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Modern Size-Exclusion Chromatography Separations of Biosimilar Antibodies at Physiological pH and Ionic Strength

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Abstract

The state of protein derived self-associated, aggregated, and fragmented impurities in therapeutics are critical quality attributes (CQA) that are widely monitored using non-denaturing Size Exclusion Chromatography (SEC). The mobile phases often used to provide an accurate quantification of these impurities on modern high sample-throughput SEC columns may often be required to deviate from the pH of the formulation buffer and be of much higher ionic strength, which may result in the dissociation of aggregated proteins during the SEC analysis.[1] Therefore, as an alternative we evaluated the use of physiological pH (~7.4) and ionic strength (~150 mM) Dulbecco's phosphate buffered saline (DPBS) as an SEC eluent for the analysis of the currently available biosimilar monoclonal antibodies on three HPLC system compatible SEC columns, a Waters XBridge Premier, 2.5 µm particle size, 250 Å pore size, a previous generation Waters BioResolve SEC mAb 200 Å, 2.5 µm column, and a Cytiva SuperDex 200 Increase Column. In addition, comparisons to the 1.7 µm particle-size UPLC version of the XBridge Premier SEC Column are presented

Benefits

- Increased capability and stability to evaluate protein therapeutic size variants using SEC with buffers at or

near physiological pH (~7.4) and ionic strength (~150 mM)

- Rapid method development and simplified routine deployment using pre-made Dulbecco's PBS buffer as an SEC eluent
- Significantly higher sample throughput as compared to dextran-particle based columns for SEC separations when using physiologically compatible PBS buffers
- Improved platform analytical SEC method versatility

Introduction

Waters XBridge Premier Protein SEC 250 Å, 2.5 µm and ACQUITY Premier Protein SEC 250 Å, 1.7 µm columns are the product of two technical advancements. First, the metallic surfaces of column body, end fittings, and frits are modified with a proprietary chemistry (MaxPeak Premier High Performance Surfaces (HPS) Technology. The purpose of this modification is to greatly reduce the ionic interactions and the formation of metal-complexes between the analyte proteins and metallic surfaces while maintaining the high efficiency and reproducible particle packing capabilities obtained when using metal column hardware. In addition, the diol bonding of the BEH SEC particle has been supplanted with hydroxy-terminated polyethylene oxide (PEO) to further minimize secondary ionic and hydrophobic interactions. These improvements have resulted in an SEC Column that provides many of the protein inertness benefits afforded by using cross-linked dextran-agarose packed in non-metallic column hardware such as SuperDx 200, which was first introduced over 30 years ago.[1] However, these modern columns also offer significantly greater sample throughput due to increased BEH particle strength and decreased BEH particle sizes. The use of bridged ethyl hybrid (BEH) particles also provides for greater long-term basic pH stability (pH range 2.5 to 8.0) versus pure silica-based SEC particles, although the upper pH range is not as high as the current Cytiva SuperDex 200 Increase Column (pH range 3 to 12).

In an attempt to take advantage of the inert properties of these new column technologies we evaluated the use of Dulbecco's phosphate buffered saline (DPBS) at physiological pH (~7.4) and ionic strength (~150 mM), and ionic strengths ranging from 120 mM (0.8X DPBS) to 300 mM (2X DPBS) as an SEC mobile phase. SEC mobile phases that deviate significantly from the pH and isotonicity of the formulation buffer may alter the state of self-association in the protein sample being assessed.[1] However, using an SEC mobile phase as close as possible to the composition to the formulation buffer of a therapeutic protein, although preferable, is often not possible. Alternatively, the use of a physiologically representative buffer in terms of pH and osmolality may more likely

provide an effective SEC separation and perhaps a more relevant assessment of protein self-association. In this regard, the use of DPBS as a mobile phase provides a pH (7.4) and tonicity consistent with serum, interstitial fluid, and lymph, the primary fluids that an intravenously or subcutaneously administered parenteral therapeutic protein can be exposed.

