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Expanding Size-Exclusion Chromatography Platform Method Versatility for Monoclonal Antibody Analysis Using Waters XBridge Premier Protein SEC Columns

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Abstract

Due to their potentially deleterious impact on immunogenic response or activity, the levels of self-associated, aggregated, and fragmented size-variant impurities in therapeutic proteins are typically considered to be critical quality attributes (CQA) throughout both pre-clinical and clinical studies, and in approved drug products. Size exclusion chromatography (SEC) is often the method deployed to monitor these impurities. Since many biopharmaceutical development companies have multiple similar candidates, such as monoclonal antibodies, in their development pipeline benefits in terms of method use, documentation, staff training, and laboratory stocking can be gained by adopting effective platform analytical methods when possible.[1,2] A platform SEC method using a single mobile phase and column should always provide high resolution and reliable separations. Consequently, the ability of an SEC column to provide these capabilities over a broader range of mobile phase conditions should improve upon the number of compounds that can be analyzed via a platform method. In addition, when a specific protein requires re-optimization of a platform method mobile phase composition a more versatile SEC column can streamline that method development.

In this study we evaluated the SEC separation effectiveness for the currently available biosimilar monoclonal antibodies using a 20 mM sodium phosphate buffer (NaPi) over a pH range of 5.8 to 7.6 and sodium chloride concentrations ranging from 50 mM to 400 mM. These highly varied SEC eluents were assessed for a Waters XBridge Premier Protein, SEC 250 Å, 2.5 µm Column and a previous generation Waters BioResolve SEC mAb, 200 Å, 2.5 µm Column.

Benefits

- Increased SEC capability and stability to evaluate protein therapeutic size variants using SEC with buffers over a wide pH range (5.8 to 7.6) and ionic strength (~70 mM to ~450 mM)
- High resolution mAb aggregate and fragment size variant separations on an high-performance liquid chromatography (HPLC) compatible SEC column
- Qbd method development and robustness testing facilitated by using Empower with Auto▪Blend Plus
- Improved platform analytical SEC method versatility

Introduction

The recently developed Waters XBridge Premier Protein SEC, 250 Å, 2.5 µm Column uses MaxPeak Premier High Performance Surfaces (HPS) hardware and hydroxy-terminated polyethylene oxide (PEO) bonded BEH (bridged-ethylene hybrid organosilica) BEH-PEO particles to greatly reduce unspecific interactions with a wide range of analyte proteins. The goal of these changes to the current Waters diol-bonded BEH SEC Columns packed in unmodified stainless steel hardware was to provide a high-efficiency SEC column of superior versatility that can be deployed for the widest range analytes and using the broadest range of mobile phase conditions, including mildly basic pH buffers. We recently demonstrated the comparable inertness of XBridge and ACQUITY Premier Protein SEC columns (2.5 µm and 1.7 µm particle size) to that of an SEC column with cross-linked dextran-agarose particles packed in non-metallic column hardware using Dulbecco's phosphate buffered saline (DPBS) at a mildly basic physiological pH (~7.4) and ionic strength (~150 mM) as an SEC mobile phase.[3] In the same study, we also observed a modest increase in method robustness for the Premier SEC column versus and an SEC column with BEH-Diol particles packed into stainless steel hardware.

In this study we compared the platform capability and versatility of the XBridge Premier Protein SEC Column to

that of a current Waters diol-bonded BEH SEC Column (Waters BioResolve SEC mAb, 200 Å, 2.5 µm). The Waters diol-bonded BEH SEC Column technology also provides an SEC column that has been shown to be platform capable.[4] The four currently available biosimilar monoclonal antibody drug products (bevacizumab, infliximab, rituximab, and trastuzumab) that are marketed in the United States of America were evaluated using a 20 mM sodium phosphate buffer (NaPi) over a wide pH range of 5.8 to 7.6 and sodium chloride concentrations ranging broadly from 50 mM to 400 mM in a full-factorial quality by design (QbD) approach. These highly varied SEC eluents cover a broad range of mAb and other recombinant protein drug product formulation buffers with respect to pH and isotonicity.

