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WP1618: Enhanced SEC-MALS analysis of therapeutic mAbs, biosimilars and AAVs with XBridge Premier columns

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Summary

SEC-MALS is widely used for the characterization of biopharmaceuticals. Coupling Wyatt's DAWN™ MALS and Optilab™ dRI detectors to Waters' Arc™ Premier HPLC system with XBridge™ Premier columns provides enhanced SEC-MALS performance. This application note demonstrates the benefits of XBridge Premier Protein and XBridge Premier GTx BEH SEC columns for multi-attribute quantitation of therapeutic antibodies, biosimilars and adeno-associated viruses (AAVs) by SEC-MALS.

Introduction

Size-exclusion chromatography coupled with multi-angle light scattering, UV, and differential refractive index detection (SEC-MALS) has long been used to quantify and characterize proteins and other biopharmaceuticals. SEC-MALS determines multiple product attributes—including absolute molar mass, aggregation, and conjugation or payload—in a single, robust assay. It is reagent-free, automatable, and can be incorporated into QA/QC environments.

Successful SEC-MALS analysis begins with successful chromatography, but finding a suitable column and optimizing a chromatographic separation method are time-consuming. XBridge Premier columns are suitable as a platform column technology that can be deployed quickly without extensive method development. These columns, combined with MALS, are found to offer significant improvements over silica-bead based columns for robust and sensitive multi-attribute quantitation of proteins and AAVs.

Materials and Methods

Instrumentation

SEC was performed using a Waters Arc Premier HPLC with 2998 PDA UV detector, DAWN MALS detector with embedded WyattQELS dynamic light scattering module and Optilab dRI detector. UV data were collected at both 280 nm and 260 nm. MALS, UV, and dRI data were collected and analyzed using ASTRA™ software.

Figure 1. A SEC-MALS system comprises HPLC modules (pump, autosampler, and UV detector) with Wyatt's MALS and dRI instruments.

Columns

XBridge Premier columns combine two novel technologies, Waters MaxPeak™ Premier high-performance surfaces, and 2.5 µm BEH-PEO particles. They are compared against traditional 5 µm silica-bead columns that are well-qualified for SEC-MALS.

Proteins

For protein applications, separation was performed using an XBridge Premier Protein SEC column, 250 Å, 7.8 mm x 300 mm, or a Wyatt Technology silica-bead SEC column, 300 Å, 7.8 mm x 300 mm. The mobile phase was Dulbecco's PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 3.1 mM NaN_3 pH 6.7). The flow rate was 0.5 mL/min. Injections consisted of

- Herceptin (trastuzumab): 10 µL at 21 mg/mL;
- Kanjinti (trastuzumab biosimilar): 10 µL at 21 mg/mL;
- Pertactin: 50 µL at 0.5 mg/mL;
- UT antibody: 50 µL at 1.0 mg/mL.

Pertactin and UT antibody were kindly provided by the University of Texas at Austin.

AAV

Samples consisted of empty and full AAV8 samples. Separation was performed using an XBridge Premier GTx BEH SEC column 450 Å, 7.8 mm x 300 mm, or a Wyatt Technology AAV column, 500 Å, 4.6 mm x 300 mm. The mobile phase was prepared as described in Wyatt's AAV SOP Guidance Manual and run at a flow rate of 0.5 mL/min. Analysis was performed according to the AAV SOP Guidance Manual using the Viral Vector Analysis method in ASTRA.

Results and Discussion

Platform column technology minimizes LC method development

Non-ideal chromatography, arising from secondary (non-steric) interactions between the sample and the column, can cause changes in elution time relative to standards, peak tailing, and other detrimental effects. These LC artifacts often cause uncertainties in traditional analytical SEC analysis. Significant time and resources may be needed to optimize the method and improve the chromatography. As demonstrated herein, with very minimal optimization, the XBridge Premier Protein SEC column can greatly improve separation over generic silica-based SEC columns.

Pertactin is a highly immunogenic virulence factor of *Bordetella pertussis*, the bacterium that causes pertussis and is used to produce the acellular pertussis vaccine. As seen in Figure 2 (top), pertactin eluted as a split peak from the generic silica-based column due to undesirable secondary interactions. However, without changing the mobile phase, flow rate, or injection amounts, pertactin eluted from the XBridge column in a single, well-defined monomer peak (Figure 2, bottom). In both cases, MALS provides absolute quantitation of the molar mass, and additional MALS quantitation confirmed that the second peak was not caused by the on-column dissociation of dimers or any other co-eluting species.

