Application Note

Pharma and Biopharma

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Altura Size Exclusion 300 Å Allows Significant Gains in Performance at Low Salt Concentrations

Authors

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Abstract

Nonspecific adsorption of biomolecules to charged surfaces leads to significant peak tailing. Size exclusion chromatography is often employed under physiological conditions (150 mM NaCl, pH 7.2)[1], which exacerbates nonspecific adsorption, leading to loss of sensitivity and resolution. Metal binding constitutes a major source of nonspecific adsorption; as a result, conventional stainless steel columns have become limiting in the high-performance analysis of biotherapeutics. Replacing stainless steel components with Ultra Inert surfaces can significantly improve separation performance at low salt concentration, improving the applicability of SEC with downstream workflows.

Introduction

Size exclusion chromatography is the gold standard approach for monitoring biotherapeutic size, including protein aggregation, confirmational change, and polymerization. Biomolecules travel through the column, with smaller molecules penetrating further into the particle pore volume and being retained longer. However, as biomolecules contain numerous reactive sites with differing pKa values, it is common for SEC to suffer from significant nonspecific interactions with charged surfaces, most commonly, exposed silanol groups as well as the metal surface of the column housing.[2] Buffer systems high in salt are often necessary to minimize nonspecific adsorption and improve separation performance.

Herein, we report the significant benefits of the new Agilent Altura SEC 300 Å column in the separation of small proteins, large monoclonal antibodies (mAbs), and antibody–drug conjugates (ADCs). Altura HPLC columns feature Ultra Inert technology that prevents metal binding, minimizing peak tailing in comparison to conventional stainless steel column casings, particularly under lower-salt mobile phase conditions. Peak tailing was reduced by over two-fold for small proteins such as lysozyme at salt concentrations as low as 25 mM, as well as mAbs such as rituximab and the antibody drug conjugate sacituzumab govitecan.

Experimental

Reagents and chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). All buffer salts were HPLC grade, proteins were purchased lyophilized from Sigma-Aldrich, all mAbs and the antibody drug conjugate sacituzumab govitecan were purchased from Evidentic. The gel filtration standard (p/n 1511901) was purchased from Biorad.

Sample preparation

All samples were diluted to 1 mg/mL with 50 mM phosphate buffer, pH 7.0.

Instrumentation

- Agilent Infinity III 1260 Multiple wavelength detector (p/n G7165A)
- Agilent Infinity III 1260 Multicolumn thermostat (p/n G7116A)
- Agilent Infinity III 1260 Bio multisampler (p/n G5668A)
- Agilent Infinity III 1260 Bio-inert pump (p/n G5654A)

Software and data processing

- Agilent OpenLab CDS Acquisition version 2.8
- Agilent OpenLab CDS Analysis version 2.8

Method conditions

Table 1. Method conditions.

Parameter	Value
Column	– Agilent AdvanceBio SEC 300Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5301) – Agilent Altura SEC 300Å, 4.6 × 300 mm, 2.7 µm with Ultra Inert technology (p/n PL1580-5301A)
Mobile Phase	Eluent A) 50 mM phosphate buffer, pH 7.0 Eluent B) 50 mM phosphate buffer, pH 7.0 + 500 mM NaCl
Flow Rate	0.35 mL/min
A:B*	Eluent conditions used to achieve set NaCl concentrations Eluent A % / Eluent B % / NaCl concentration (mM) 95 / 5 / 25 90 / 10 / 50 80 / 20 / 100 50 / 50 / 250 20 / 80 / 400 0 / 100 / 500
Column Temperature	25 °C
Injection Volume	1 µL
Total Run Time	20 min
UV	220 nm
Response Time	10 Hz

* Salt conditions were varied by mixing eluent A and B; the gel filtration standard sample was run using 150 mM phosphate buffer, pH 7.0.

Table 2. pI values of analyte proteins.

Analyte	pI
α-Chymotrypsinogen	9.1
Lysozyme	11.4
Myoglobin	7
Ovalbumin	5.1
Sacituzumab govitecan	8.5
Infliximab	7.6
Rituximab	9.4
Eculizumab	6.1

Results and discussion

Altura coating minimizes peak tailing under low salt conditions

To measure the gain in performance due solely to the Altura Ultra Inert technology, the same lot of SEC material was packed into either stainless steel or Altura hardware.

