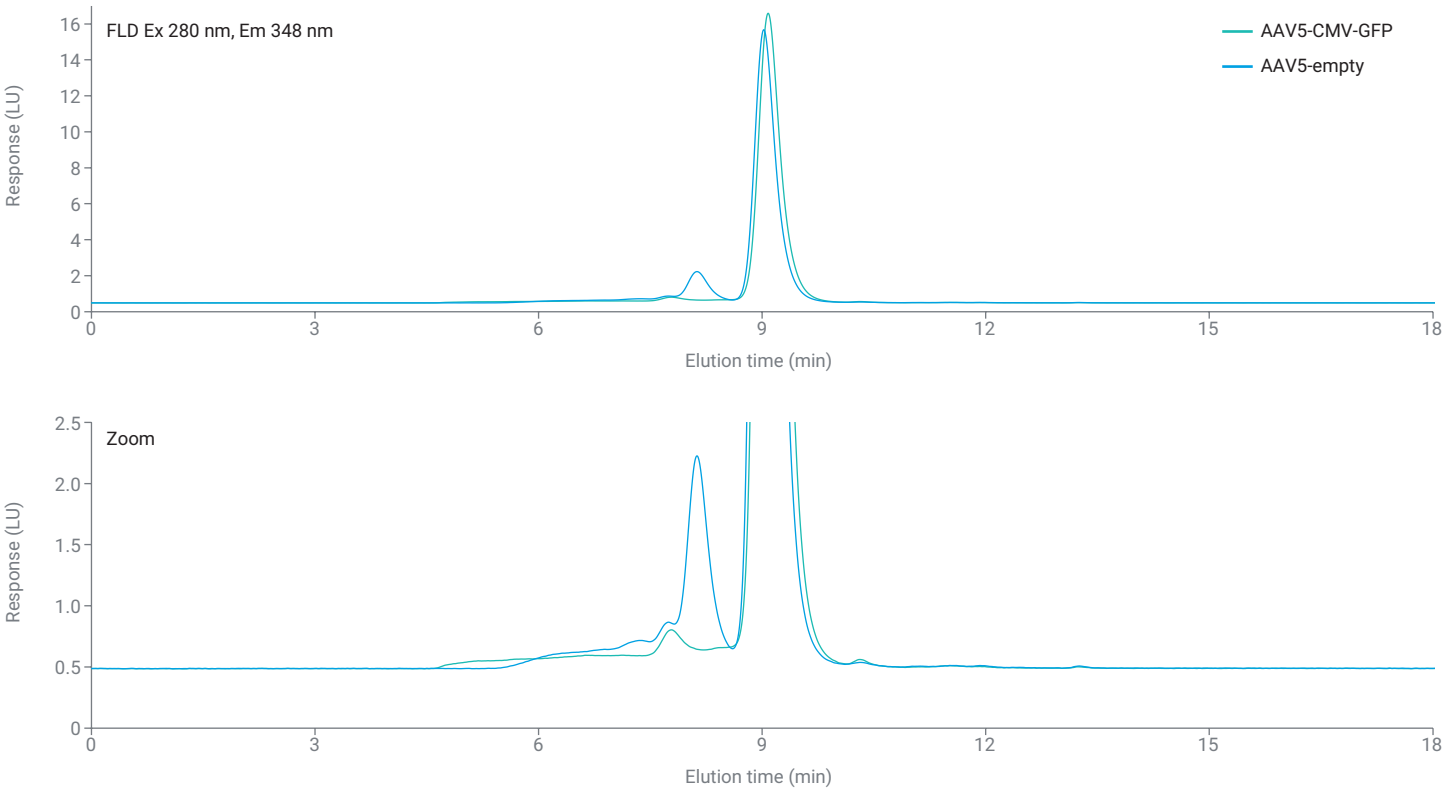


**Figure 3.** Separation of an AAV5-CMV-GFP sample with encapsulated CMV-GFP promotor and genome (cyan) and AAV5-empty sample (no genome) (blue) using FLD on an Agilent AdvanceBio SEC 500 Å column.