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) BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm (p/n: 176004595)
Column temp.:	25 °C
Sample temp.:	6 °C
Injection volume:	2–4 µL
Flow rate:	0.75 mL/min
Mobile phase A:	100 mM monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), Sigma-Aldrich BioXtra (71507) (0.1 µm sterile filtered)
Mobile phase B:	100 mM dibasic sodium phosphate dihydrate ($NaH_2PO_4 \cdot H_2O$), Sigma-Aldrich BioUltra (71643) (0.1 µm sterile filtered)
Mobile phase C:	1.00 M sodium chloride (NaCl) BioUltra (71376) (0.1 µm sterile filtered)
Mobile phase D:	Milli-Q 18 MΩ water (0.1 µm sterile filtered)

Data Management

Chromatography software	Empower 3 (FR 4) with Auto•Blend Plus

Results and Discussion

Impact of pH and NaCl Concentration on XBridge Premier Protein SEC and BioResolve SEC mAb Column Performance

A full response surface experimental design was employed to evaluate the effects pH and NaCl concentration on the SEC separation performance for four biosimilar mAb samples (bevacizumab, infliximab, rituximab, and trastuzumab) when using the BioResolve SEC mAb Column or the XBridge Premier SEC Column. A 7.8 x 300 mm column size was evaluated at a flow rate of 0.75 mL/min for both columns. Using Empower with Auto•Blend Plus software with concentrated sodium phosphate (100 mM NaH_2PO_4 and 100 mM Na_2HPO_4) and sodium chloride (1.00 mM NaCl) a series of sixteen 20 mM sodium phosphate buffers were generated with pH values of 5.8, 6.4, 7.0, and 7.6 and NaCl concentrations of 50 mM, 100 mM, 200 mM, and 400 mM. The predicted ionic strengths for these buffers are provided in Table 1.

	pH			
mM NaCl	**5.8**	**6.4**	**7.0**	**7.6**
400	420	428	441	452
200	220	228	241	252
100	120	128	141	152
50	70	78	91	102

Table 1. Predicted ionic strengths of 20 mM sodium phosphate buffers with different concentrations of sodium chloride (mM NaCl).

Injections of 2.0 µL were used for bevacizumab (Mvasi, 25 mg/mL) and trastuzumab (Herceptin, 21 mg/mL), while 4.0 µL of infliximab (Avsola, 10 mg/mL) and rituximab (Ruxience, 10 mg/mL) were injected. The pH

conditions were run in the order 5.8, 7.6, 6.4, and 7.0 and at each pH the NaCl concentrations were analyzed in the order 200 mM, 100 mM, 400 mM, and 50 mM. Only single analyses were used for each condition evaluated and a molecular weight standard was evaluated at each condition to confirm consistent column performance over the course of the analysis. The results for the BioResolve SEC mAb Column are shown in Figures 1, 3, 6, and 7, and those for the XBridge Premier Protein SEC Column are shown in Figure 2, 4, 6, and 8. For these samples, HMW2 and HMW1 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 LMW1 and LMW2. LMW1 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 LMW2 is principally comprised of single Fab and Fc domains.

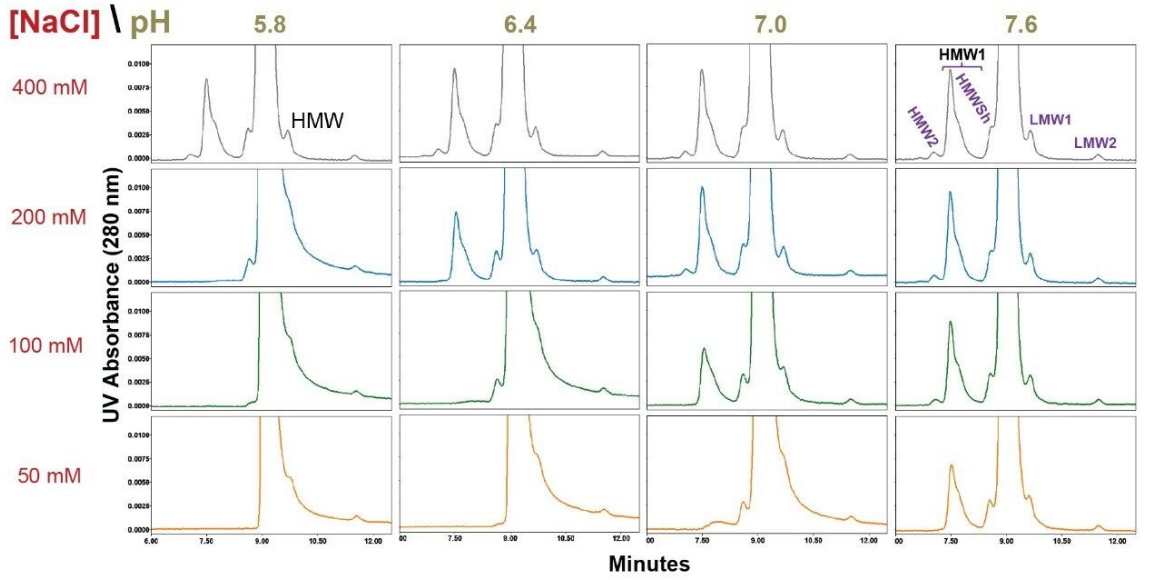

Figure 1. Bevacizumab drug product SEC separations using a BioResolve SEC mAb Column (200 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower with Auto▪Blend Plus empirical data.