The columns selected for this study were a Waters BioResolve SEC mAb Column (diol bonded BEH, 200 Å, 2.5 µm particle size, stainless steel hardware), the Waters XBridge Premier Protein SEC 250 Å, 2.5 µm (PEO bonded BEH, MaxPeak HPS hardware) Waters XBridge Premier, 2.5 µm particle size, 250 Å, pore size column, and a Cytiva SuperDex 200 Increase Column packed with 8.6 µm crosslinked dextran-agarose particles packed in glass and polymer hardware. In addition, comparisons to the 1.7 µm particle-size UPLC ACQUITY version of the XBridge Premier Column are presented. SEC separations for the four monoclonal antibodies (mAbs) currently available in the United States of America as biosimilars were evaluated.

Experimental

Sample Description

Biosimilar mAbs were bevacizumab (Mvasi, 25 mg/mL), infliximab (Avsola, 10 mg/mL), rituximab (Ruxience, 10 mg/mL), and trastuzumab was the originator biologic (Herceptin, 21 mg/mL). All samples were analyzed neat following one or more freeze-thaw cycles.

LC Conditions

LC system:	ACQUITY UPLC H-Class Bio with CH-30A APH Column Heater
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelengths: 280 nm and 214 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit

	PTFE/Silicone Septum, 300 µL volume, 100/pk (p/n: 186002639)
Column(s):	XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 x 300 mm, Column plus mAb Size Variant Standard (p/n: 176005070) ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm, Column plus mAb Size Variant Standard (p/n: 176005072) BioResolve SEC mAb Column, 200 Å, 2.5 µm, 7.8 x 300 mm (p/n: 176004595) Cytiva Superdex 200 Increase 10/300 GL Column, 10 x 300 mm (p/n: 28990944)
Column temp.:	Ambient
Sample temp.:	6 °C
Injection volume:	2–10 mL
Flow rate:	0.25 – 0.5 mL/min
Mobile phase A:	Phosphate-buffered saline (DPBS, 10X), Dulbecco's formula 10X (Alfa Aesar, J61917) (0.1 µm sterile filtered)
Mobile phase B:	Milli-Q 18 MΩ water (0.1 µm sterile filtered)

Data Management

Chromatography software:	Empower 3 (FR 4)

Results and Discussion

A. Impact of DPBS Concentration on XBridge Premier SEC and BioResolve SEC mAb Column Performance

The four biosimilar mAb samples were evaluated at varying concentrations of DPBS for the BioResolve SEC mAb and XBridge Premier SEC columns. For both columns a 7.8 x 300 mm column size was evaluated at a flow rate of 0.5 mL/min. UV absorbance at 214 nm was used for improved sensitivity for low abundance size variants versus UV absorbance at 280 nm. However, this was not effective for quantification at the sample loads used due to the monomer peak being above the linear range of the detector. The results for the BioResolve SEC mAb Column are shown in Figure 1, and those for the XBridge Premier SEC Column are shown in Figure 2. For these samples HMWS2 and HMWS1 are presumed to predominately represent multimeric forms of the mAb and dimeric self-associated forms of the mAb. Antibody fragmentation was also observed in these samples as LMWS1 and LMWS2. LMWS1 is presumed to be primarily the result of a single cleavage in the mAb hinge region yielding an approximately 100 KDa fragment comprised of a covalent Fc domain and a single Fab domain, while LMWS2 is principally comprised of single Fab and Fc domains.

