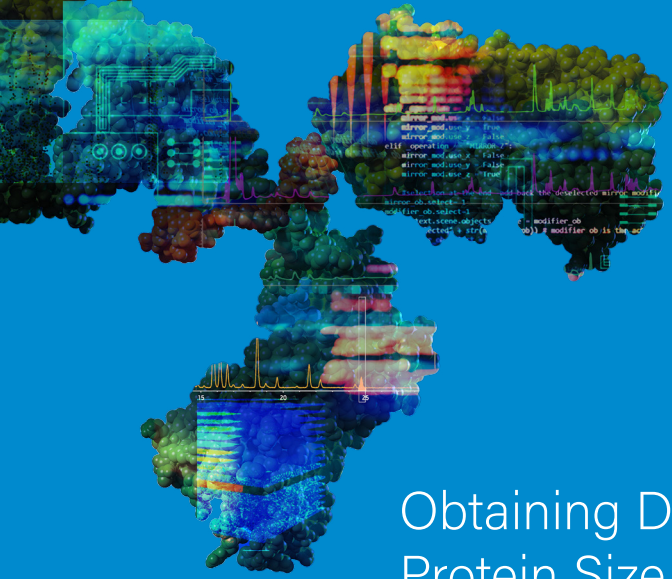
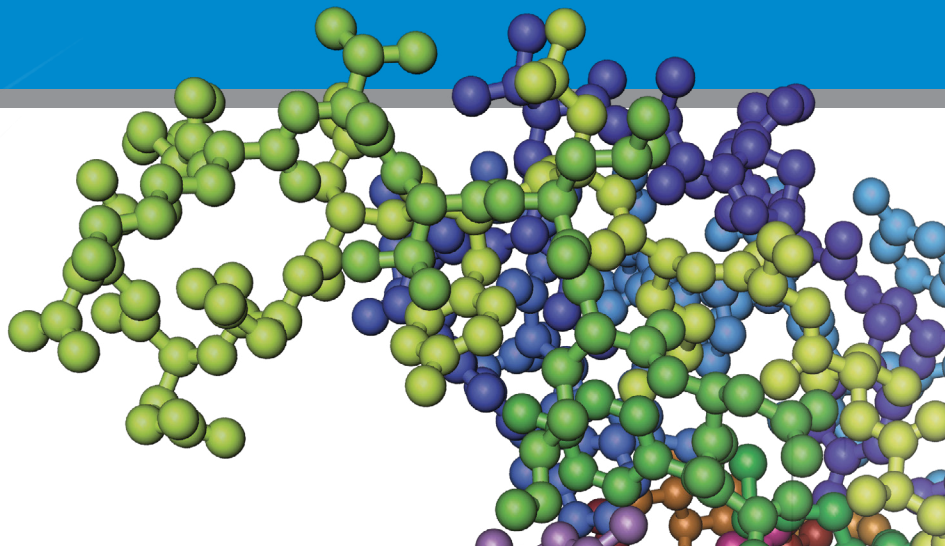


Obtaining Desired mAb, ADC, and Bi-Specific Protein Size Variant Separations and Maximizing SEC Column Life