Table 3 and Figure 1 demonstrate the change in peak tailing for a set of representative proteins, for proteins with a pI value above 7 there is at least a two-fold increase in symmetry at the lowest salt conditions investigated. Representative chromatograms of lysozyme and sacituzumab govitecan are shown in Figures 2 and 3, showing a general gain in peak symmetry at salt concentrations below 250 mM with the Altura SEC column; as the salt concentration is increased to 500 mM, the metal binding present in the stainless steel is largely mitigated by the high salt, and both columns give similar performance. However, we observed a general shift to later elution times with increasing salt, indicating induced hydrophobic interactions. Higher-salt mobile phases also risk more wear and tear on the LC system, particularly if using non-bio LCs.

Table 3. Tailing factor versus salt concentration for representative proteins. N/A denotes extremely high tailing.

Sample	Na (mM)	Peak Tailing SS	Peak Tailing Altura	Symmetry gains with Altura
α-Chymotrypsinogen	25	2.93	1.02	2.87
α-Chymotrypsinogen	50	1.62	0.95	1.70
α-Chymotrypsinogen	100	1.05	0.90	1.18
α-Chymotrypsinogen	250	0.85	0.82	1.03
α-Chymotrypsinogen	400	0.80	0.78	1.02
α-Chymotrypsinogen	500	0.77	0.75	1.02
Ovalbumin	25	0.98	0.98	1.02
Ovalbumin	50	0.97	0.96	1.02
Ovalbumin	100	0.95	0.94	1.02
Ovalbumin	250	0.92	0.91	1.02
Ovalbumin	400	0.92	0.90	1.02
Ovalbumin	500	0.92	0.90	1.02
Lysozyme	25	9.25	4.46	2.07
Lysozyme	50	5.45	2.23	2.45
Lysozyme	100	2.49	1.41	1.76
Lysozyme	250	1.15	1.04	1.11
Lysozyme	400	1.06	1.01	1.04
Lysozyme	500	1.05	1.01	1.04
Sacituzumab govitecan	25	N/A	6.15	N/A
Sacituzumab govitecan	50	N/A	5.96	N/A
Sacituzumab govitecan	100	13.70	1.90	7.22
Sacituzumab govitecan	250	1.54	1.23	1.25
Sacituzumab govitecan	400	1.31	1.19	1.10
Sacituzumab govitecan	500	1.27	1.18	1.08
Rituximab	25	NA	1.98	N/A
Rituximab	50	4.56	1.28	3.57
Rituximab	100	1.25	1.09	1.15
Rituximab	250	1.08	1.07	1.01
Rituximab	400	1.07	1.06	1.01
Rituximab	500	1.07	1.06	1.01
Eculizumab	25	1.08	1.07	1.01
Eculizumab	50	1.08	1.07	1.01
Eculizumab	100	1.08	1.06	1.01
Eculizumab	250	1.07	1.06	1.01
Eculizumab	400	1.08	1.06	1.01
Eculizumab	500	1.08	1.06	1.01