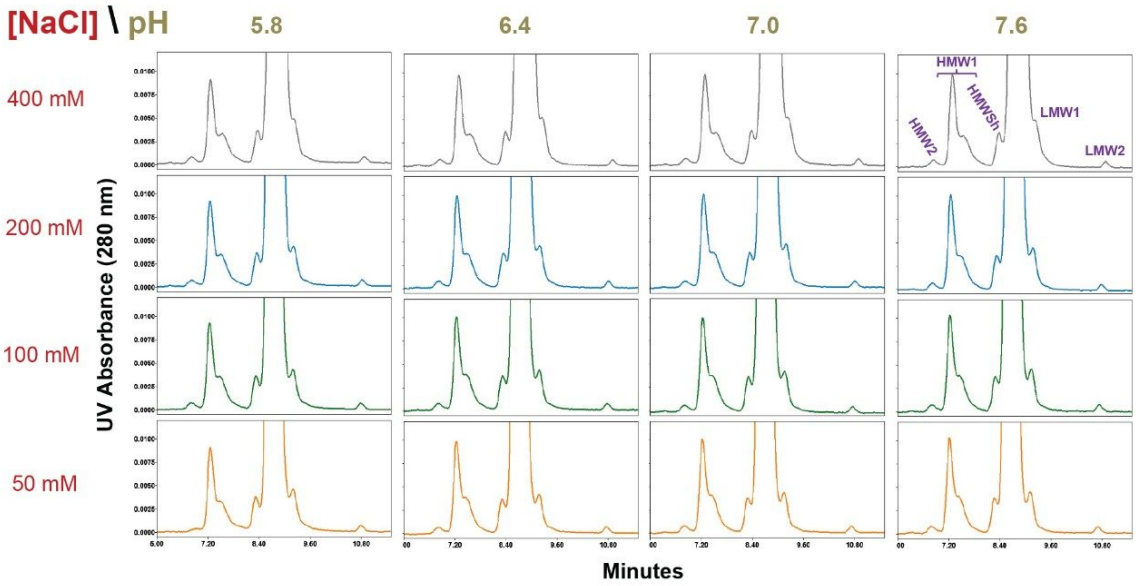

Figure 2. Bevacizumab drug product SEC separations using an XBridge Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower with Auto•Blend Plus empirical data.

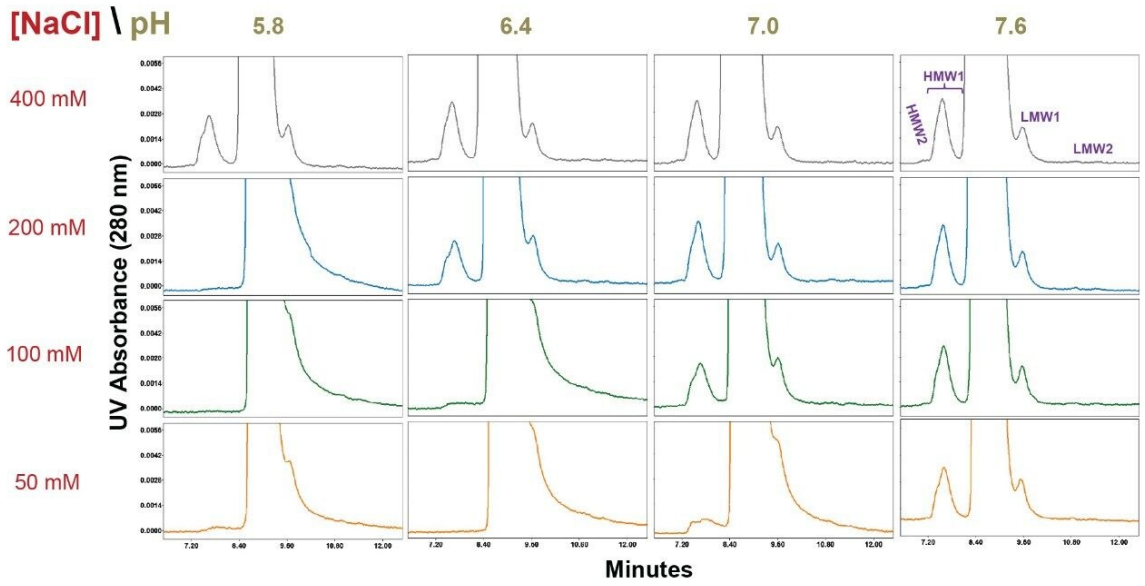

Figure 3. Infliximab drug product SEC separations using a BioResolve SEC mAb Column (200 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower with Auto•Blend Plus empirical data.