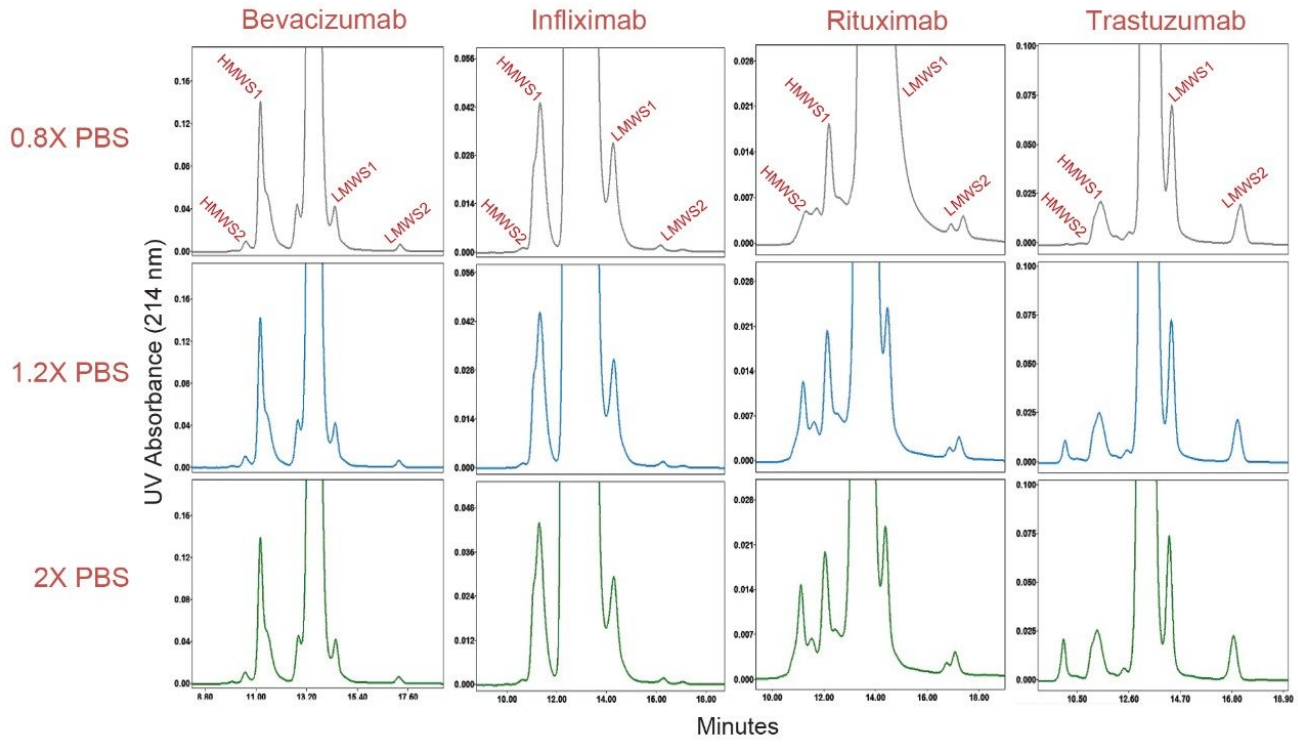

Figure 1. Stacked Plots of biosimilar mAb samples at varying concentrations of Dulbecco's phosphate buffer saline using a BioResolve SEC mAB, 200 Å, 2.5 µm, 7.8 x 300 mm, column are shown. Experimental conditions provided in text.