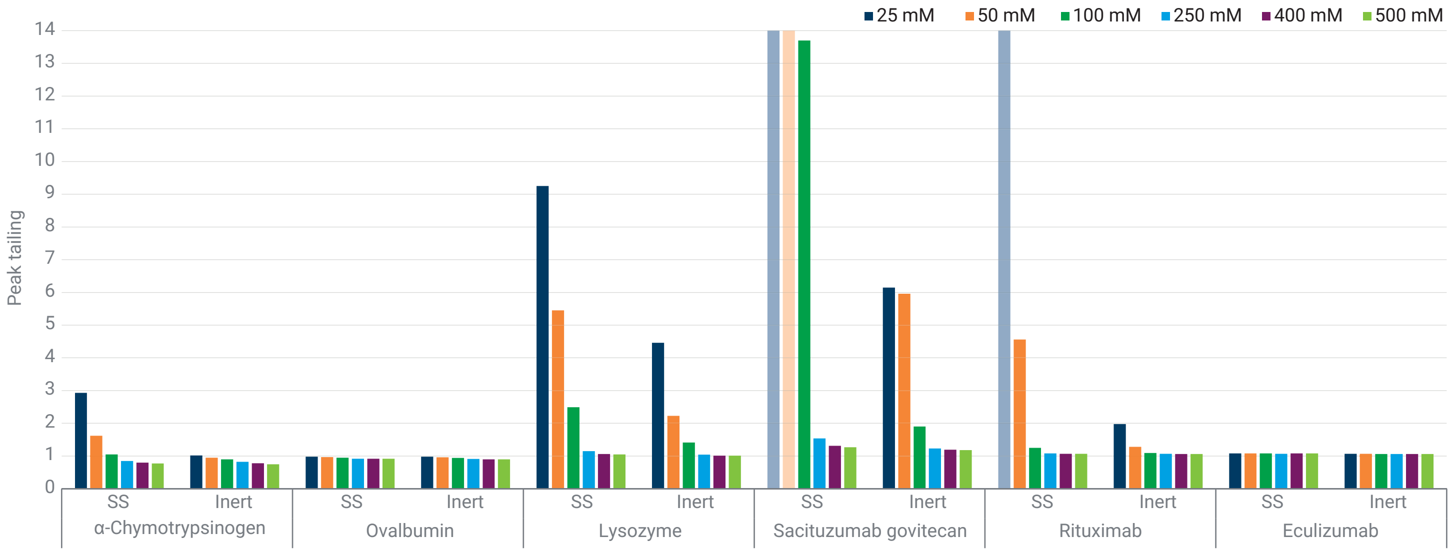

Figure 1. Global impact of NaCl concentration on peak tailing of proteins, mAbs, and ADCs outlined in Table 1. Shaded bars represent extremely high tailing off scale of other conditions.

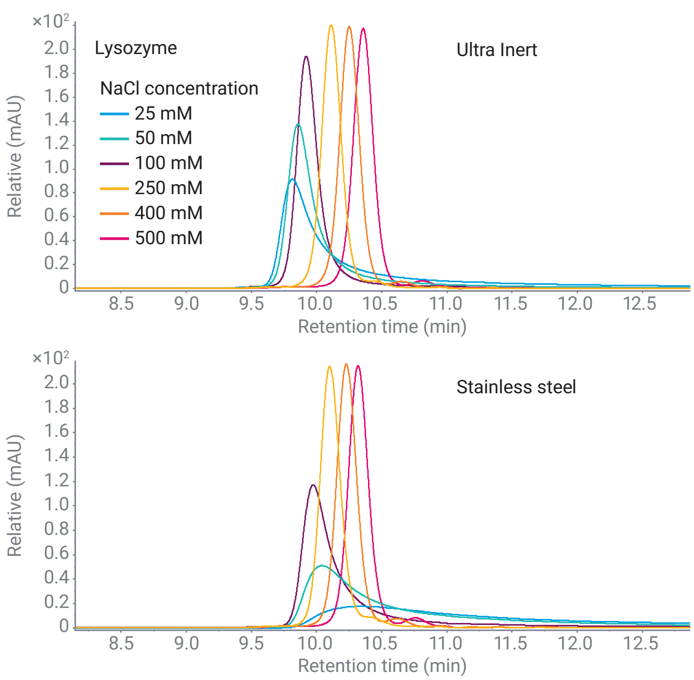

Figure 2. Representative chromatograms obtained for lysozyme using Ultra Inert columns versus stainless steel columns at varying NaCl concentrations added to 50 mM phosphate buffer.

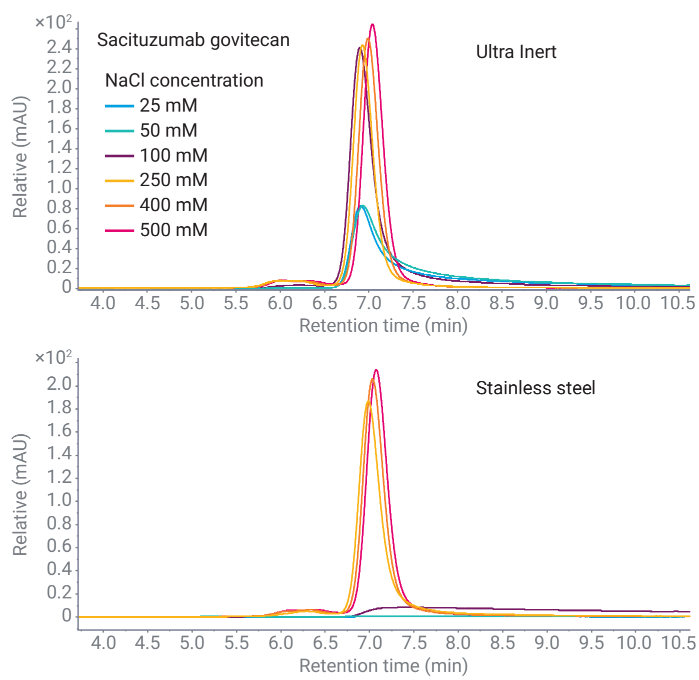

Figure 3. Representative chromatograms obtained for the ADC sacituzumab govitecan using Ultra Inert columns versus stainless steel columns at varying NaCl concentrations added to 50 mM phosphate buffer.