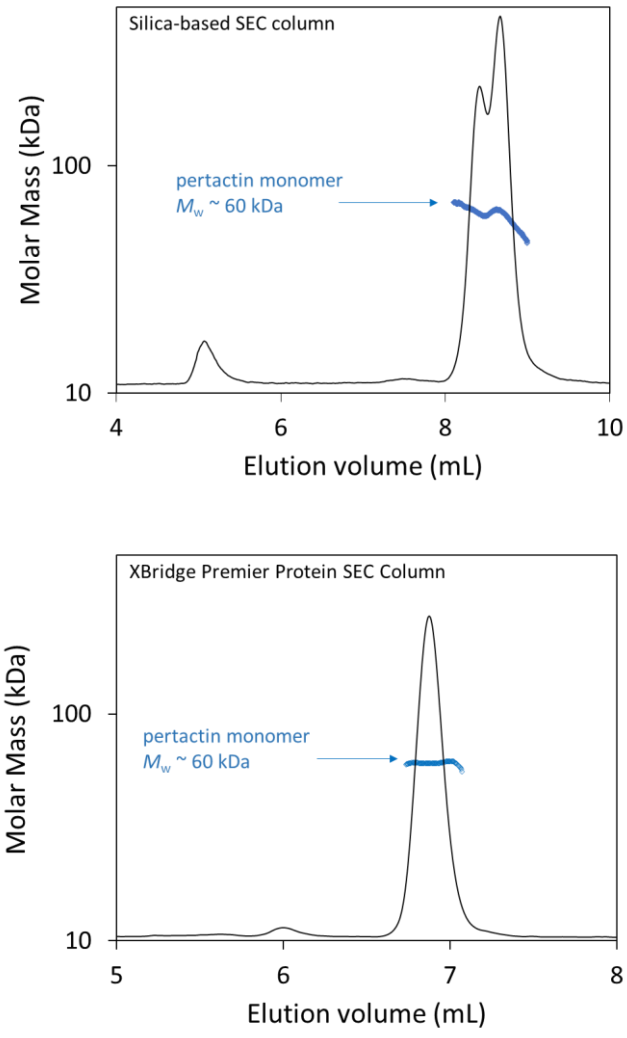

Figure 2. XBridge Premier column promotes an ideal separation. Pertactin eluted from a generic silica-based SEC column (top) and Waters XBridge Premier Protein SEC column (bottom). Molar mass determined by MALS is overlaid with the light scattering chromatograms.

Benefits of MALS for protein analysis

Although the XBridge Premier column provided well-formed peaks and high resolution, the pertactin monomer (60 kDa) unexpectedly eluted well before the BSA monomer (66 kDa), as seen in Figure 3. In order to understand if this apparent anomaly arises from different protein conformations, it is useful to examine DLS measurements provided by the DAWN's QELS module. The hydrodynamic radius R_h of the BSA monomer was found to be 3.5 nm, while the R_h of the monomer pertactin was 4.0 nm (Figure 3). This correspondence of R_h to elution order (larger sizes elute earlier) confirms that the separation occurred under ideal SEC conditions wherein molecules are separated solely by diffusion size, i.e., hydrodynamic radius. Notably, the difference in size between these two proteins does not correlate to their molar masses, highlighting the value of MALS for absolute molar mass determination.

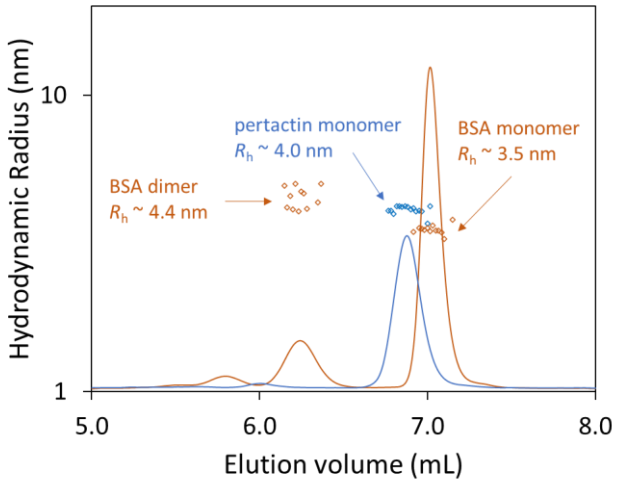

Figure 3. Hydrodynamic radius comparison between BSA and pertactin. R_h values (determined by the WyattQELS module) are overlaid with the light scattering chromatograms.

