Application Note

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Evaluation of SEC Columns for Analysis of ADC Aggregates and Fragments

Choosing the best stationary phase to overcome nonspecific interactions

Authors

Andrea Angelo P. Tripodi
Agilent Technologies, Inc.
Church Stretton, UK

Arnaud Delobel
Quality Assistance S.A.
Donstiennes, Belgium

Abstract

Antibody drug conjugates (ADCs) use the specificity of a monoclonal antibody (mAb) to ensure that cytotoxic drugs are delivered to the right target. The use of ADCs is a highly efficient way of tackling various diseases. There are already ten ADC biotherapeutics that are approved and commercialized with many more in development. An example ADC is illustrated in Figure 1.

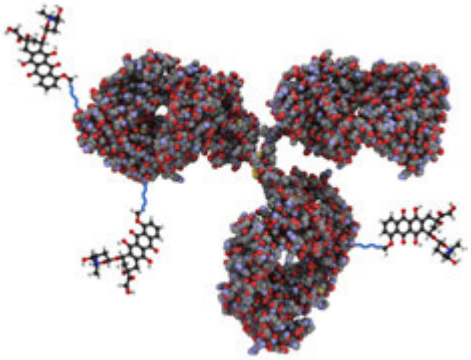

Figure 1. General structure of an antibody drug conjugate, courtesy of Quality Assistance S.A.

As with any biotherapeutic, the analysis of critical quality attributes is paramount. However, this analysis is complex due to the presence of the cytotoxic drugs attached to the antibody. Size exclusion chromatography (SEC) is effective, but still challenging, for the quantification of aggregates and fragments. ADCs are frequently more hydrophobic than mAbs alone and are therefore more susceptible to nonspecific interactions. This application note compares the performance of some of the commercially available SEC columns for the analysis of ADCs.

Introduction

Currently there are ten approved commercially available ADCs. Brentuximab vedotin and trastuzumab emtansine belong to the two main classes of ADCs: Cys-linked and Lys-linked. Both employ an IgG1 monoclonal antibody as the vehicle. Brentuximab is an anti-CD30 chimeric mAb, and trastuzumab is an anti-HER2 humanized mAb. However, the approach taken to conjugate the small molecule drug differs in these ADCs.

In trastuzumab emtansine, the DM1 drug is conjugated to lysine (Lys) side chains using a noncleavable succinimidyl linker (SMCC). The resulting configuration is a random distribution of drug molecules across the surface of the protein. There are 90 Lys residues throughout the trastuzumab molecule, and each molecule may contain up to eight DM1 conjugates. In brentuximab vedotin, partial reduction of the intrachain disulfide bridges allows for up to eight monomethyl auristatin E (MMAE) drug molecules to be conjugated through cleavable valine-citrulline linkers.

Both approaches modify the hydrophobicity of the underlying mAb, but also present other problems. Trastuzumab emtansine is a heterogeneous mixture due to the random distribution of the drug molecules. Whereas brentuximab vedotin has a more specific distribution but at the expense of the loss of the stabilizing intrachain disulfide bonds.

These features make the analysis of such molecules more complex. Even size exclusion chromatography, the preferred method for quantification of aggregates, is more challenging due to more nonspecific interactions.

This application note explores the differences in stationary phase construction and particle size, both of which can aid the analysis of ADCs. It also determines which products exhibit fewer secondary interactions in the absence of any organic modifier. Using organic modifier to reduce some nonspecific interactions could also alter the level of aggregation observed.

For the purposes of this investigation, a range of commercially available SEC columns were chosen that have features designed to make them more suitable for analysis of complex molecules, like ADCs. Most contain a hydrophilic coating to mask interactions with the underlying silica particles. Some, such as column B from Vendor 1, have differences in the way this coating is attached, which alters the orientation on the surface.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Instrumentation

A UHPLC instrument with UV detection was used. Method parameters are listed in Table 1.

Table 1. Chromatographic conditions.