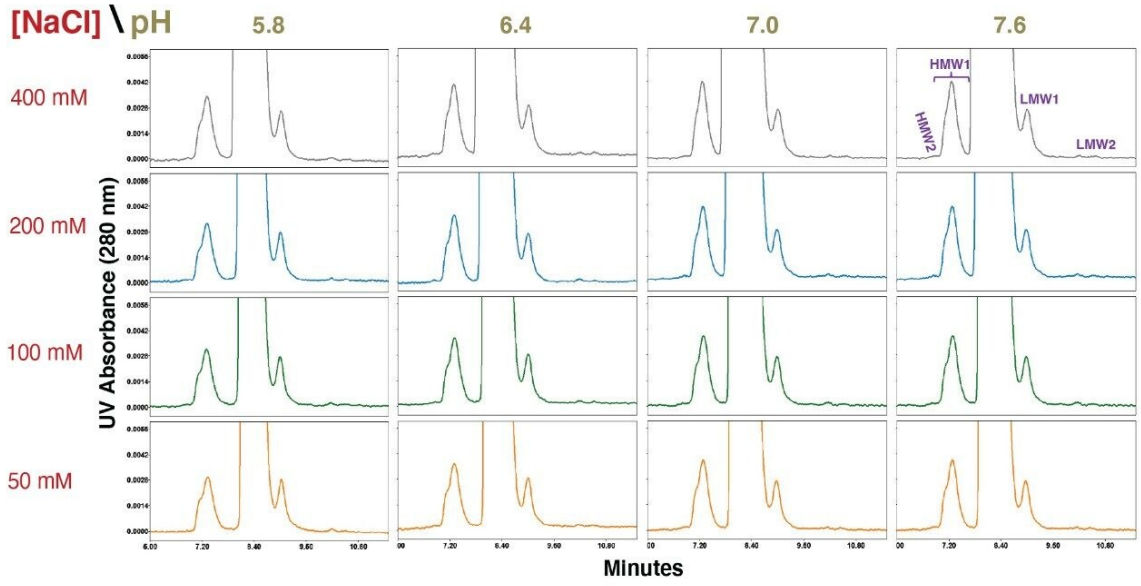

Figure 4. Infliximab drug product SEC separations using an XBridge Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Peak identities are labeled for pH 7.6, 400 mM NaCl chromatogram. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower with Auto▪Blend Plus empirical data.

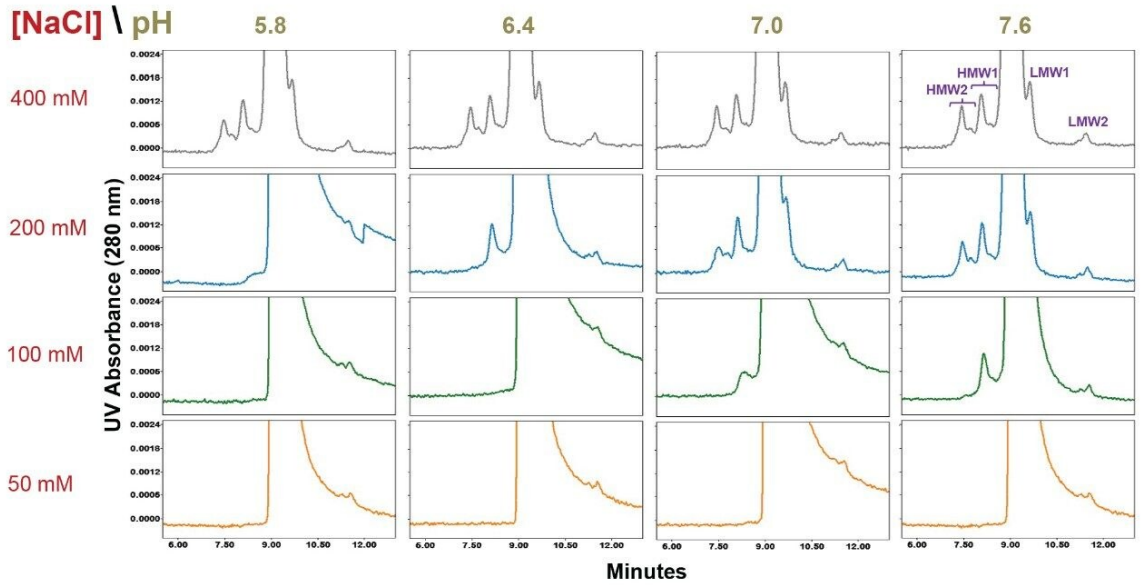

Figure 5. Rituximab drug product SEC separations using a BioResolve SEC mAb Column (200 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Peak identities are labeled for pH 7.6, 400 mM NaCl chromatogram. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower with Auto▪Blend Plus empirical data.