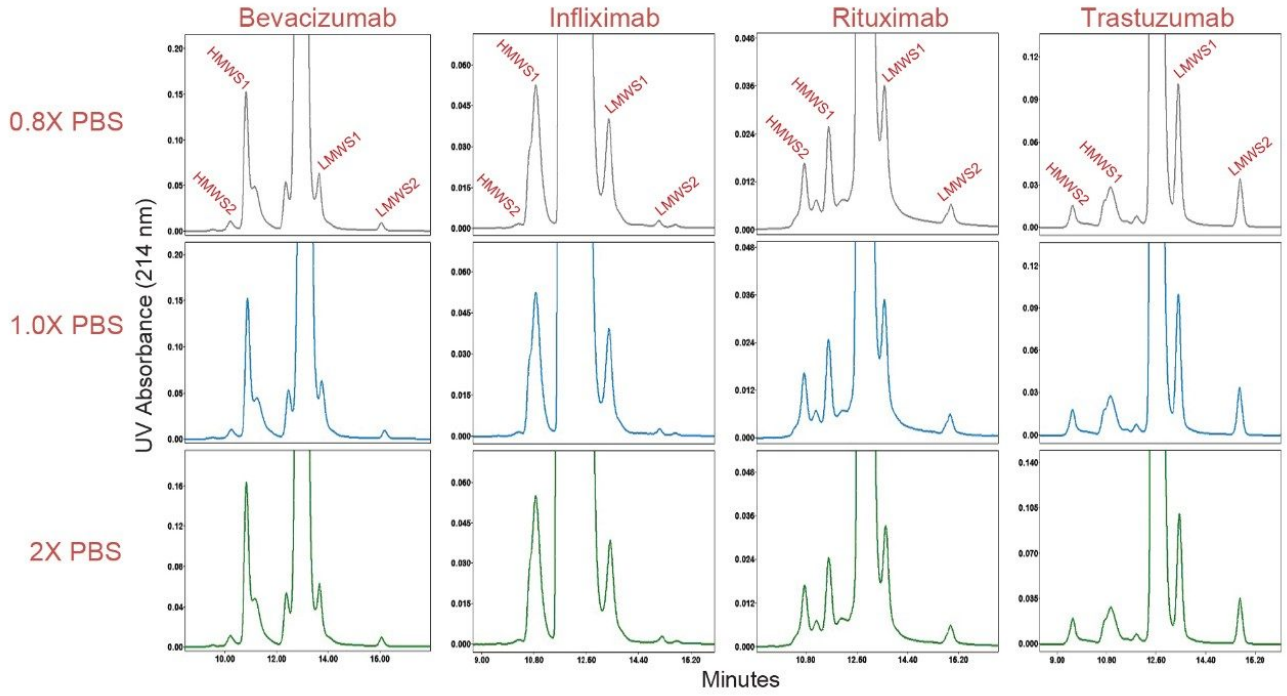

Figure 2. Stacked plots of biosimilar mAb samples at varying concentrations of Dulbecco's phosphate buffer saline using an XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 x 300 mm column are shown. Experimental conditions provided in text.

For bevacizumab and infliximab, equivalent results with respect to HMWS recoveries and LMWS1 fragment resolution are observed from a range of 0.8X DPBS to 2X DPBS on both columns. However, for rituximab and trastuzumab a significant loss in the recovery of the multimeric HMWS (HMWS2) and reduced resolution of the LMWS1 fragment at the 0.8X DPBS condition are seen on the BioResolve Column. For rituximab, the deleterious effects on the BioResolve Column are practically resolved at a 1.2X DPBS concentration, while for trastuzumab we observe maximum recovery of HMWS2 with 2X DPBS, albeit that the trend observed indicates that full recovery would likely be achieved between at a 1.5X DPBS concentration. In contrast, consistent results for rituximab and trastuzumab are observed on the XBridge Premier Column from 0.8X DPBS to 2X DPBS. These results indicate that the surface chemistries of the XBridge Premier Column hardware and particles have reduced the unspecific interactions with the four biosimilar mAbs in comparison to the previous generation diol-bonded BEH SEC Columns using stainless-steel hardware. More specifically, this result may correlate with the isoelectric points (pI) of these mAbs. The reported measured pI values of trastuzumab (pI=9.1) and rituximab

(pI=9.4) are significantly more basic than bevacizumab (pI=8.3) and infliximab (pI=7.6) which may be leading to greater to cationic interaction with low abundance negative charges such as silanol on the packed particles.[2] These results indicate that the Premier SEC Columns provide a broader range of conditions for which they can deliver effective separations versus previous generation diol-bonded BEH SEC Columns and, as a result, should be more amenable for use in platform analytical SEC methods.

