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Improving SEC-MALS Data Quality with Ethylene Bridged Hybrid HPLC Size-Exclusion Columns

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APPLICATION BENEFITS

- Substantially improved performance of a Waters™ XBridge™ Protein BEH SEC Column versus an industry standard silica-based SE-HPLC column in an SEC-MALS experiment
- Significantly reduced column conditioning requirements prior to use in SEC-MALS experiments
- Improved resolution of monoclonal antibody fragments in an SE-HPLC separation

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class System

Waters XBridge Protein BEH SEC Column

Waters BEH200 SEC Protein Standard Mix

KEYWORDS

Size-exclusion, SEC-MALS, HPLC, proteins, IgG, adalimumab (Humira®)

INTRODUCTION

The assessment of protein aggregation is an essential part of any testing plan for recombinant protein based biotherapeutic products. Protein aggregation or self-association can impact both the efficacy and the safety profile of these therapies. Specifically, the primary safety concern is the increased risk of an immunogenic response due to the presence of multivalent epitopes.[1] Size-exclusion high-performance liguid chromatography (SE-HPLC) is most commonly relied on for monitoring protein aggregation in drug substance and drug product samples. SEC using a UV absorbance detector provides reproducible results for these analyses that can be relied upon once the veracity of the results are demonstrated with complementary analyses such as analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). Additionally, the online molecular weight characterization of peaks observed in SEC can be facilitated through the use of multi-angle light scattering detectors (SEC-MALS).[2] SEC-MALS has also been reported as a common method in biosimilarity assessments,[3] and in the analysis of heparin molecular weights as a qualified method.[4]

The goal of this application was to evaluate and compare the performance of an industry standard diol-bonded silica-based SE-HPLC column to a diol-bonded ethylene bridge hybrid (BEH) organo-silica based column. The quality of data obtained, and ultimately the accuracy of molecular weight assignments, from an SEC-MALS experiment is greatly impacted by both particulates within the sample, the mobile phase and particulates that may originate from the column itself. These particulates or fines may either be "released" as a bolus from the column during a pressure transient that occurs during the injection of the sample or gradually shed from the column throughout the course of the separation. In the former case, a large light scattering peak may be observed very early in the chromatogram while in the latter case the baseline noise of the light scattering channels throughout the chromatogram may have higher noise levels.

EXPERIMENTAL

Sample description

BEH200 SEC Protein Standard Mix was reconstituted in 500 µL of SEC mobile phase to yield the following:

Analyte	pI	MW
Thyroglobulin, 3 mg/mL	4.6	660,000
IgG, 2 mg/mL	6.7	150,000
BSA, 5 mg/mL	4.6	66,400
Myoglobin, 2 mg/mL	6.8, 7.2	17,000
Uracil, 0.1 mg/mL	N/A	112

A mAb sample of adalimumab (Humira) was used past expiry at a diluted concentration of 1.0 mg/mL.

Method conditions (unless noted otherwise):

LC conditions

System:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 nm
Columns:	XBridge Protein BEH SEC, 200Å, 3.5 µm, 7.8 × 300 mm (p/n: 186003596) and BEH200 Protein Standard Mix, (p/n: 186006518); Diol-bonded silica-based SEC, 250Å, 5 µm, 7.8 × 300 mm
Column temp.:	25 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	1.0 mL/min
Mobile phase:	Fisher phosphate buffered saline prepared 2x to a final concentration of 20 mM phosphate, 5.4 mM KCl, and 274 mM NaCl, pH 7.4, 0.2 µm sterile filtered
Sample vials:	Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL volume (p/n: 186002640)
MALS detector:	Wyatt miniDAWN TREOS