The pitfalls of traditional column calibration, wherein some samples undergo secondary interactions with the column, are further illustrated in Figure 4. SEC-MALS correctly measured the molar mass of the UT antibody monomer, BSA monomer, and BSA dimer (Figure 4, top). However, the elution order of the UT antibody monomer relative to the BSA dimer is unexpected.

Unlike the pertactin example, online DLS shows that, in this case, the separation is not purely due to differences in hydrodynamic size. As seen in Figure 4 (bottom), the UT antibody sample (R_h = 5.6 nm) is larger than the BSA dimer BSA (R_h = 4.4 nm). Therefore, the late elution of the UT antibody monomer relative to the BSA dimer is likely due to secondary interactions with the column.

Optimizing LC conditions to reduce secondary interactions is time-consuming and labor-intensive for many samples. MALS allows for confidently measuring the molar mass directly, regardless of elution time, while online DLS confirms whether elution order is ideal. Hence SEC-MALS eliminates the need for extensive LC condition optimization, saving time and resources.

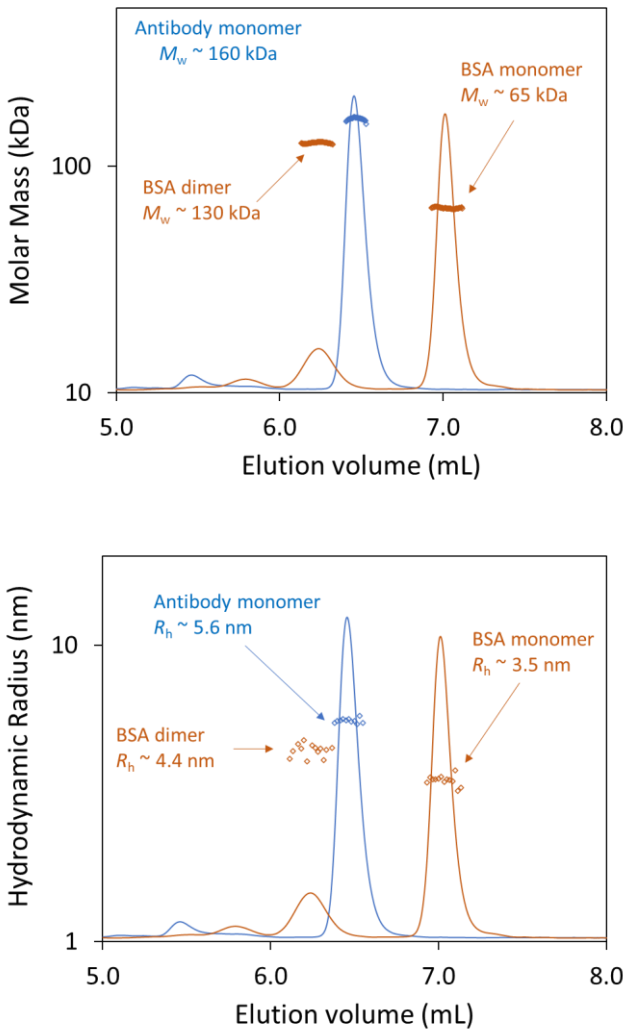

Figure 4. MALS enables accurate molar mass determination regardless of elution orders. Top; molar mass determined by MALS overlaid with light scattering chromatogram. Bottom; hydrodynamic radius determined by DLS overlaid with light scattering chromatogram.

SEC-MALS to assess biosimilarity

One barrier to entry for biosimilar antibody drugs is proving their biosimilarity compared to the innovator

drug. A biosimilarity study involves extensive *in vivo* testing, thus driving up the cost and preventing some life-saving drugs from reaching patients. Better *in vitro* biosimilar testing can offload some pressure from *in vivo* testing, thereby decreasing the time, cost, and resources needed to take biosimilar antibodies to market[1].