Figure 4 highlights how the Ultra Inert hardware impacts chromatograms at low-salt concentrations. Globular proteins such as lysozyme and A-CT suffer from tailing with the stainless steel column at 25 mM NaCl, which is largely attenuated by the Altura Ultra Inert technology. Critically, mAbs such as rituximab and the ADC sacituzumab govitecan show either extremely low signal or no signal at all at 25 mM when using stainless steel, while peaks can be seen (albeit with some tailing) on the Altura hardware. These data suggest that the Altura Ultra Inert technology allows for a significant reduction in salt concentrations when running SEC of metal-sensitive biomolecules.

While proteins with an overall positive surface charge benefit dramatically from Ultra Inert column hardware, proteins and mAbs with a more acidic surface charge are far less sensitive to nonspecific adsorption. For example, eculizumab with a pI of 6.1 and ovalbumin with a pI of 5.1 did not benefit from either the Ultra Inert hardware or increasing salt concentrations. Finally, an interesting observation was the apparent peak fronting that occurred at the highest salt concentrations for α-chymotrypsinogen (Figure 5), demonstrating the need to optimize SEC conditions for individual proteins, which the Altura hardware can provide by reducing the dependency on salt to eliminate metal binding.

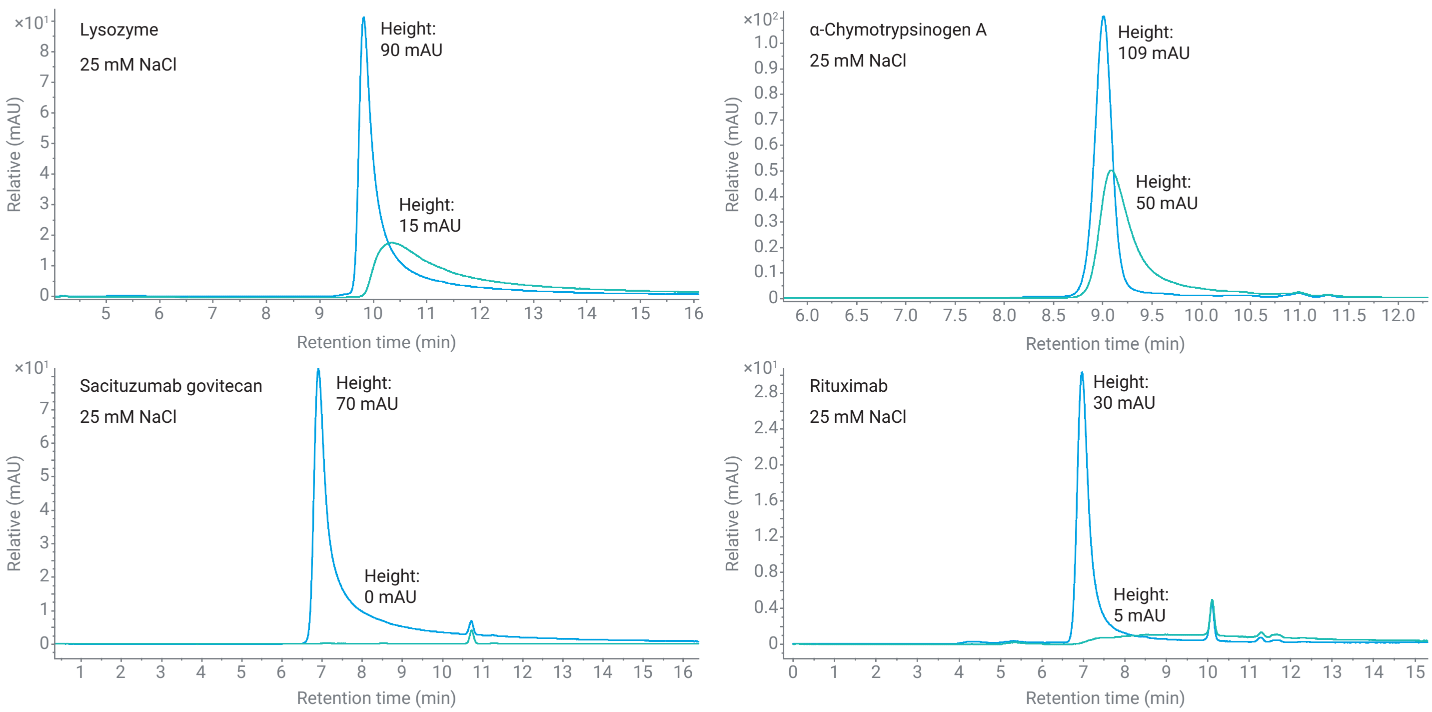

Figure 4. Representative chromatograms obtained from Ultra Inert versus stainless steel columns at low salt concentrations (50 mM phosphate buffer + 25 mM NaCl).

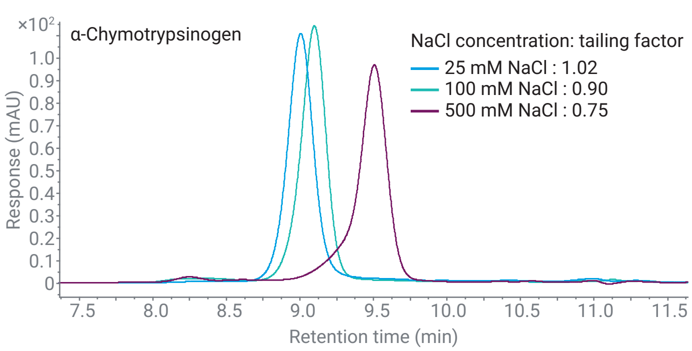

Figure 5. α-Chymotrypsinogen peak tailing factor versus NaCl concentration on an Agilent Altura SEC column.