HPLC Conditions	
Columns	**A:** Agilent AdvanceBio SEC 300 Å, 4.6 × 300 mm, 2.7 µm **B:** Vendor 1 200 Å, 4.6 × 300 mm, 1.7 µm **C:** Vendor 2 300 Å, 4.6 × 300 mm, 1.8 µm
Mobile Phase	Phosphate buffered saline (PBS)
Flow Rate	0.35 mL/min
Column Temperature	30 °C
Injection Volume	5 µL Trastuzumab, 10 µL Brentuximab
Total Run Time	20 minutes

Results and discussion

It is immediately obvious from the shape of the peaks (tailing or even multiple components) where secondary interactions are causing issues. Column A, the Agilent AdvanceBio SEC 300 Å, 2.7 µm column, performs better than columns B and C, despite both having a smaller particle size. Figure 2 highlights how the peak shape and dimer/monomer resolution is superior with the AdvanceBio SEC 300 Å for analyzing the Lys-linked trastuzumab emtansine, compared to the competitor columns B and C. Column B exhibits increased secondary interactions, as shown by a loss of peak resolution. The loss of resolution is probably due to the different type of stationary phase chemistry on column B. Column C gives a slightly narrower peak shape, but the resolution is also inferior versus the AdvanceBio column (Table 2).

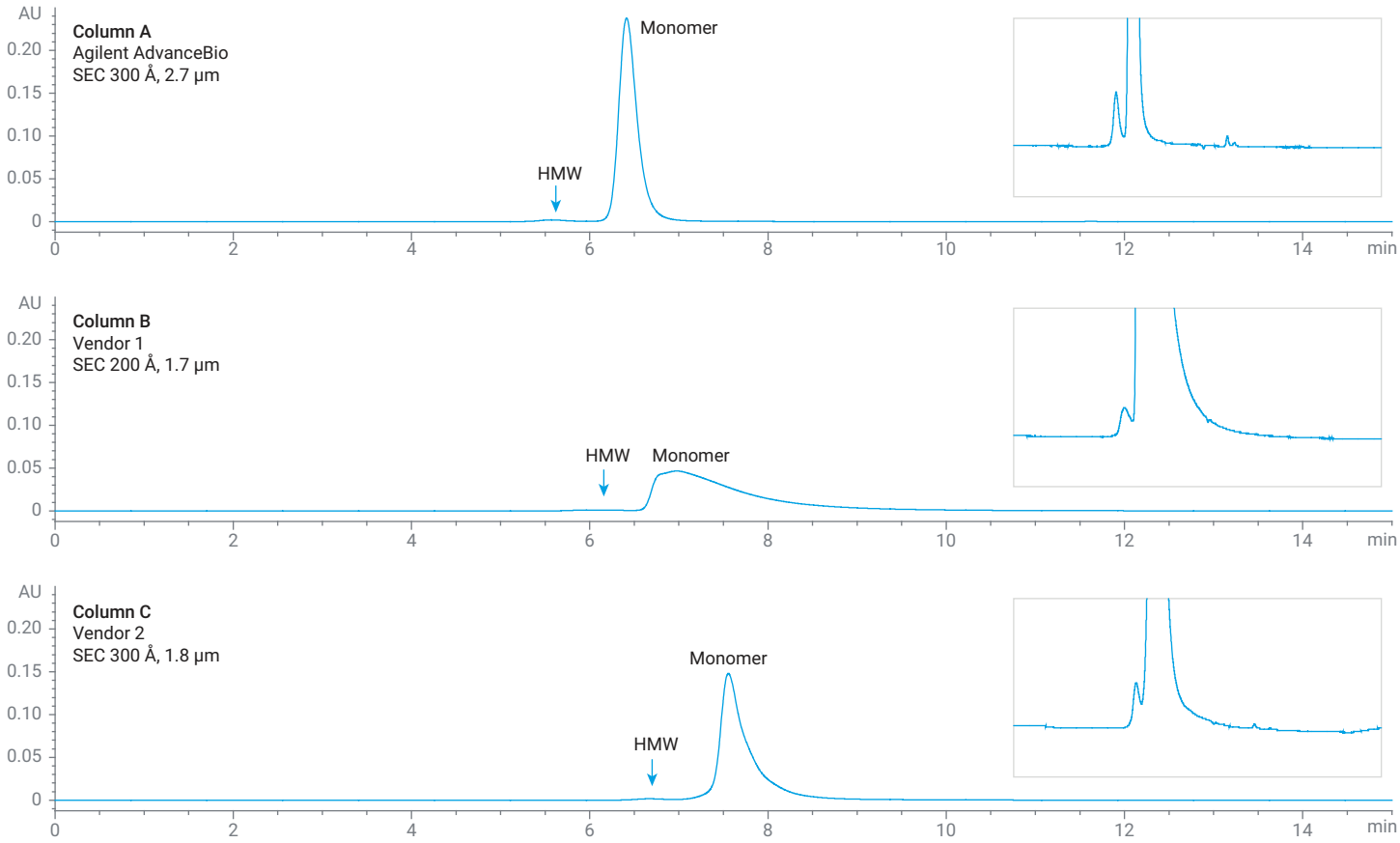

Figure 2. SEC of trastuzumab emtansine using 4.6 × 300 mm SEC columns with PBS, pH 7.4 at 0.35 mL/min. Inset shows full chromatogram zoomed into baseline region (0 to 0.02 mAU).