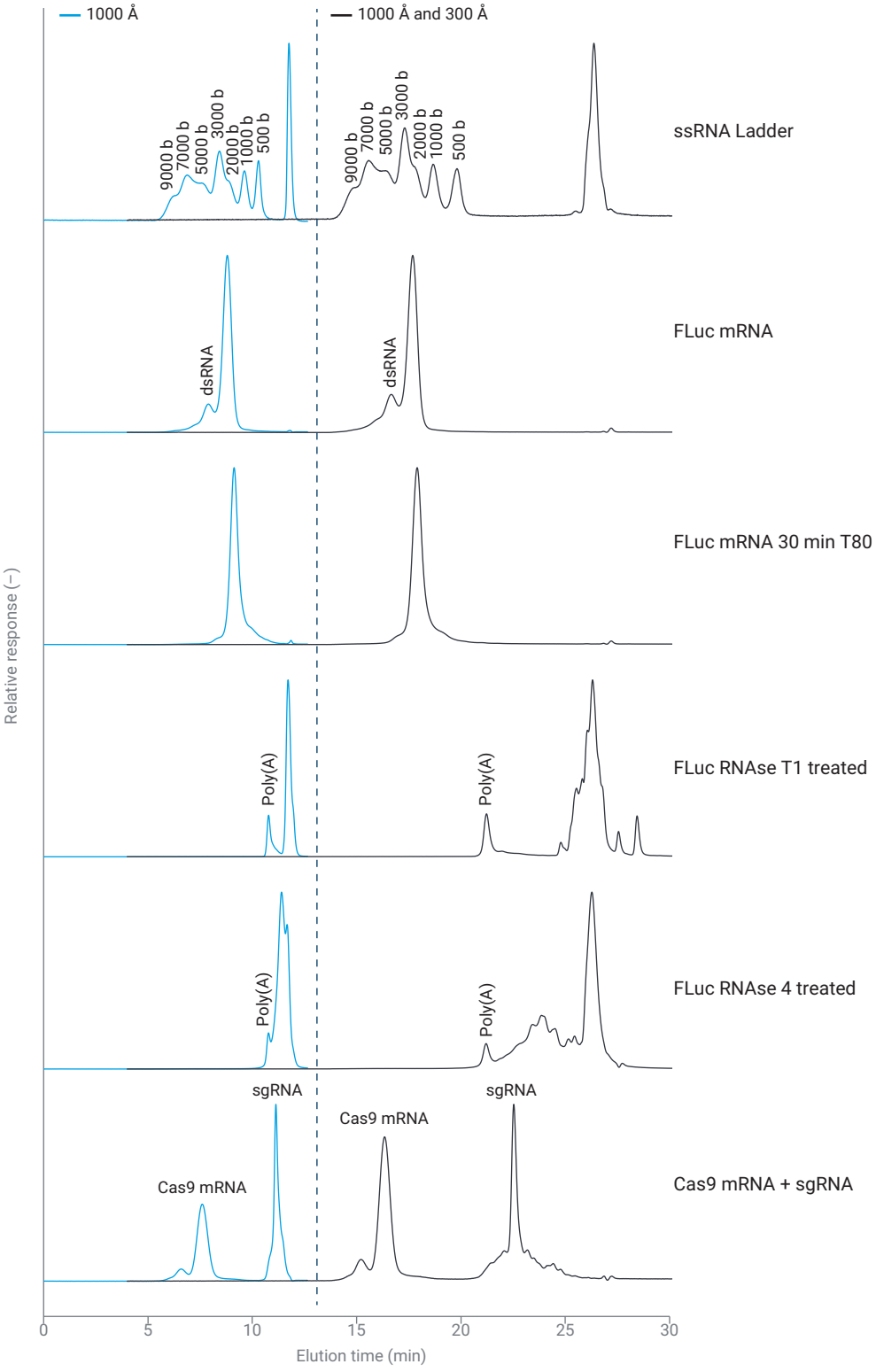


**Figure 4.** Evaluation of different mRNA samples on a single Agilent AdvanceBio SEC 1000 Å column (blue chromatograms) and an AdvanceBio SEC 1000 Å column serially coupled with an AdvanceBio SEC 300 Å column (black chromatograms). For easy comparison, the elution times of the black traces were offset with 4 minutes.