Altura coating enables high-sensitivity quantification of protein aggregation

Protein aggregation into dimers and trimers is a critical quality attribute (CQA) uniquely probed by SEC; the ICH stipulates that the total high molecular weight (HMW) and low molecular weight (LMW) peak areas must be quantified and rationalized.[3] It is therefore necessary that aggregate quantitation is accurate and highly sensitive.

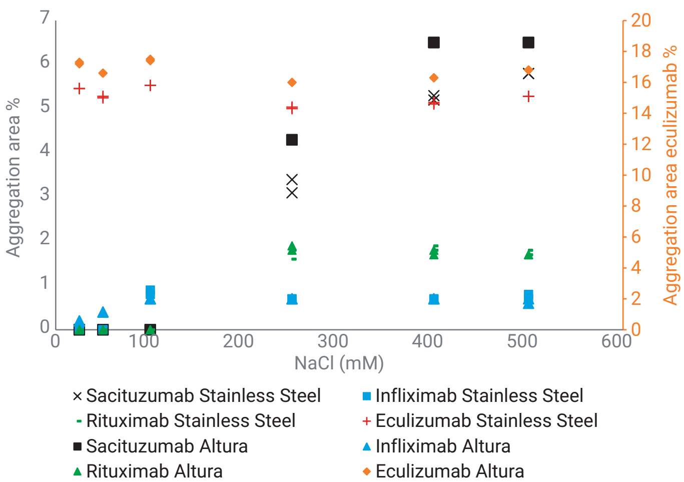

Figure 6. Protein aggregation versus salt concentration for mAbs and ADCs.

Figure 6 highlights that for all mAbs and ADCs evaluated in this study, the Altura columns had a higher sensitivity for the degree of protein aggregation than the stainless steel columns, providing a more accurate measure of aggregation. Figure 7 shows a representative chromatogram for the ADC sacitzumab govetican, wherein the Altura column provides a more accurate quantification of HMW aggregation than the stainless steel column. Interestingly, the gain in peak shape for the aggregates allows observation of dimer as well as larger multimer peaks, while the stainless steel column cannot discriminate between the two; the resolution between the main peak and the aggregate peaks with the Ultra Inert column was 1.41 while the stainless steel column was 1.33, showing improved separation power. Additionally, the mAb rituximab, which also has a high pI value, gained resolution with the Ultra Inert column showing 1.72, while the stainless steel provided 1.58 at 250 mM NaCl. Conversely, eculizumab did not benefit significantly. To reduce the amount of aggregation observed, it is possible to operate at lower salt conditions, which Altura Ultra Inert technology facilitates.