Waters™

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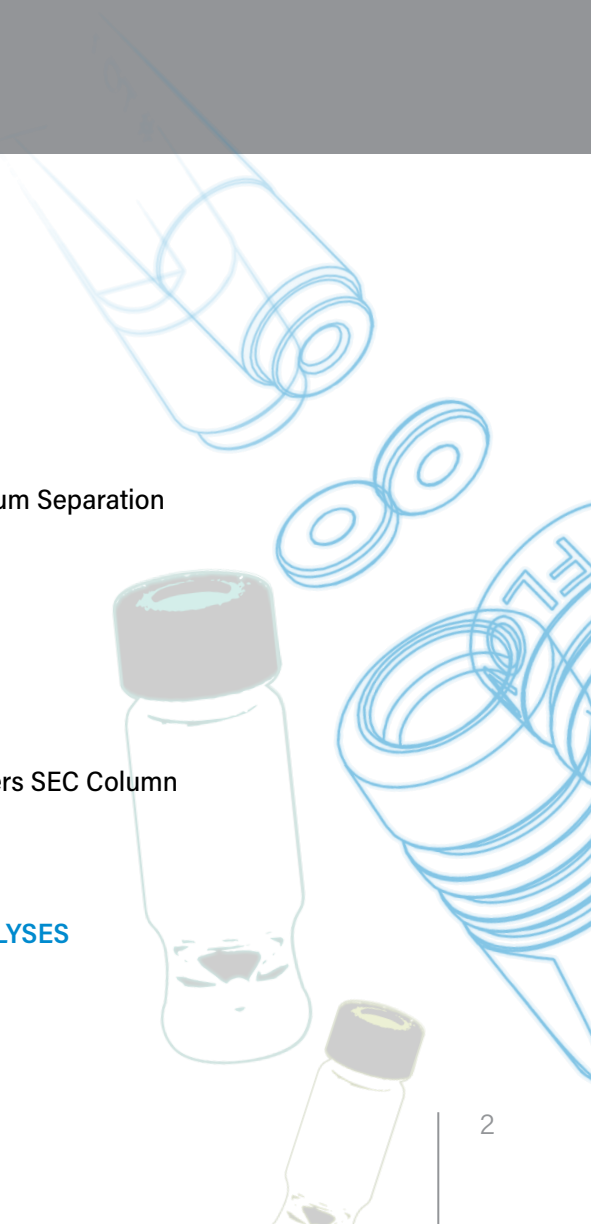
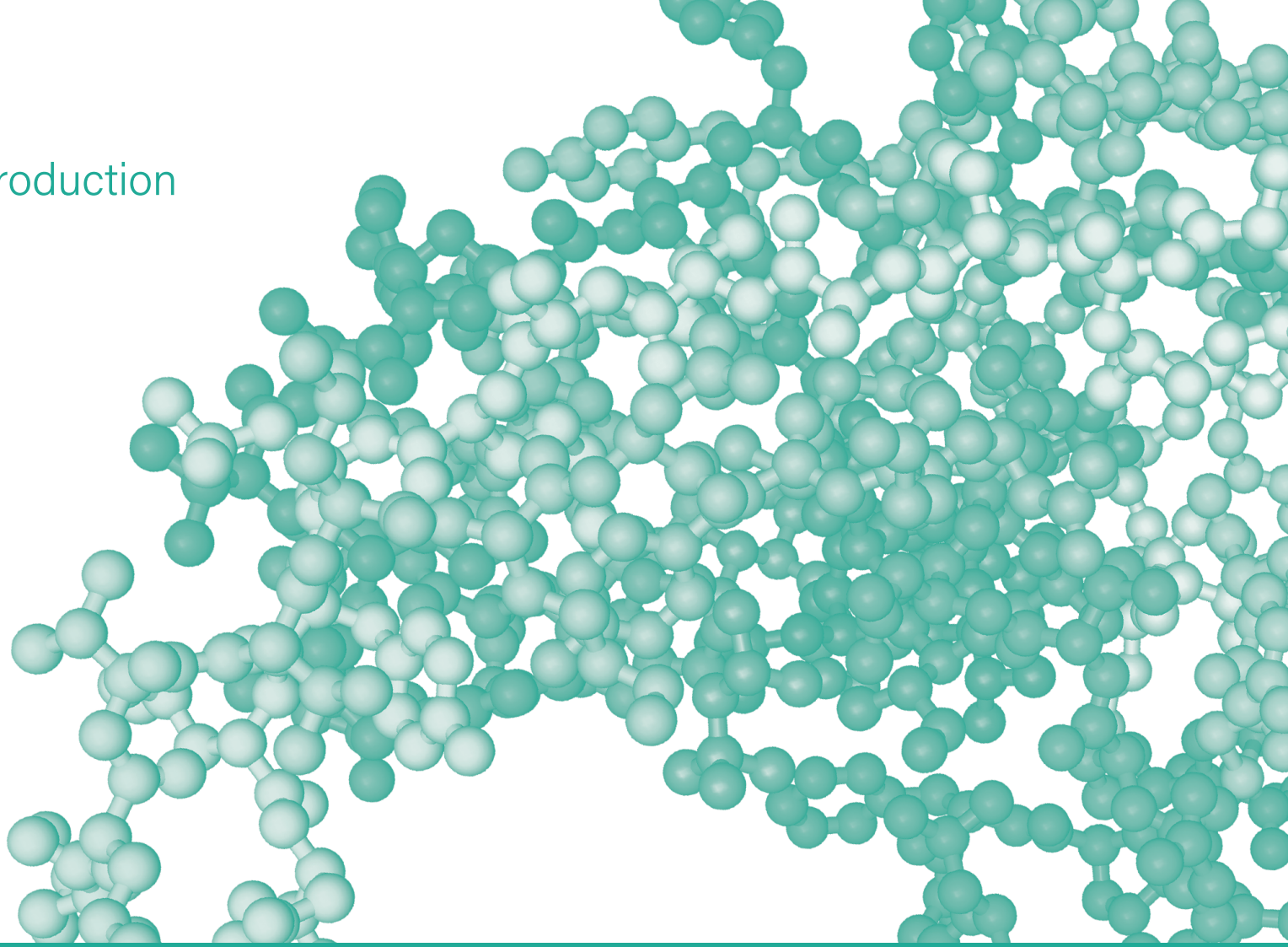


I. Introduction



I. Introduction

Several different analytical techniques have been used for separation of accurate quantitation of aggregates (>450,000 Daltons), monomers (150,000 Daltons) and fragments (<100,000 Daltons) contained on analyzed biotherapeutic monoclonal antibody (mAb) and associated antibody drug conjugates (ADCs). However, data for size-exclusion chromatography (SEC) has been favored in the research, development, and QC testing of these species due to the technique's accuracy, reproducibility, and relative ease of use.