Table 2. SEC comparison values for trastuzumab emtansine: peak width at half height, tailing factor, percentage of aggregation and resolution.

Column	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
A: Agilent AdvanceBio SEC 300 Å, 2.7 µm	0.22	1.35	98.7	1.3	1.82
B: Vendor 1 200 Å, 1.7 µm	0.98	3.70	98.8	1.2	0.65
C: Vendor 2 300 Å, 1.8 µm	0.28	1.93	98.3	1.7	1.42

Looking at the Cys-linked brentuximab vedotin in Figure 3, column B is divided into two peaks, which indicates that the stationary phase is interacting with the sample. For column C, a sharp peak for the monomer was obtained but the aggregate percentage confirms that the AdvanceBio SEC column has superior performance when evaluating the HMW species, almost absent in column C (Table 3).

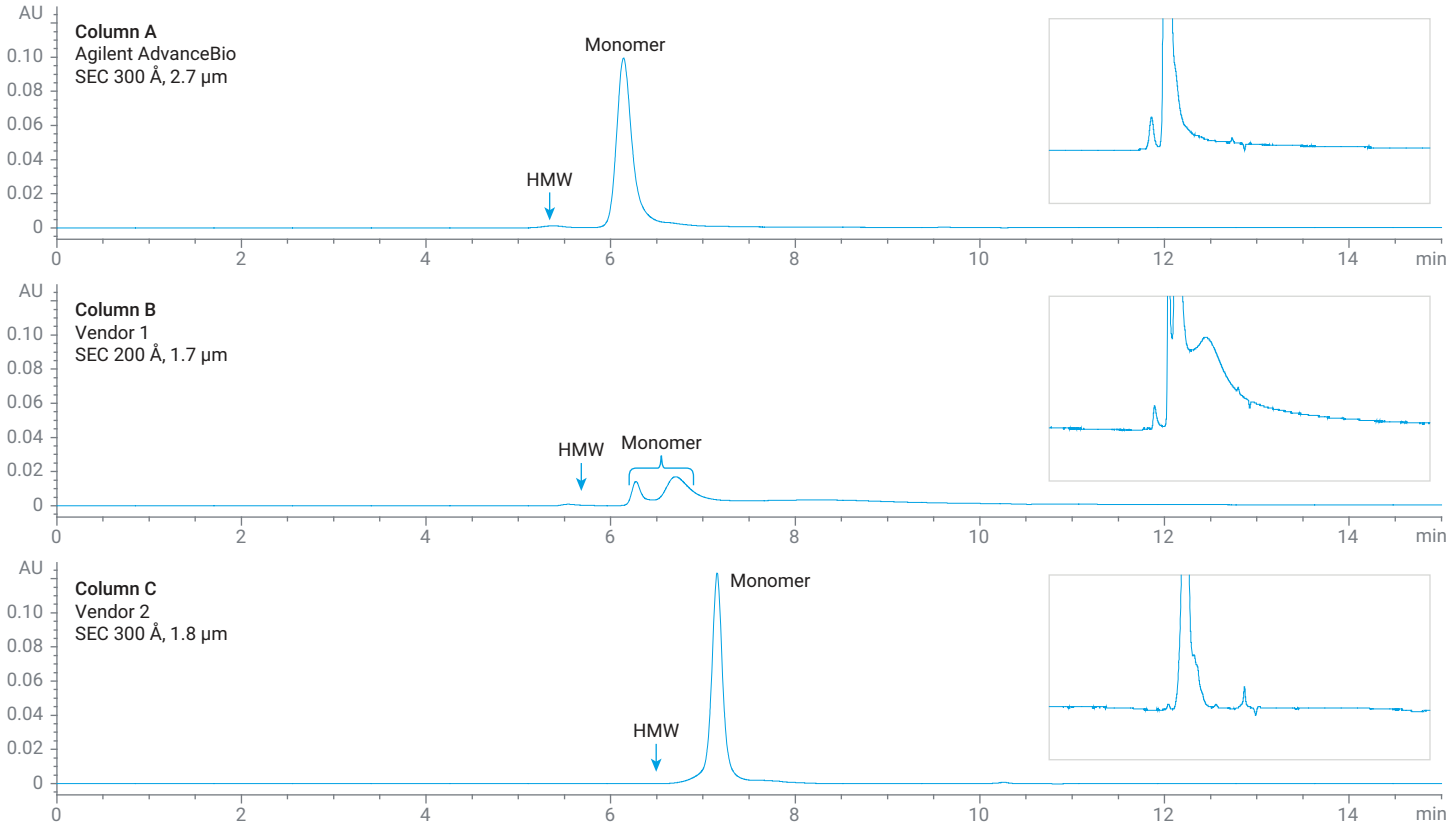

Figure 3. SEC of brentuximab vedotin using 4.6 × 300 mm SEC columns with PBS, pH 7.4 at 0.35 mL/min. Inset shows full chromatogram zoomed into baseline region (0 to 0.02 mAU).

Table 3. SEC comparison values for brentuximab vedotin: peak width at half height, tailing factor, percentage of aggregation and resolution.

Column	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
A: Agilent AdvanceBio SEC 300 Å, 2.7 µm	0.17	1.47	98.3	1.7	2.11
B: Vendor 1 200 Å, 1.7 µm	Not applicable	Not applicable	99.0	1.0	Not applicable
C: Vendor 2 300 Å, 1.8 µm	0.12	1.0	99.8	0.2	3.62

The AdvanceBio SEC 300 Å, 2.7 µm column was also tested under several mobile phase conditions to better understand the chromatography, and to improve peak shape and resolution for both ADCs. This evaluation confirms the influence of pH ranging from 6.8 to 7.4, as shown in Figures 4 and 5. Higher resolution gives more accurate dimer/monomer detection and is achieved at pH 7.4 (Tables 4 and 5).