In this study, the innovator trastuzumab antibody drug Herceptin and its biosimilar Kanjinti were compared by SEC-MALS. As shown in Figure 5 (left), the molar masses measured for monomeric and dimeric antibodies are identical between Herceptin and Kanjinti, at ~160 kDa and ~300 kDa, respectively. Furthermore, both products contain 98% monomer and ~1% dimer by mass. As shown in Figure 5 (right), the XBridge Premier column resolved the monomer and low molecular weight (LMW) species. Both products contain ~1% LMW; however, the molar masses of these species are different, with the Kanjinti LMW species at 47 kDa and the Herceptin LMW species at 60 kDa.

Interestingly, light scattering data also reveal differences in high-molecular-weight (HMW) species. Since light scattering intensity is proportional to both concentration and molar mass, MALS is uniquely suited for detecting and quantitating protein aggregates, which may not be visible by UV or RI alone. In this case, Herceptin exhibits a unique HMW species not present in Kanjinti. This HMW species, with R_h of 11 nm and M_w of 1.4 MDa, constitutes just 0.1% of the total mass but is clearly identified by MALS.

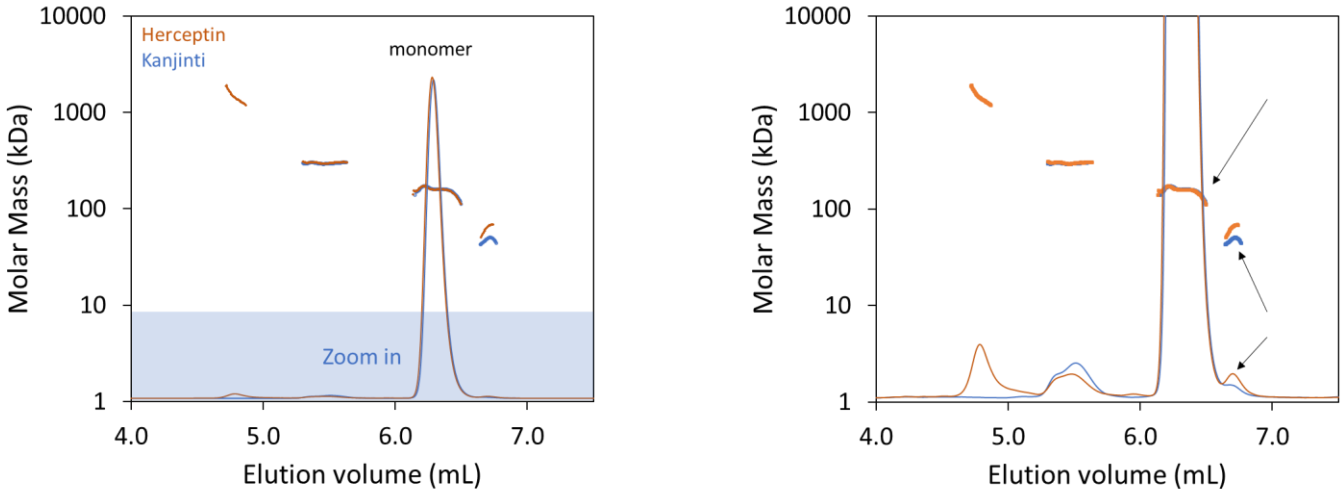

Figure 5. MALS enables detailed *in vitro* comparison of the biophysical similarity of mAbs. Left, LS chromatogram with molar mass overlay as measured by MALS. Right, LS chromatograms zoomed in.

Enhanced multi-attribute quantitation (MAQ™) of AAV with GTx gene therapy column and SEC-MALS

Adeno-associated virus (AAV) is a single-strand DNA virus that has become popular as a gene therapy delivery vehicle. Critical quality attributes (CQAs) of AAV products that are quantified by SEC-MALS include viral titer, full capsid ratio and aggregate content. SEC-MALS offers a robust and easy-to-deploy means for measuring these CQAs along with extended characterization, at under 30 minutes per sample[2,3].

Figure 6 shows chromatograms of full AAV samples from two batches, analyzed with SEC-UV-MALS-RI. By combining light scattering and differential refractive index data with UV absorption at 280 nm and 260 nm, we can determine the composition of the eluting peak in terms of the proteinaceous capsid and encapsidated DNA molar masses, and quantify the capsid titer and payload at each point across the chromatogram. Both full and empty AAVs eluted well from the XBridge Premier GTx column and were resolved from dimer and higher-order aggregates.