In the early 1970s, scientists used of LC-based SEC columns in stainless steel hardware containing >15 µm porous silica-based particles that were functionalized with a DIOL coating to make the particles "waters wettable" and to minimize any undesired ionic interactions between free silanols on the silica particles and charged amino acid groups on the protein. Separations were based ideally on the differing hydrodynamic volumes (i.e., relative size in solution or Stokes Radii) of the separated proteins as they entered and exited the pores contained in the SEC particles. However, studies showed that undesired hydrophobic interactions could occur between the sample and the hydrophobic DIOL coating (e.g., with ADC samples) and / or ionic interactions between the protein and stainless-steel hardware column hardware that included the inlet and outlet frits as well as column wall.

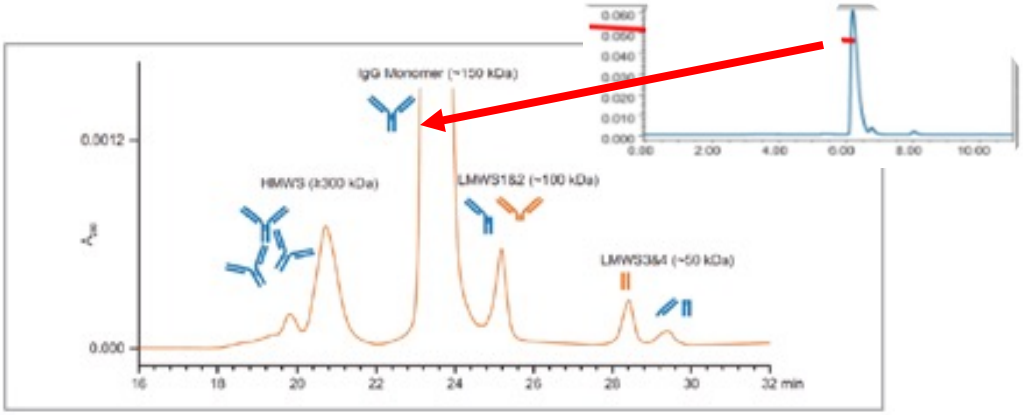
In 2010, Waters™ introduced the use of SEC columns containing porous hybrid organic/inorganic particles for protein-based SEC. Compared to silica-based, DIOL coated particles, SEC columns containing bridged ethylene hybrid (BEH) particles with surface modified diol groups provided a significant reduction in any charged silanol activity, thus reducing undesired ionic interactions between SEC particles and proteins. In addition, the high mechanical strength of the BEH-DIOL coated particles containing 200Å pores provided the ability to pack stable SEC columns containing 1.7 µm particles in 4.6 mm ID hardware for outstanding mAb separations in an appropriately configured, low dispersion LC capable of withstanding backpressure of up to 15,000 psi (Figure 1). Comparable mAb separation performing BEH-DIOL, 200Å SEC column, containing 2.5 µm and 3.5 µm particles were also available in 7.8mm ID for use on more traditional HPLC Systems where high sample throughput was not a desired performance attribute.

In 2022, Waters scientists recognized two remaining customer needs related to SEC analysis of mAb and ADC. The first involved the desire to further reduce non-desired hydrophobic and ionic interactions occurring between the separated proteins and the DIOL-Coated SEC particles or traditional stainless steel columns. The second request was a SEC offering where a generic "platform" SEC eluent formulation could be used to successfully analyzed many different mAb and associated species.

This document will introduce scientists to the latest performance advantages seen using Waters MaxPeak Premier Protein SEC 250Å column offerings vs previous generations of SEC columns for mAb analyses. It will also include various "tips and tricks" to help users obtain desired separation performance as well as guidelines of how to maximize SEC column life.