To obtain better insights into size distribution of the digested samples, the 1000 Å SEC column was serially coupled with a 300 Å column to enhance the separation. The total back pressure increased to 280 bar when running two columns in tandem, which is well within the 400-bar pressure limit of this SEC column. This arrangement leaves the separation of the 0.5–9 kb ssRNA ladder largely unaffected. The advantage of the smaller-pore-size column is most evident in the separation of RNase digests. Compared to RNase T1, RNase 4 generates larger oligonucleotide fragments, which can be explained by their specificity, as RNase T1 cuts at the 3' side of guanine whereas RNase 4 allows for more targeted digestion as it cleaves at the 3' side of uridine/guanine or uridine/adenine. The dual-pore-size SEC configuration is also beneficial for the separation of a 1:1 (wt:wt) mixture of 4.5 kb Cas9 mRNA and 104 b sgRNA coformulated for CRISPR-Cas-mediated genome editing. The sgRNA standard is seen to contain many shortmer/longmer impurities that are poorly resolved solely using the 1000 Å column.

## Profiling of pDNA topologies using 1000 Å AdvanceBio SEC column

pDNA can appear in several topological forms, such as supercoiled (SC), nicked or open-circular (OC), linear, and multimers. These are typically assessed by anion-exchange chromatography (AEX), agarose gel electrophoresis or capillary electrophoresis (CE). SEC is emerging as an alternative method to study pDNA topology, where the selection of particle pore size is important (see Figure 1). In general, the linear form is larger in size than the other topologies, which translates to different elution times on SEC, but typically requires ultrawide pore sizes. Figure 5 presents the measurement of 7.5 kbp pDNA at varying flow rates on a 1000 Å SEC column. Due to the immense length of the molecule, with size often exceeding 100 nm, pDNA cannot enter the pores of the column, thereby making the column suboptimal to exhibit SEC of this pDNA sample.