As shown in Figure 6, the SEC-MALS method measures a consistent molar mass for the capsid and the nucleic acid across the monomer peak. In addition to quantifying the molar masses of the capsid and DNA, ASTRA's Viral Vector Analysis simultaneously quantifies the total AAV concentration (Cp) and full-to-total ratio (Vg/Cp). These

CQAs are summarized in Table 1. Although both full AAV samples have a similar capsid content (Vg/Cp ~ 1), the total concentration of Full Batch #1 is seen to be half that of Batch #2.

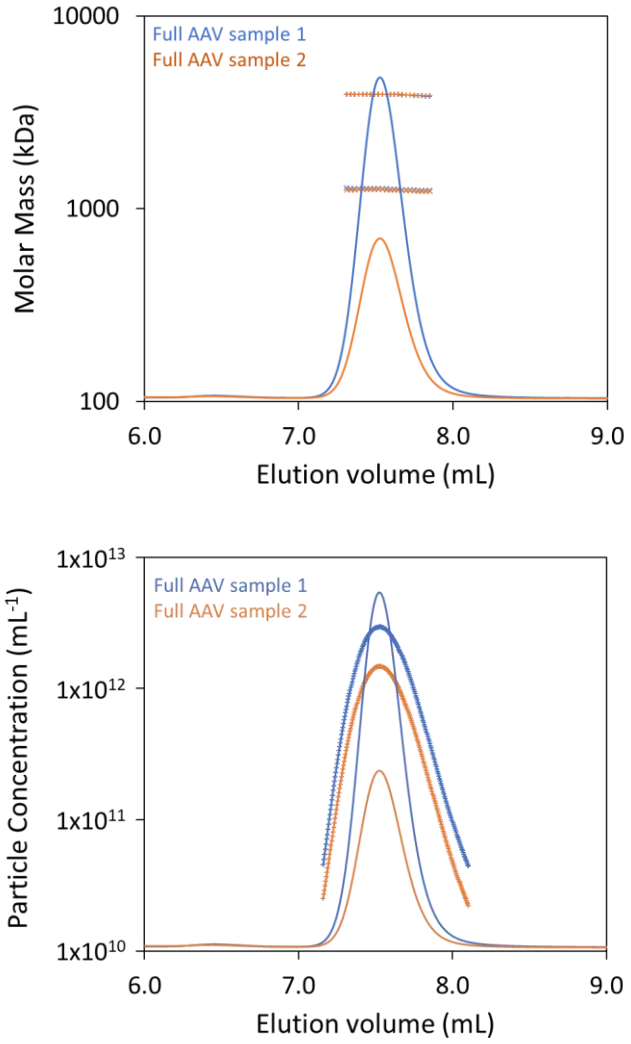

Figure 6. AAV multi-attribute quantitation for two full AAV samples are overlaid with the light scattering chromatogram. Top, LS chromatogram with molar mass overlay. Bottom, LS chromatograms with particle concentration overlay.

The XBridge Premier GTx column also offers higher sensitivity than that found for SEC-MALS with conventional silica-bead columns. As seen in Figure 7, the measurement of eluted viral titer closely matches the injected viral titer even at values below the current LOQ, 5 x 10^{10} particles/mL (specified in the ASTRA AAV SOP, as determined with conventional silica-based columns with a 7.8 mm inner diameter x 300 mm column). This improved sensitivity helps conserve precious samples or improve signal-to-noise, increasing the method's robustness.

Table 1. Multiple CQAs measured by combined SEC-UV-MALS workflow. AAV8 samples provided by Baylor College of Medicine.

	Empty AAV	Full AAV, Batch 1	Full AAV, Batch 2
Total concentration, Cp (particles/mL)	2.1 x 10^{12}	5.2 x 10^{13}	1.0 x 10^{14}
Full concentration, Vg (particles/mL)	NA	5.4 x 10^{13}	1.1 x 10^{14}
Capsid content, Vg/Cp	0.01	1.05	1.06
M_{capsid} (MDa)	3.70	3.95	3.94
M_{DNA} (MDa)	0.05	1.26	1.27
R_h (nm)	11.9	13.7	13.6