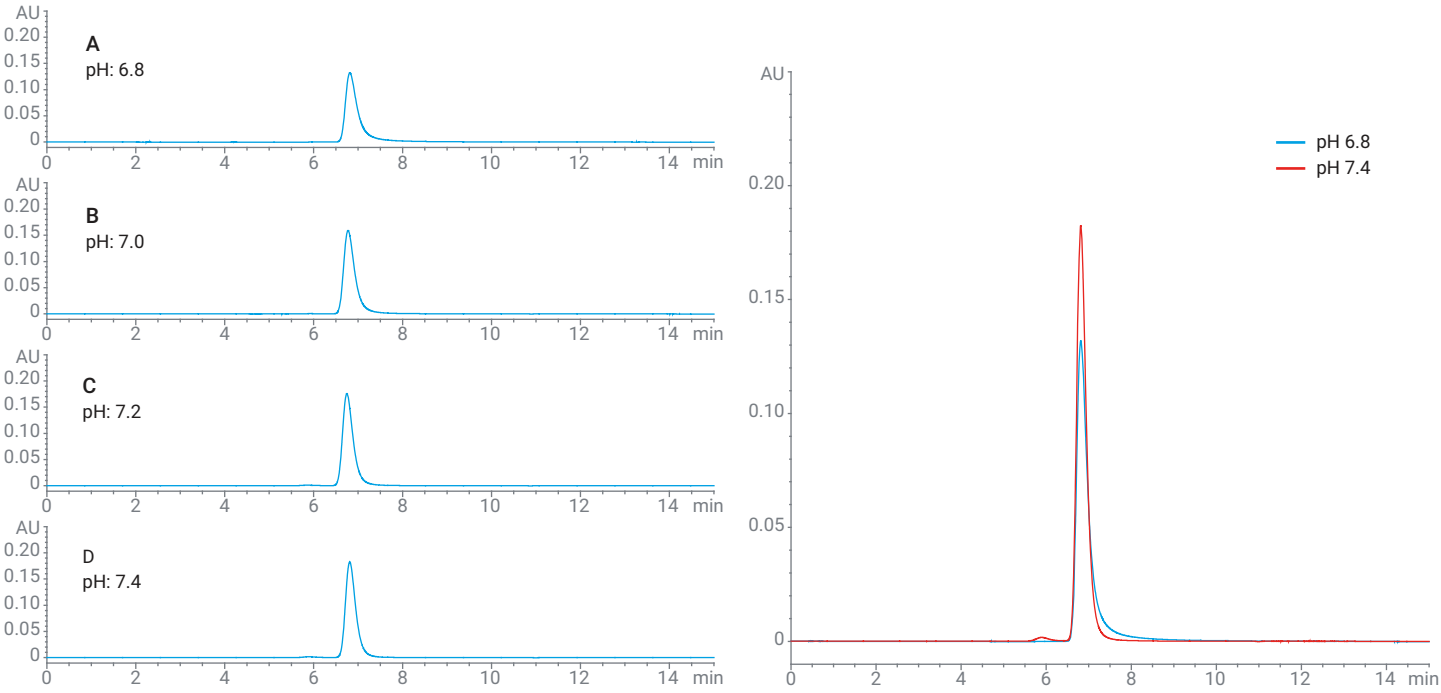

Figure 4. SEC of trastuzumab emtansine using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column running at 0.35 mL/min at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4.

Table 4. Effect of pH on chromatography for trastuzumab emtansine on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

pH	RT (min)	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