Data management

Chromatography software:	Empower™ 3
MALS software:	Wyatt Astra 7

RESULTS AND DISCUSSION

The extent of column conditioning required prior to performing an SEC-MALS experiment was evaluated for each of the columns using the same mobile phase and on the same LC-MALS system. Each column was installed and washed at 1 mL/minute for one hour with mobile phase. The system back pressure for silica-based SEC column was 193 bar (2800 psi) with a pressure drop across the column of approximately 41 bar (600 psi) and for the XBridge SEC Column the pressures were 228 bar (3300 psi) and 76 bar (1100 psi). Following conditioning, a series of four 10 µL injections of the mobile phase was performed, followed by analysis of the protein standard mix and the adalimumab sample. Following the protein samples, three additional mobile phase blanks were injected in order to re-evaluate the baseline noise. A comparison of the entire baseline for Channel 1 of the miniDAWN TREOS (43.6° scattering angle) for the two columns is shown in Figure 1. Channel 1 was selected for these comparisons as it exhibits the highest noise. These results show that the peak induced by the pressure transient during the injection of the sample is significantly greater for the silica-based SEC column and while it is observed to gradually decrease upon subsequent injections it is still present at a height of ~4 mV for the fourth blank. In comparison, the initial blank run with the XBridge BEH SEC Column is observed with a peak height of ~0.1 mV which is reduced to ~0.02 mV upon the fourth blank injection.

A comparison of the third blank injection chromatograms produced by the two columns after sample analysis is also shown in Figure 1. For the silica-based SEC column the pressure transient induced peak is still detected at a height of ~1 mV while this peak is no longer observed for the XBridge BEH SEC Column.

In addition to the pressure transient induced baseline disturbance, another SEC-MALS method suitability concern is the noise level of the baseline. This measurement was determined for the first two minutes of the fourth blank analysis (Figure 2). In this comparison the noise level of the baseline observed for the silica-based SEC column was approximately four times greater than that of the XBridge BEH SEC Column. This decrease in baseline noise increases the sensitivity of the SEC-MALS data for lower molecular weight proteins and for lower abundance proteins and protein aggregates. This is demonstrated in the analysis of myoglobin (17 kDa) in the standard protein mix (Figure 3) and in the analysis of the high molecular weight species (HMWS) of adalimumab (Figure 4).

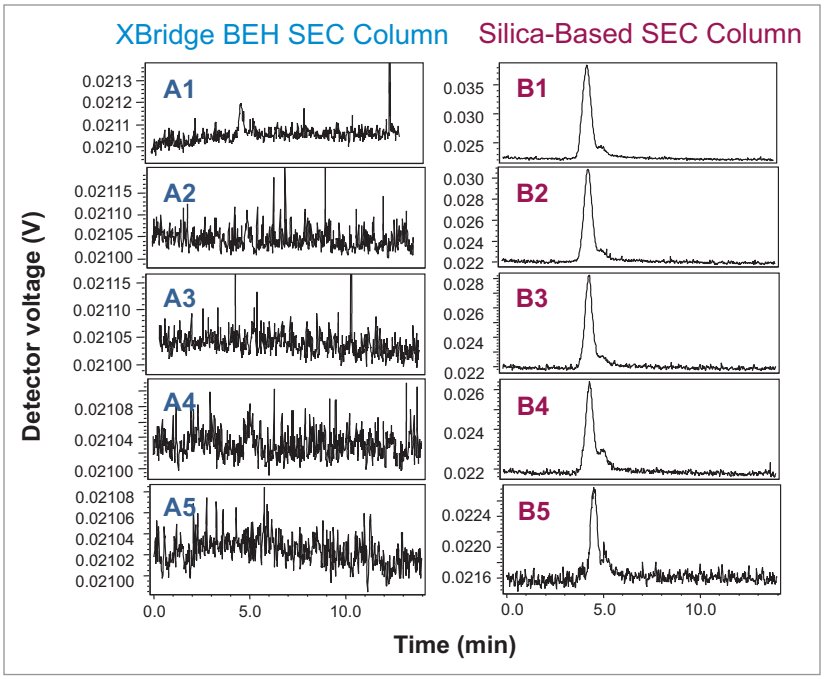

Figure 1. SEC-MALS baseline noise profiles for Channel 1 (43.6°) of a Wyatt miniDAWN TREOS MALS detector for the columns evaluated. Profiles A1 through A4 and B1 through B4 represent the first four blank injections on a new column after one hour of conditioning. Profiles A5 and B5 represent the third blank after a series of six samples.