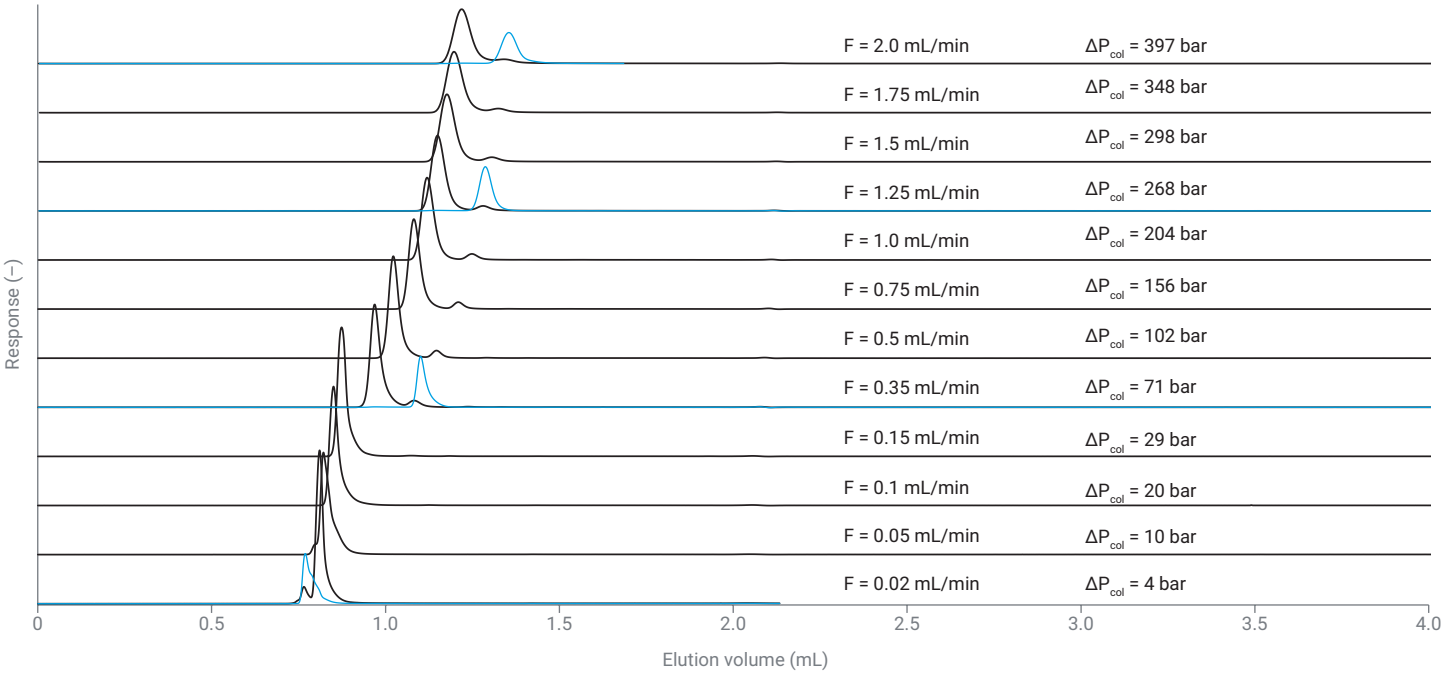


**Figure 5.** UV 260 nm chromatograms of 7.5 kbp pDNA (black trace) and linearized pDNA (blue trace) at different flow rates on a 4.6 × 150 mm Agilent AdvanceBio SEC 1000 Å column.

This means that HDC is the dominating separation mechanism. By plotting the chromatograms in elution volume, it is possible to easily compare the separation over a large flow-rate range. As expected, low flow rates result in smaller peak widths attributed to improved separation efficiency when moving closer to the van Deemter optimum linear velocity. A small shift to increased elution volume is observed at higher flow rates, which is indicative of nonSEC elution behavior (e.g., slalom chromatography). A minor impurity peak precedes the main peak at low flow rates and appears to retain more at increased flow rates. To investigate which topology this is related to, the pDNA sample was incubated with a restriction enzyme to generate the linearized form and injected at selected flow rates (see blue chromatograms). Supercoiled pDNA is stiffer and hence, less stretchable than linear pDNA, making it less prone to exhibit additional retention due to slalom chromatography.[8] This is best demonstrated by the relative change in elution volume, which is 51% for the supercoiled pDNA sample and 78% for linearized pDNA, showing the latter to be much more prone to shear stretching at elevated flow rates.

## Conclusion

SEC is ideally suited to study size heterogeneity associated with biopharmaceuticals. Careful selection of pore size allows for optimal separation of different prophylactic or therapeutic modalities, which can vary over several orders of magnitude in MW and coincidentally also in hydrodynamic diameter. To demonstrate the applicability of the Agilent AdvanceBio SEC columns, intriguing cases were selected related to mAbs, AAVs, mRNA, and pDNA. The same running buffer was used for all applications, allowing analytical scientists to select a go-to choice of mobile phase which can be further fine-tuned for a given application. FLD allows for the sensitive detection of aggregate species in AAV samples. A 1000 Å–300 Å dual-pore size combination of columns can greatly improve the resolution of mRNA coformulations or digests. By profiting from the slow elastic relaxation time of linear pDNA under high shear-flow conditions, topological profiling of pDNA samples can be achieved by slalom chromatography.

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