6.8	6.81	0.27	2.01	100	0	Not applicable
7.0	6.77	0.26	1.60	99.5	0.5	1.23
7.2	6.74	0.25	1.46	98.9	1.1	1.60
7.4	6.81	0.25	1.41	98.7	1.3	1.80

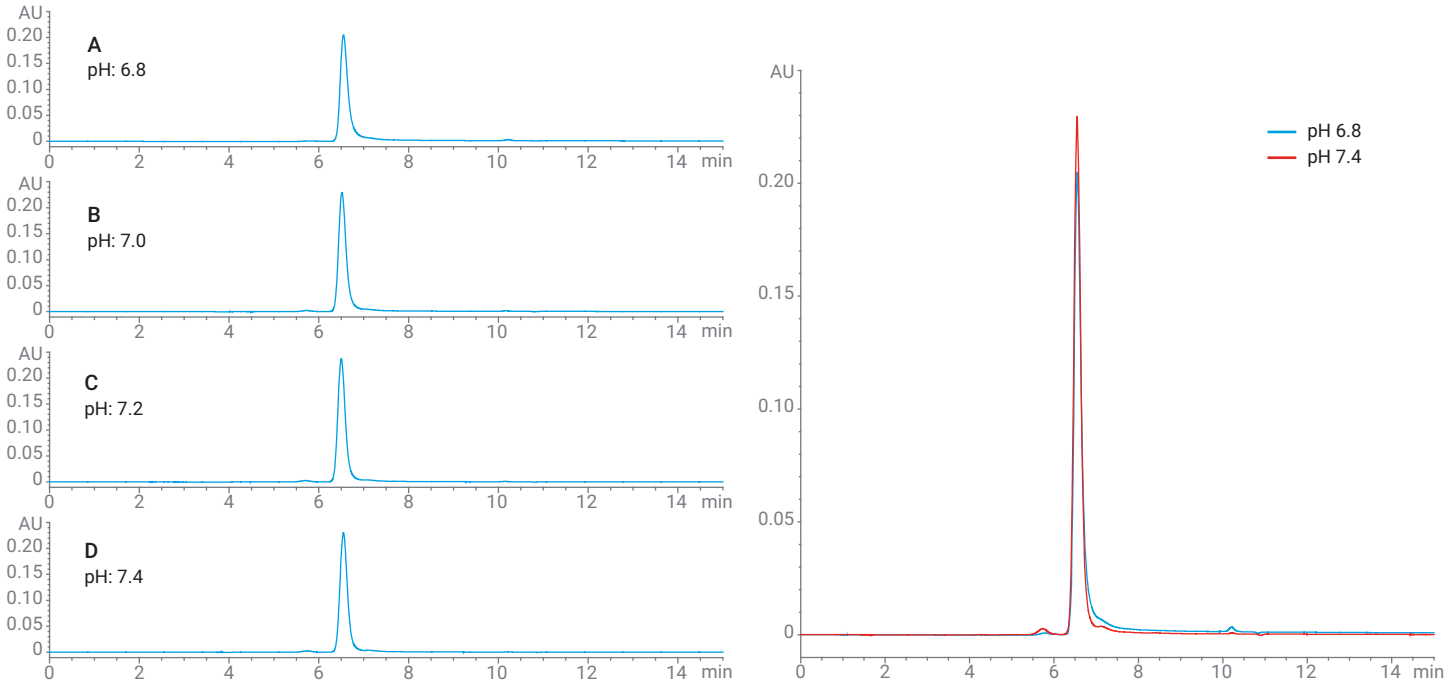

Figure 5. SEC of brentuximab vedotin using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column running at 0.35 mL/min at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4.