B. SuperDx 200 Increase. XBridge Premier, and ACQUITY Premier Biosimilar mAb Separations with DPBS

In addition to the XBridge Premier, the four biosimilar mAbs were analyzed using a DPBS (1X) mobile phase with a Cytiva SuperDx 200 Increase 10/300 GL (8.6 mm, 10 x 300 mm) column and an ACQUITY Premier 1.7 mm, 4.6 x 300 mm, SEC Column (Figure 3). The SuperDx column technology using dextran-agarose cross-linked particles and biocompatible column hardware has been commonly used for the purification and analysis of proteins and peptide at or near physiological conditions for over 30 years. Also, while the large particle size and lower flow rate limits, due to the more compressible nature of the packed particles, of the SuperDx columns result in longer analysis times compared to 5 mm and smaller particle size silica-based columns, they exhibit superbly low levels of unspecific interactions with proteins and peptides. Additionally, the larger size packed particles are less likely to filter out larger multimeric forms of the proteins or alter the state of protein self- association due to pressure effects.

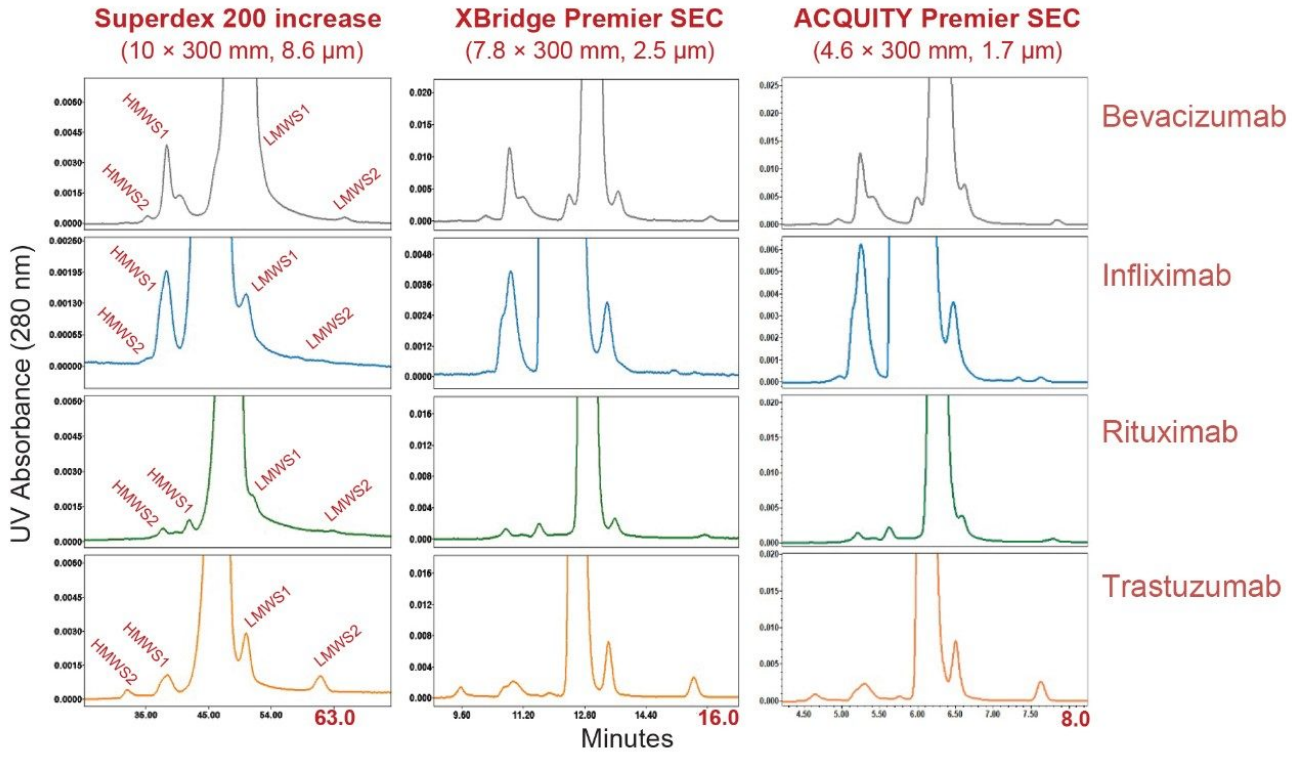

Figure 3. Stacked plots of SEC separations on selected columns for biosimilar mAb sample using 1X Dulbecco's phosphate buffer saline as a mobile phase are shown. Experimental conditions provided in text.