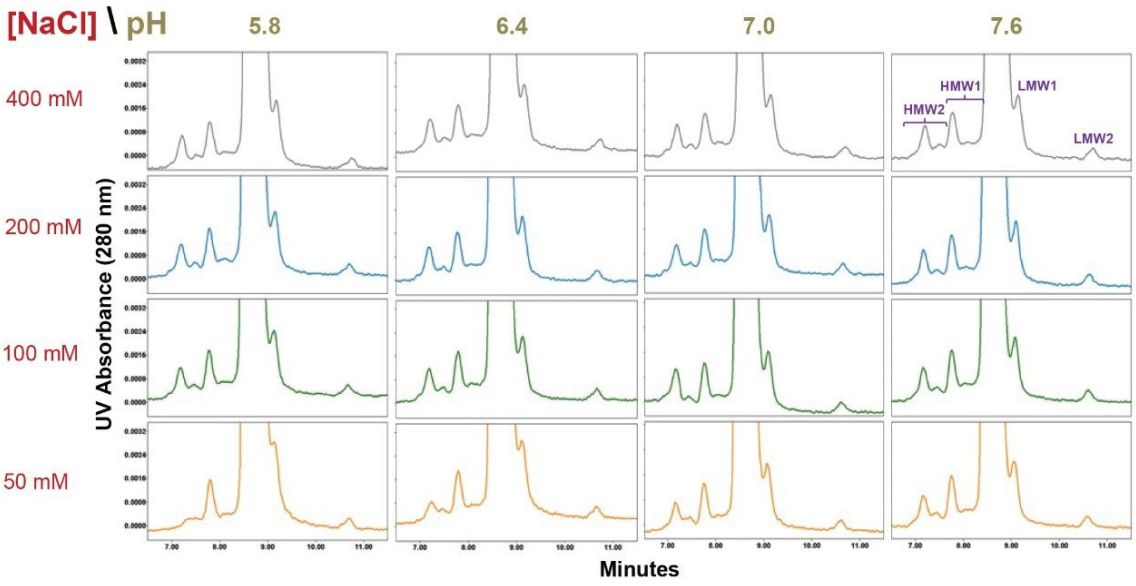

Figure 6. Rituximab drug product SEC separations using an XBridge Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Peak identities are labeled for pH 7.6, 400 mM NaCl chromatogram. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated Empower with Auto▪Blend Plus empirical data.