Table 5. Effect of pH on chromatography for brentuximab vedotin on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

pH	RT (min)	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
6.8	6.55	0.18	1.70	99.5	0.5	1.92
7.0	6.51	0.18	1.37	98.7	1.3	2.18
7.2	6.50	0.18	1.28	98.5	1.5	2.20
7.4	6.54	0.18	1.24	98.4	1.6	2.25

The effect of salt concentration was also evaluated with SEC chromatograms of both ADCs, trastuzumab emtansine (Figure 6) and brentuximab vedotin (Figure 7). 50 mM sodium phosphate pH 7.4 was used with the addition of 100, 200, and 300 mM of NaCl. For both ADCs, the use of high salt concentrations does not noticeably improve the performance, see Tables 6 and 7 respectively.

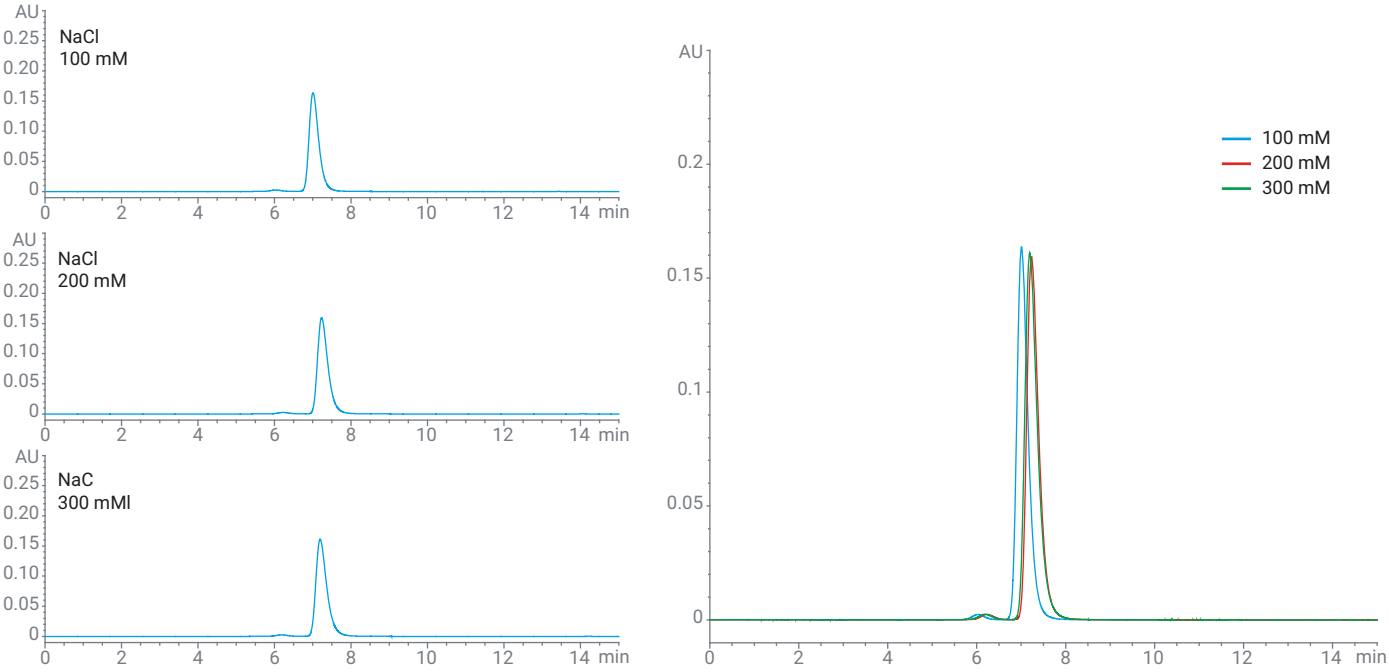

Figure 6. SEC chromatograms of trastuzumab emtansine using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column with PBS, pH 7.4 at 0.35 mL/min, showing the effect of NaCl concentration.