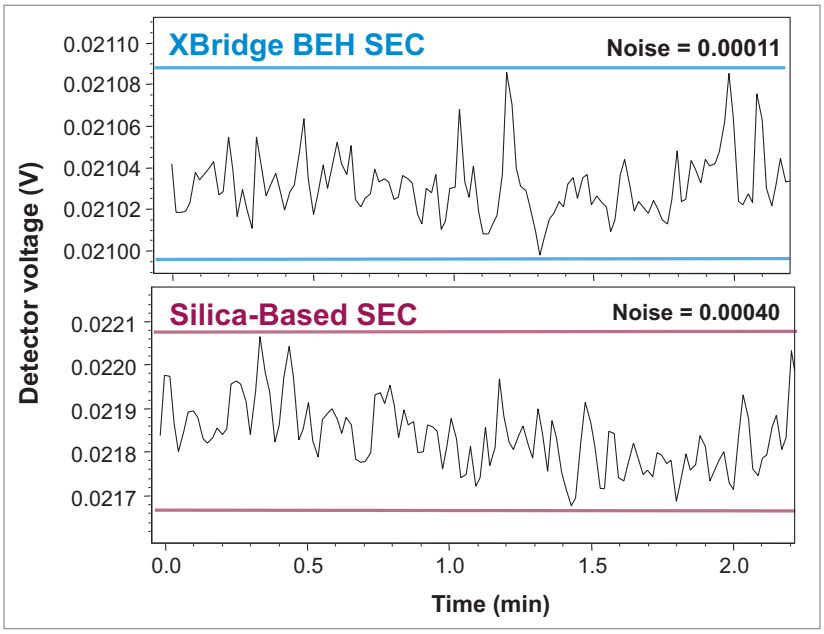

Figure 2. Zoomed-in view of the SEC-MALS baseline noise profiles for Channel 1 (43.6°) of a Wyatt miniDAWN TREOS MALS detector from the fourth blank run on a previously unused column.

Comparing the chromatograms obtained for the standard protein mix (Figure 3) shows several differences in both the overall chromatographic separation quality and in the MALS signal. Under the conditions tested and using uracil as a low molecular weight marker (Peak E) the calculated USP plate count was 45,000 for the XBridge BEH SEC Column and 32,000 for the silica-based SEC column. This 41% improvement is consistent with the 43% increase predicted based on 3.5 µm and 5 µm particle sizes of the respective columns. Chromatographically the XBridge BEH SEC Column provides greater resolution (Rs = 2.62) between the IgG monomer (Peak B2) and BSA (Peak C) peaks than what is observed for the silica-based SEC column (Rs = 1.89). Additionally, comparisons of the peak-to-valley (P/V) measurements between the IgG HMW (Peak B1) and the IgG monomer (Peak B2) are also modestly improved for the XBridge BEH SEC Column (P/V = 1.99) versus the silica-based SEC column (P/V = 1.84). However, the P/V measurements between the IgG HMW (Peak B1, ~300 kDa) and the thyroglobulin monomer (Peak A2, ~660 kDa) were equivalent for the XBridge BEH SEC Column (P/V = 1.20) and silica-based SEC column (P/V = 1.19). This is the result of the 250Å pore size of the silica-based SEC column being more optimal for proteins in this size range than the 200Å pore size of the XBridge BEH SEC Column. In comparing the MALS data of the standard protein mix for the two columns, we will compare the signal-to-noise (S/N) measurements for the lowest molecular weight protein in the standard mix, myoglobin (17 kDa) which produces that lowest MALS signal intensity per unit mass. The lower baseline noise levels observed for the XBridge BEH SEC Column directly translate into improved S/N levels for this low molecular weight protein (S/N = 25) in contrast to the silica-based SEC column (S/N = 9). This improved S/N may provide more accurate and reproducible molecular weight assignments.