For this work, the SuperDx column was run at a flow rate of 0.25 mL/min to maximize resolution. It has been reported that despite the low levels of protein-column interactions afforded using SuperDx and similar SEC columns that some protein size variants are not fully recovered due to unspecific ionic interactions.[3] Therefore, the biosimilar mAbs were evaluated on this column at DPBS concentrations ranging from 0.8X to 2X and demonstrated reproducible recoveries (data not shown). As a result, in the absence of additional corroborative data such as analytical ultracentrifugation (AUC), the HMWS content observed using the SuperDx column was used to evaluate the effectiveness of the XBridge Premier SEC and ACQUITY Premier SEC Columns. The flow rate deployed for the XBridge Premier SEC Column was 0.5 mL/min as previously described. The flow rate for the ACQUITY Premier SEC was near maximum at 0.35 mL/min, which is twice the linear velocity used for the XBridge Column. As a result, the analysis times for the SuperDx 200, XBridge Premier, and ACQUITY Premier Columns were 100 min, 25 min, and 12.5 min, respectively.

Consistent chromatographic profiles for the biosimilar mAbs (Figure 3), and comparable quantitative results for HMWS1 and HMWS2 (Figure 4) were observed for the SuperDx, XBridge Premier, and ACQUITY Premier Columns with the predominant differences in resolutions resulting from column efficiencies or LC system dispersion. By reducing the flow rate on the SuperDx column to 30% of the linear velocity deployed on the XBridge Premier Column, effective separation of the HMWS variants is observed and the LMWS1 fragments are partially resolved, however, run times are increased approximately 4-fold. To achieve the separation efficiency of the XBridge Premier Column it would be predicted that three SuperDx columns would need to be run in series at a 3-fold reduction in linear velocity resulting in analysis times approximately nine times longer (experiment not performed).