Table 6. Effect of NaCl concentration on chromatography for trastuzumab emtansine on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

NaCl Concentration	Monomer %	Aggregate %	Resolution
100 mM	98.3	1.7	1.98
200 mM	98.2	1.8	1.92
300 mM	98.2	1.8	1.85

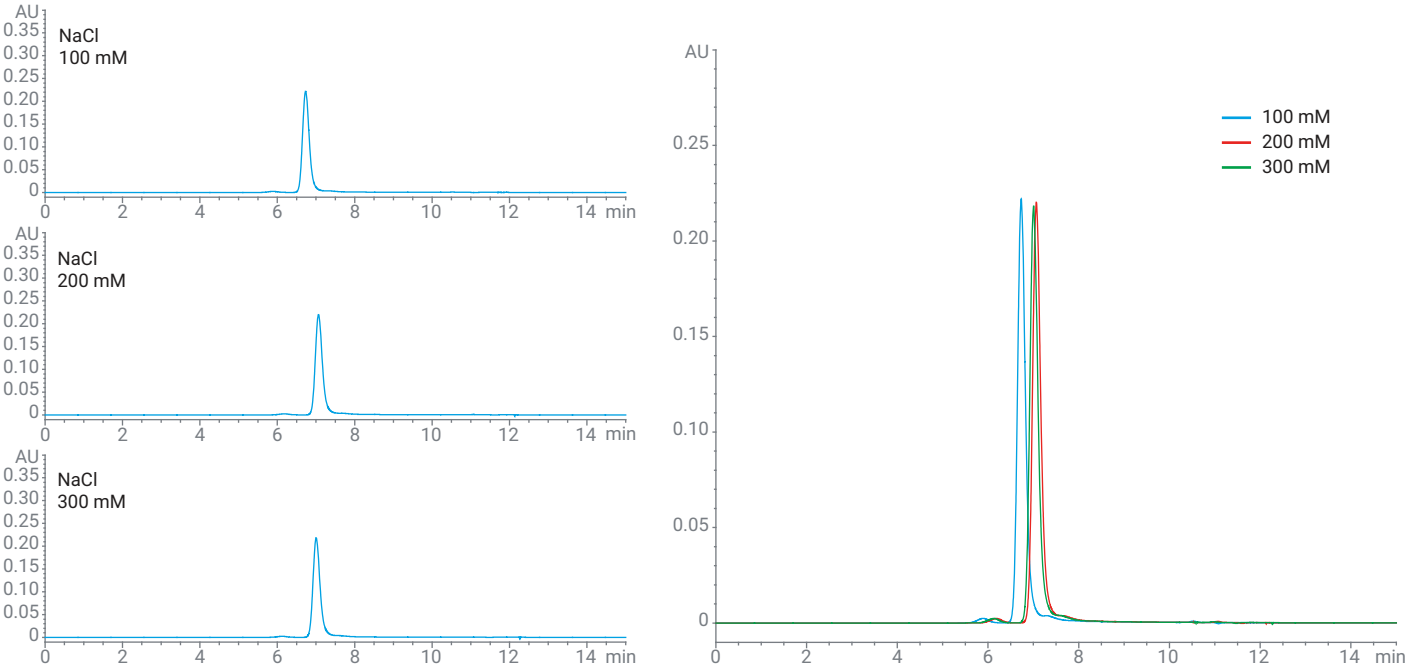

Figure 7. SEC of brentuximab vedotin using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column with PBS, pH 7.4 at 0.35 mL/min, showing the effect of NaCl concentration.

Table 7. Effect of NaCl concentration on chromatography for brentuximab vedotin on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

NaCl Concentration	Monomer %	Aggregate %	Resolution
100 mM	98.3	1.7	2.15
200 mM	98.2	1.8	2.15
300 mM	98.2	1.8	2.15

Conclusion

The choice of size exclusion stationary phase can have a marked impact on the efficacy for aggregate analysis of more challenging molecules such as ADCs. Choosing a sophisticated stationary phase with optimized hydrophilic polymer coating helps to overcome nonspecific interactions. Avoiding nonspecific interactions can lead to improved peak shape and resolution in the absence of organic modifiers in the mobile phase.

In addition, mobile phases having different pH values may have an important effect on the peak resolution, as shown with the Agilent AdvanceBio SEC 300 Å, 2.7 µm column. Moreover, it was shown that the concentration of NaCl does not particularly affect the separation, even when using 300 mM of salt.

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