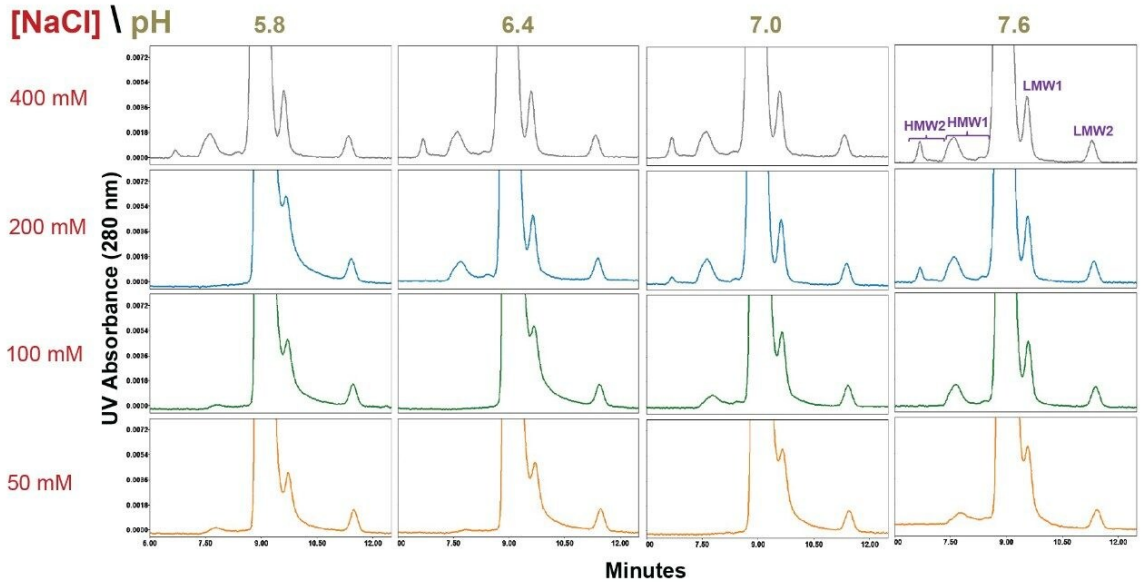

Figure 7. Trastuzumab drug product SEC separations using a BioResolve SEC mAb Column (200 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Peak identities are labeled for pH 7.6, 400 mM NaCl chromatogram. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower AutoBlend+ empirical data.

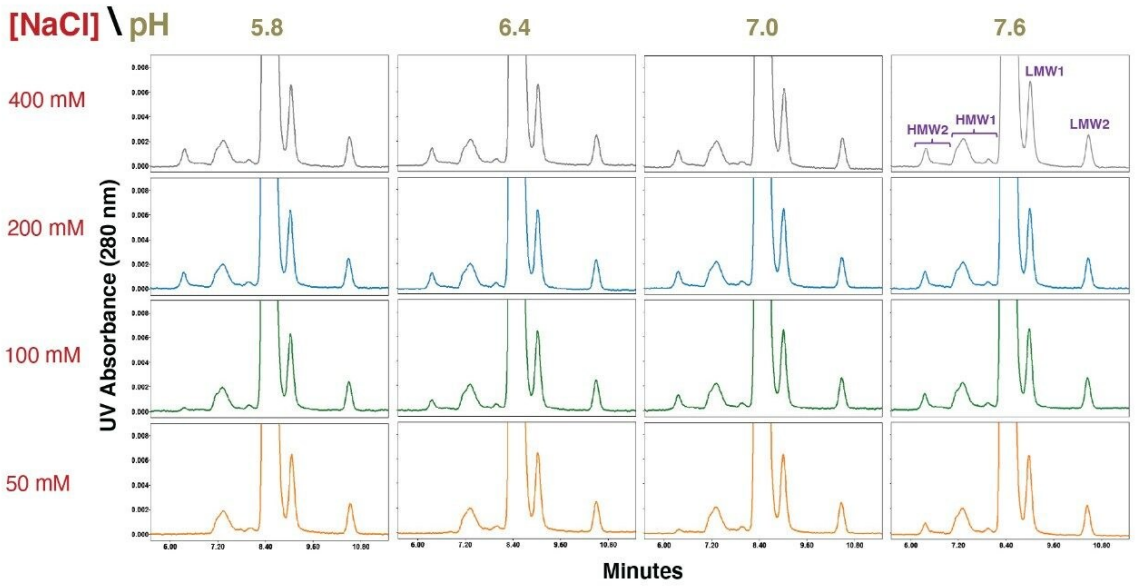

Figure 8. Trastuzumab drug product SEC separations using an XBridge Premier Protein SEC Column (250 Å, 2.5 µm, 7.8 x 300 mm) are shown for pH values ranging from 5.8 to 7.6 (20 mM sodium phosphate) and sodium chloride (NaCl) concentrations ranging from 50 mM to 400 mM. Peak identities are labeled for pH 7.6, 400 mM NaCl chromatogram. Flow rate was 0.75 mL/min (15 minute analysis time), and pH and NaCl concentrations were generated using Empower AutoBlend+ empirical data.

Data analysis for each mAb evaluated included a determination of the relative peak areas for HMW2, HMW1, LMW2, and LMW1 and the resolution for LMW1 as determined by peak-to-valley ratio (P/V). Exceptions included the additional evaluation of a HMW shoulder (HMWSh) for bevacizumab, and no evaluation for HMW2 or LMW2 for infliximab due to the low abundances (<0.02%) and signal-to-noise (<2) of those size variants. To assess the effective operating range for each mAb and column combination, visually similar chromatograms with respect to the resolution and relative peak areas of the observed HMW and LMW variants were integrated and the median values for the relative abundance of the HMW and LMW forms determined. A pH and NaCl concentration buffer composition were considered effective for HMW1 and LMW1 if the percent peak areas were within 10% of their median values, in addition the peak-to-valley (P/V) resolution for LMW1 was required to be greater than 1.05 for consistent drop-baseline integration. Due to the lower abundances of HMW2 and LMW2, percent peak areas were required to be within 20% of the median for their separation to be considered effective. To visualize the

versatility of the two columns, compilations of the mAb results were generated. In the first compilation (Figure 9) the number of mAbs for which HMW2 (when applicable), HMW1, and LMW2 (when applicable) were all effectively quantified were summed for each NaCl concentration and pH condition and are presented as a heat map. For the second compilation, in addition to HMW2, HMW1, and LMW2, effective analysis of LMW1 was also required (Figure 10).