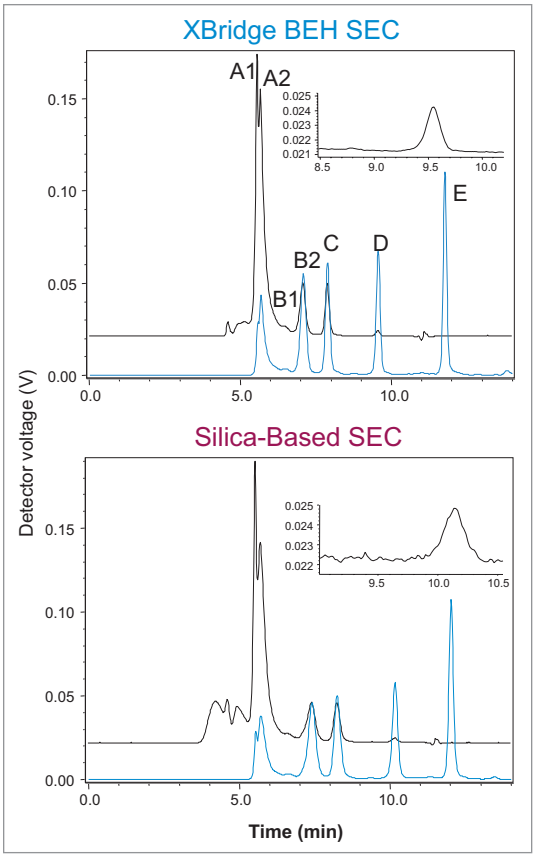

Figure 3. SEC-MALS UV (280 nm, blue) and Channel 1 (43.6°, black) MALS chromatograms for the protein standard mix tested on both columns. The peak identities are: (A1) thyroglobulin dimer 1.32 kDa, (A2) thyroglobulin 660 kDa, (B1) IgG dimer 300 kDa, (B2) IgG 150 kDa, (C) BSA 67 kDa, (D) myoglobin 17 kDa, (E) uracil 112 kDa. Shown in the insert are zoomed views of the MALS myoglobin peak.

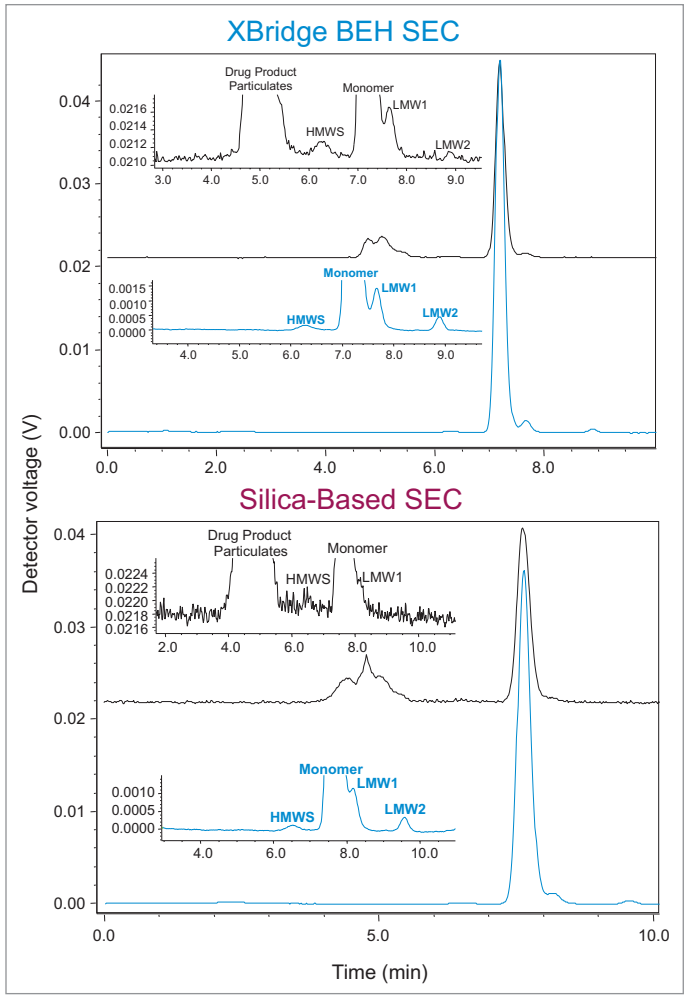

Figure 4. SEC-MALS UV (280 nm, blue) and Channel 1 (43.6°, black) MALS chromatograms for the adalimumab at 1 mg/mL. The peak identities are described in the text. Shown in the insert are zoomed views of the MALS channel.