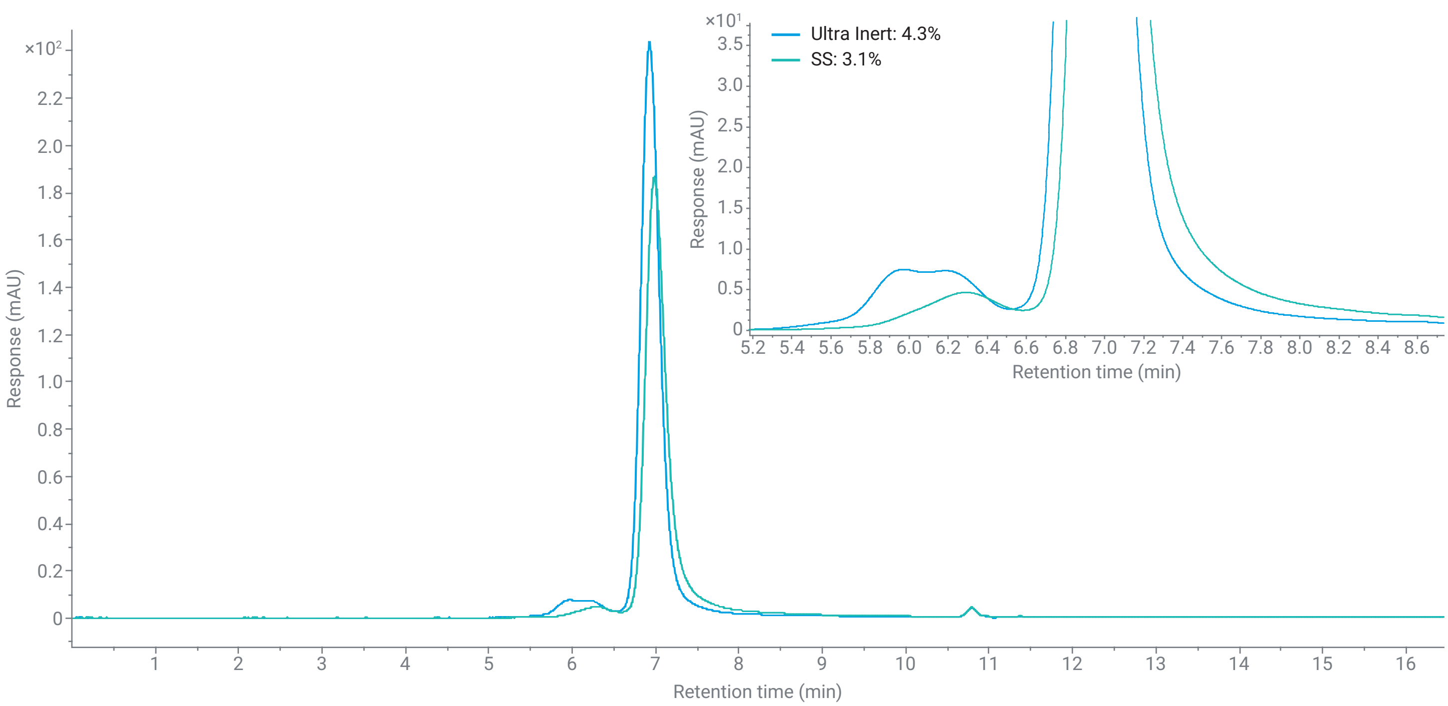

Figure 7. Representative chromatogram of sacituzumab govitecan, wherein the Agilent Altura column shows over 1% more protein aggregation than the stainless steel. The separation was performed at 250 mM NaCl. Ultra Inert TF: 1.23, Rs: 1.4; stainless steel TF: 1.54, Rs: 1.33.

Conclusion

While SEC is a critical mode of LC for the evaluation of size-based CQAs, the deleterious impact of nonspecific adsorption and the large salt concentrations necessary to ensure separation performance limit the applicability of the technique.

This application note highlights that Agilent Altura Ultra Inert technology provides a significant attenuation of nonspecific metal binding, resulting in reduced peak widths and higher sensitivity at lower salt concentrations than comparative stainless steel hardware. We have demonstrated that most proteins experience an optimal range of salt concentrations; too low results in deleterious adsorption, while too high can incur hydrophobic interactions that reduce performance and induce additional aggregation. Subsequently, the Altura hardware will allow for further versatility in method development by increasing the effective range of salt conditions that can be employed. Effective mitigation of metal–analyte interactions supports a more robust method for platform use across a variety of samples, with less need for sample rework.

References

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