Figure 9. Heat-map compilation of HMW1, HMW2, and LMW2 size variant SEC analysis effectiveness of four biosimilar mAb drug products (Figures 1 through 8) is shown for both the BioResolve SEC mAb and XBridge Premier Protein SEC Columns. Effective separations with respect to both quantification and resolution were scored as 1 or 0, resulting in a maximum score of 4. Additional chromatographic and data analysis details are provided in the text.

Figure 10. Heat-map compilation of HMW1, HMW2, LMW2, and LMW1 size variant SEC analysis effectiveness of four biosimilar mAb drug products (Figures 1 through 8) is shown for both the BioResolve SEC mAb and XBridge Premier Protein SEC columns. Effective separations with respect to both quantification and resolution were scored as 1 or 0, resulting in a maximum score of 4. Additional chromatographic and data analysis details are provided in the text.

When comparing the heat-maps generated for the BioResolve SEC mAb Column and the XBridge Premier Protein SEC Column highlights the significant gain in operating range of the XBridge Premier Column for the analysis of total HMW (HMW2 and HMW1) and the LMW2 size variant (Figure 8). Since in all conditions where LMW2 analysis failed HMW analysis also failed, the heat-maps in Figure 8 are also applicable to SEC methods being used only for HMW analysis.

When adding the additional requirements for the determination LMW1 reduced the number of successful analyses for both columns (Figure 9). The most notable observation was the failures of LMW1 analysis of bevacizumab with the XBridge Premier SEC Column (Figure 2) with 400 mM NaCl at all pH levels, where although the quantifications of LMW1 met the requirements, the P/V ratios were below the requirement (1.05). Since there was no change in elution profile or retention times from 200 mM NaCl to 400 mM NaCl for trastuzumab monomer and LMW1 on the XBridge Premier Protein SEC Column changes in column characteristics such particle pore diameter due to particle or bonded phase shrinking or swelling were ruled out as contributing factors for these failures. Therefore, it is likely that the failed P/V values are mostly due to the maximum P/V for bevacizumab on the XBridge Premier SEC Column (1.33) being the lowest observed in this study in combination with a small increase

in unspecific interactions at 400 mM NaCl, a change in protein conformation, or both.

In general, the two SEC columns performed similarly when using the higher ionic strength (higher NaCl concentration) and higher pH mobile phases. These performance characteristics are also consistent with previous results for these columns when using physiological pH (~7.4) and ionic strength (~150 mM) Dulbecco's phosphate buffered saline (DPBS) as an SEC eluent for the analysis of the same biosimilar monoclonal antibodies.[3] Also, both columns generated comparable and reproducible quantitative results for the HMW and LMW size variants when analyzed under effective conditions (Figure 11). However, in comparison to the BioResolve SEC Column the XBridge Premier SEC Column substantially improved upon the utility of the column when using both lower pH and lower ion strength mobile phases, indicative of a column with low levels of undesired ionic interactions. The wide pH range capability and low ionic interaction levels of the XBridge Premier SEC Column may potentially be used to advantage to develop versatile platform SEC methods at either a mildly basic physiological pH or the mildly acidic pH mobile phase conditions at which many therapeutic mAb and other protein-based drug products are formulated while also being able to operate at an ionic strength more representative of either physiological conditions or the formulation buffer.