In a final comparison we will evaluate the chromatograms and MALS data observed for a 1 mg/mL sample of monoclonal antibody (mAb) adalimumab (Humira) shown in Figure 4. Chromatographically the XBridge provides significantly greater resolution of the LMW1 species. This fragment is the result of the hydrolytic cleavage of one of the antigen binding fragment (FAb) arms of the mAb to yield a fragment with a molecular weight of ~100 kDa that consists of one FAb and Fc fragment. The P/V ratio measured for the LMW1 species was nearly two times greater for the XBridge BEH SEC Column (P/V = 2.04) versus the silica-based SEC column where this peak was marginally separated (P/V = 1.11). For the HMW species observed, the USP resolution observed for the XBridge BEH SEC Column (2.14) was slightly higher than that observed for the silica-based SEC column (2.05). Noted in the MALS data for both columns is a large peak eluting before the HMW peak. This peak is not observed in the UV trace and is assumed to be predominantly silicone oil and other protein and non-protein related particles in the drug product.[5] In comparing the MALS data, it is visually clear that the S/N for this separation is superior on the XBridge BEH SEC Column (S/N ~3) in comparison to the silica-based SEC column when we focus on the low abundance (~0.6%) HMW species. This low level signal allowed for the reasonable average molecular weight assignment of this peak as a mAb dimer (Figure 5) at 270 kDa ±5% while the average molecular weight for the monomer was determined as 140 kDa ±0.4%. Molecular weight assignments were made based on a reported UV extinction coefficient of 1.39 mg mL^{-1} cm^{-1} (US Patent 20070292442 A1) and a dn/dc of 0.1815 mL $gm.^{-1}$ A molecular weight assignment could not be made for the HMW peak with the silica-based SEC column experiment.

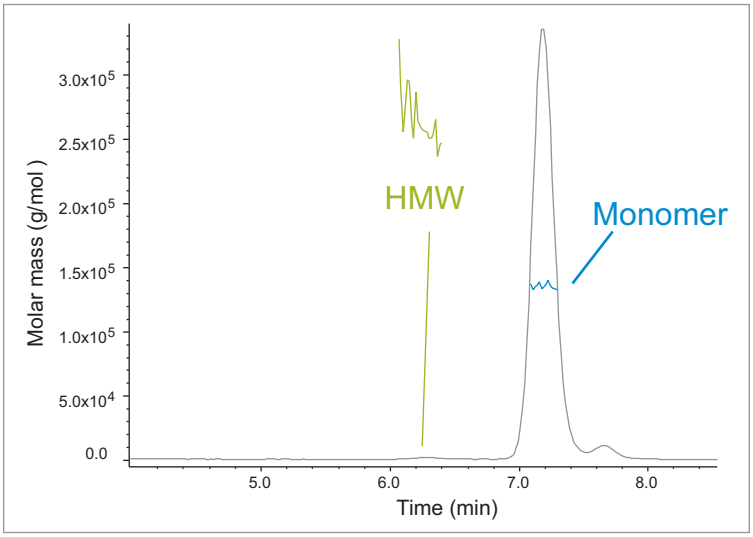

Figure 5. Molecular weight assignments for the HMW (green) and monomer peaks (blue) observed on the XBridge BEH SEC Column for the adalimumab at 1 mg/mL. The UV profile (280 nm) is shown in gray.

CONCLUSIONS

The HPLC compatible XBridge Protein BEH SEC Column with a pore size of 200Å and a particle size of 3.5 µm offers significant improvements in both the quality of MALS data and chromatographic resolution and that can be obtained during the SEC-MALS analysis of small proteins and mAbs in comparison to a standard silica-based SEC column with a pore size of 250Å and a 5 µm particle size. The BEH SEC column was observed to be conditioned much more rapidly; and following the conditioning, the level of both continuous and sample-injection pressure pulse related baseline noise was dramatically lower for the XBridge BEH SEC Column. These performance attributes can be beneficial allowing for the molecular weight determination of low abundance protein species and in the reliability of SEC-MALS data for protein species that elute near the baseline disturbance. The higher efficiency of the XBridge BEH SEC Column also afforded significant improvements in chromatographic resolution of protein species up to a molecular weight of approximately 660 kDa. The impact of this improved efficiency was readily observed in the profiles of the molecular weight standards BSA (66 kDa) and myoglobin (17 kDa). In addition, the XBridge BEH SEC Column provided greater resolution among the protein fragments, monomer, and dimer HMW species present in the mAb drug product sample.

References

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