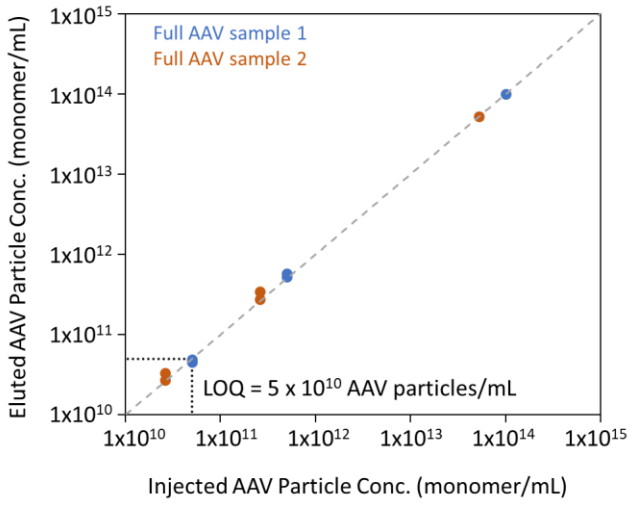

Figure 7. Limit of quantitation of AAV using 7.8 x 300 mm XBridge premier GTx columns is found to be lower than value previously determined with conventional silica columns of the same size.

Quantifying large AAV aggregates

As seen in Figure 8, the silica-based 4.6 mm SEC column can only partially resolve AAV aggregates from the monomer. In contrast, the 7.8 mm GTx SEC column fully separated the aggregates from the main monomer peak and even provided resolution between subspecies of aggregates. This improvement in aggregate resolution helps to better identify and quantify aggregation and particulate contamination and so is useful in AAV QC.

However, SEC may disrupt or otherwise remove large aggregates. The most accurate quantitation of aggregate species is accomplished with FFF-MALS (field-flow fractionation coupled to MALS, dRI and UV). With no stationary phase, FFF is an alternative to SEC for size-based

separation which does not shear or filter out large aggregate. Furthermore, it has been shown that quantitation of large aggregates by UV or fluorescence tends to greatly overestimate their abundance while MALS signals provide more accurate quantitation.[4,5]

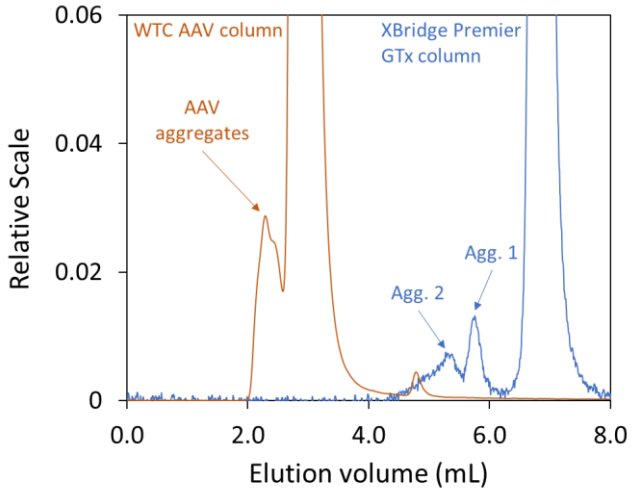

Figure 8. A zoomed-in view of the aggregates. Full AAV serotype-8 batch 1 sample from Baylor College of Medicine is used in this comparison. WTC AAV column is a silica-based 4.6 mm x 300 mm column. XBridge Premier GTx is a 7.8 mm x 300 mm column.

Conclusions

The enhanced chromatographic capabilities of XBridge Premier SEC columns provide optimal separation and high-quality light scattering data. For protein workflows, the XBridge Premier columns offer outstanding resolution and minimize unwanted column interactions. Requiring little to no method optimization, they can serve as a platform column for a wide variety of analytes, from therapeutic antibodies to vaccine antigens. Taking advantage of enhanced *in vitro* biosimilarity analysis enabled by MALS and high-resolution chromatography, manufacturers can quickly identify useful trends and attributes, saving time and resources ahead of *in vivo* testing.

For AAV applications, the XBridge Premier GTx column ensures sensitivity, high mass recovery, and impeccable light scattering data quality required for successful AAV MAQ. Traditionally, these CQAs would require multiple dedicated instruments such as ELISA for measuring Cp, qPCR for measuring Vg, or AUC for empty/full ratio. With SEC-MALS, these CQAs can be measured simultaneously in a single platform.

Overall, the enhanced chromatographic performances of the XBridge Premier Protein SEC column and the XBridge Premier GTx column complement Wyatt's MALS solutions for characterizing biotherapeutic proteins and AAVs. High-performing SEC columns mean that MALS can offer multi-attribute quantitation (MAQ) to even greater details and robustness.

Acknowledgments

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