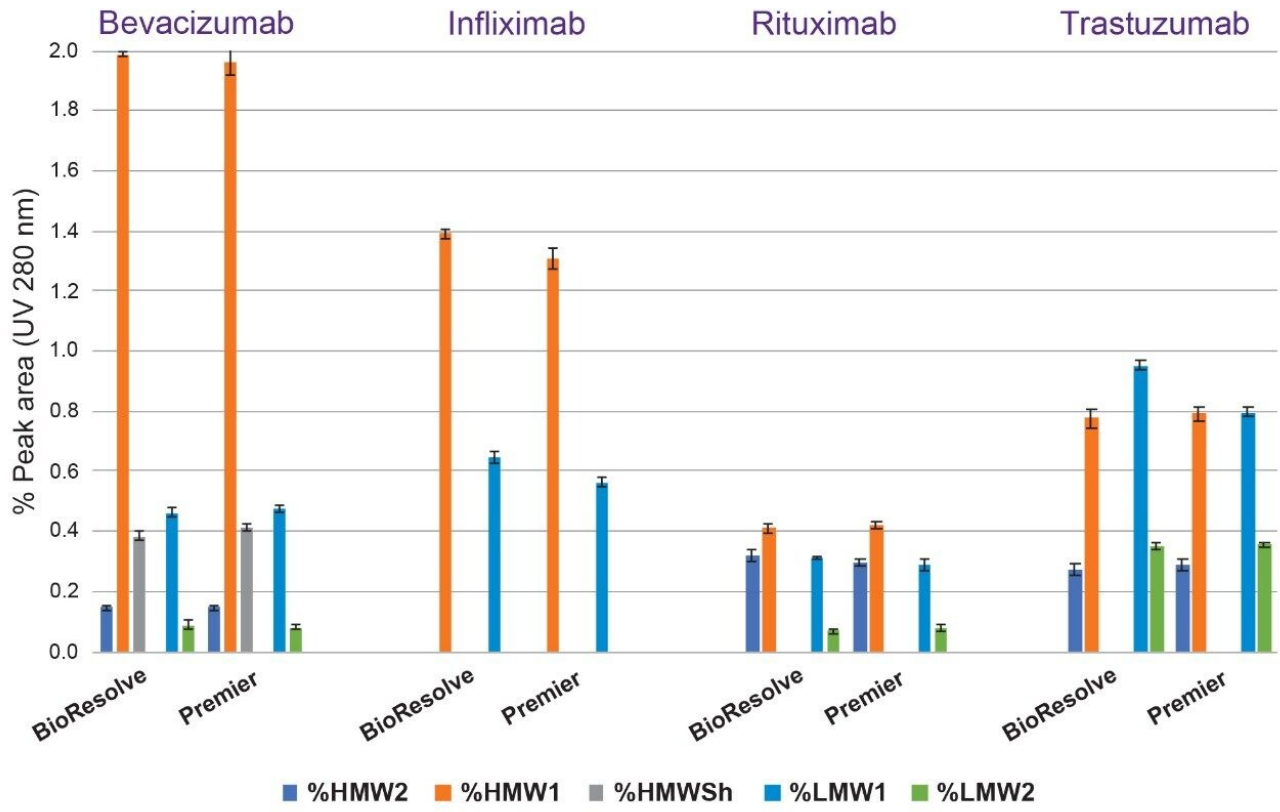

Figure 11. Comparisons of the average relative quantifications of HMW1, HMW2, LMW2, and LMW1 size variants observed in four biosimilar mAb drug products (Figures 1 through 8) are shown for both the BioResolve SEC mAb and XBridge Premier SEC columns. Only results from mobile phase conditions with which all four mAbs could be effectively separated were averaged. Mobile phases conditions selected for HMW2, HMW1, and LMW2 averages and standard deviation error bars were based on the heat-maps shown in Figure 9 (BioResolve n=4, and XBridge Premier n=10). LMW1 analyses were based on the heat-maps shown in Figure 10 (BioResolve n=4, and XBridge Premier n=6). Additional details are provided in the text.

Conclusion

We evaluated the mAb SEC platform method capabilities of the XBridge Premier Protein SEC, 250 Å, 2.5 µm 7.8 mm x 300 mm Column. The technological advancements in both the hardware and packed particle chemistry of

this column were successfully employed to produce a column that can provide high throughput analyses while providing reduced unspecific protein-column interactions in comparison to the prior generation diol-bonded BEH SEC Column, which has been previously shown to be mAb SEC platform method capable. The demonstrated improved capabilities and stability (recommended pH range: 2.5–8.0) of BEH-PEO particles at basic pH levels may also allow for the evaluation of mAb and potentially other protein therapeutic HMW and LMW impurities using SEC with buffers at or near physiological pH (~7.4) or the mildly acidic pH (~6) of many proteins' therapeutic formulations and physiological ionic strength (150–200 mM) and lower depending on the protein.

The XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 mm x 300 mm Column chemistry can provide platform analytical SEC method versatility or rapid bespoke method development while the 7.8 mm internal diameter of the column, which is compatible with HPLC, UHPLC, and UPLC chromatographic systems, offers additional simplified routine method deployment and transfer capabilities.[5,6] In addition, the ACQUITY Premier Protein SEC, 250 Å, 1.7 µm, 4.6 mm x 300 mm Column, which has been noted to have inertness comparable to that of the XBridge Premier SEC Column, can produce comparable results and an estimated approximately 30% increase in sample throughput when deployed on a low dispersion UPLC chromatographic system.

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720007500, January 2022