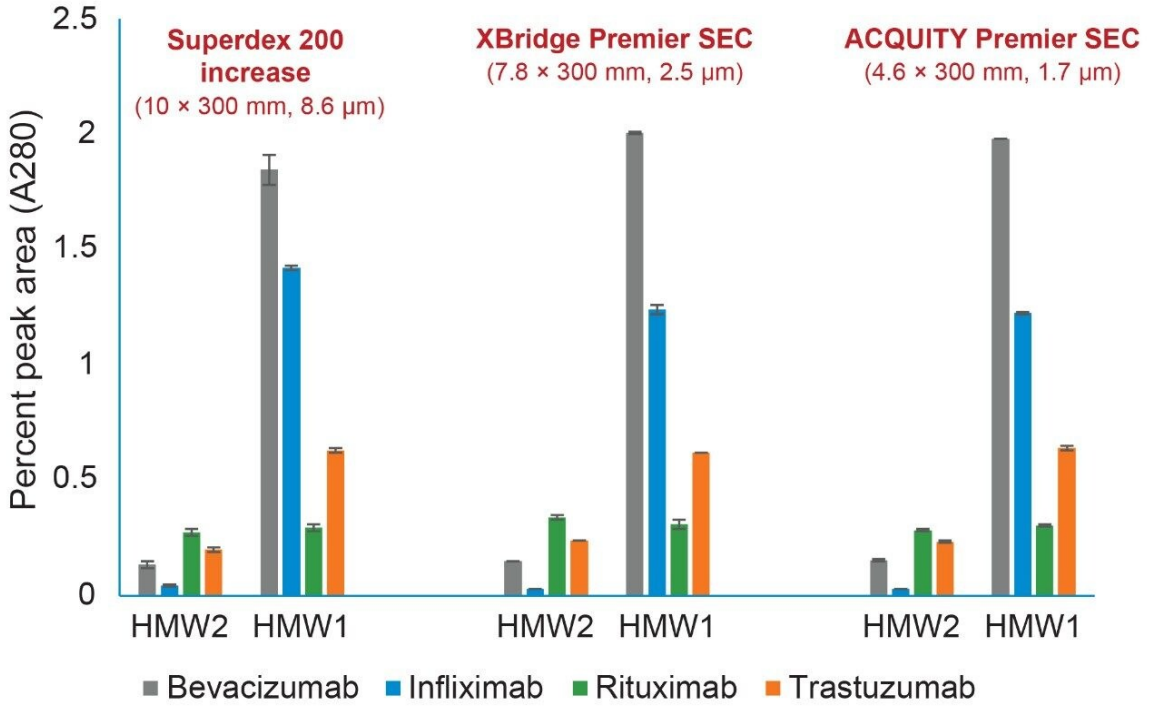

Figure 4. Compared are the measured relative abundances of biosimilar mAb HMW1 And HMW2 size variants on SuperDx 200, XBridge Premier 250 Å, and ACQUITY Premier 250 Å, SEC columns using 1X Dulbecco's phosphate buffer saline as a mobile phase. Chromatograms are presented in Figure 3. Error bars represent the range of duplicate measurements. Experimental conditions provided in text.

The ACQUITY Premier SEC Column, which was run at twice the linear velocity of the XBridge Premier SEC Column to maximize sample throughput, also provided effective separation and quantification of the HMWS

impurities. However, due to the higher reduced linear velocity and the 5-sigma system dispersion (18 µL) of the ACQUITY UPLC H-Class Bio (with a CH-30 column heater) significantly reduced resolution of the LMWS1 fragment is observed in comparison to the XBridge Premier due to the larger internal diameter and subsequent larger peak volumes of the later.[4]

These results demonstrate that an effective and robust SEC separation for the evaluated biosimilar mAbs can be achieved using a 1X DPBS physiological buffer (pH 7.4, ionic strength 150 mM) as a mobile phase with the XBridge and ACQUITY Premier SEC Columns but with significantly higher SEC sample throughputs in comparison to dextran-agarose particle-based columns. While it is not expected that 1X DPBS will be an effective mobile phase for SEC analyses of all proteins, the use of DPBS at varying concentrations offers the potential for simplified method development, transfer, and ease of use for mAb and potentially other proteins. For these studies, the DPBS concentrate was 0.1 µm sterile filtered for use on a 1.7 µm particle size column, however, for a 2.5 µm or larger particle size column 0.2 µm filtered buffers are generally acceptable. As a final note, this data demonstrates that the MaxPeak Premier HPS and BEH-PEO technologies applied to the development of these SEC Columns have resulted in a column with reduced levels of unspecific protein-column interactions that may also provide benefits when developing and deploying methods using other buffer systems.

Conclusion

With the noted technological advancements in both the column hardware and packed particle chemistry, the XBridge and ACQUITY Premier 250 Å, SEC columns provide reduced unspecific protein-column interactions in comparison to the prior generation diol-bonded BEH SEC Column. This increased capability may allow for the evaluation of protein therapeutic HMWS and LMWS using SEC with buffers at or near physiological pH (~7.4) and ionic strength (~150 mM) as demonstrated by the biosimilar mAbs tested. For these studies, with respect to unspecific interactions the XBridge and ACQUITY Premier SEC columns performed comparably to a SuperDx 200 SEC column packed with dextran-agarose cross-linked particles while providing significantly higher sample throughput. The XBridge and ACQUITY Premier SEC columns may provide platform analytical SEC method versatility, rapid method development, and simplified routine deployment using either pre-made Dulbecco's PBS or other buffer systems for mAbs and other therapeutic protein size variants ranging from approximately 10 KDa to 650 KDa.

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