Figure 1

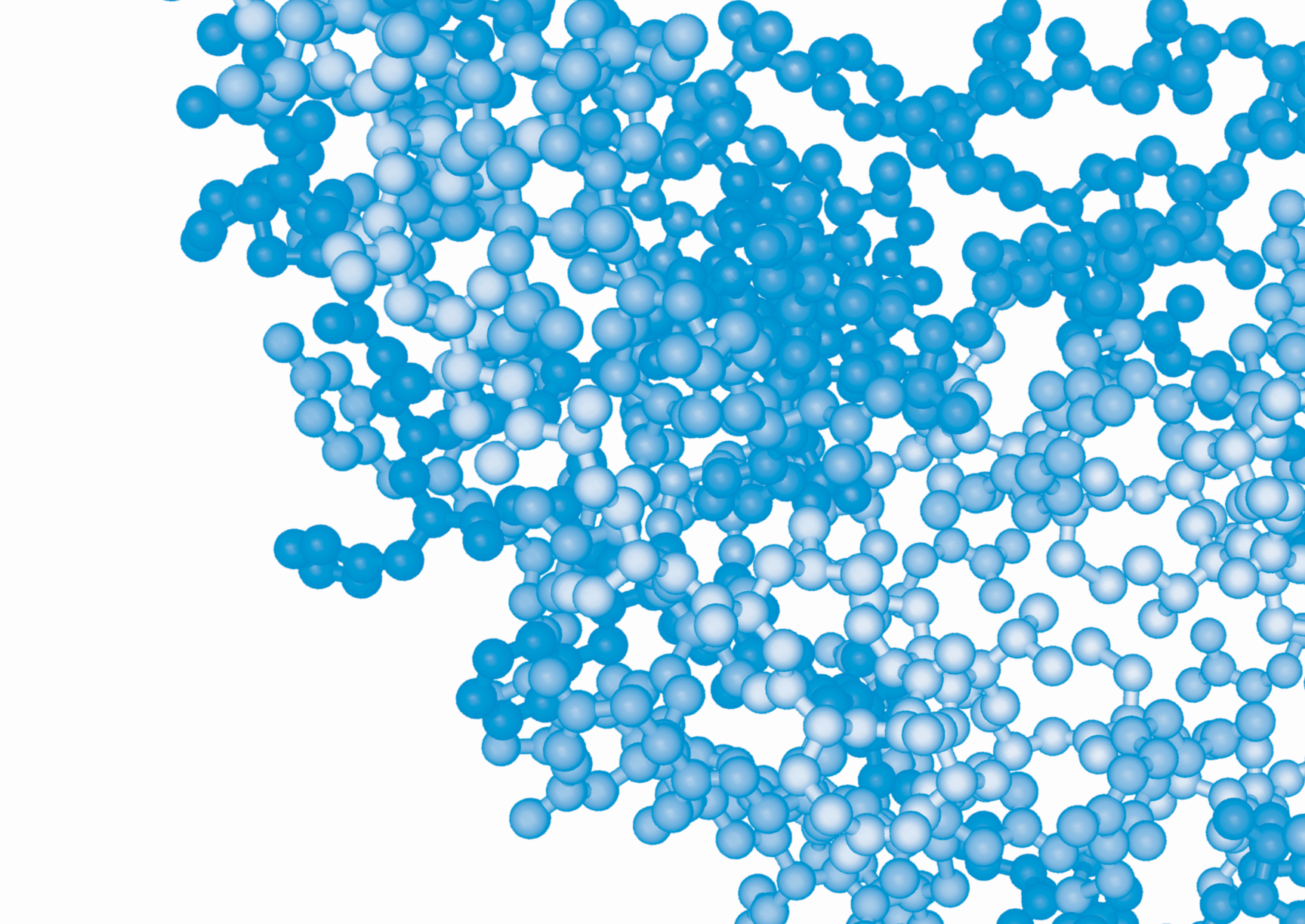
Size Exclusion Chromatography (SEC) is considered today as the reference method for the separation and quantitation of HMWS and low molecular weight species (LMWS) from the main isoform of therapeutic monoclonal antibodies based on the ability to obtain reliable data in relatively short analyses times.



Conditions

System:	ACQUITY UPLC H-Class Bio
Column:	ACQUITY Premier Protein SEC, 250Å, 1.7 µm, 4.6 x 300 mm (p/n: 186005226)
Injection:	5 µL
SEC eluent:	1X PBS (25 mM sodium phosphate with 0.15 M sodium chloride, pH 6.8)
Flow rate:	0.4 mL/min
Temp.:	30 °C
UV detection:	280 nm

Figure 1. Differences between the intact monomer and its size variants for the Waters mAb Size Variant Standard. In particular, the hydrolytic degradant fragment (Fab/c) for the NISTmAb sample like those for other mAbs appears slightly larger than the IdeS digestion fragment although they are of similar molecular weight.



II. Importance of Addressing Undesired mAb Ionic and Hydrophobic Interactions Using MaxPeak Premier Protein SEC 250Å Offerings

II. Importance of Addressing Undesired mAb Ionic and Hydrophobic Interactions Using MaxPeak Premier Protein SEC 250Å Offerings

One of the major limitations in protein size-exclusion chromatography (SEC) is the presence of undesired secondary interactions stemming from a lack of packing material and column hardware inertness. Due to their highly active surfaces, proteins (including mAbs, ADC's, and other biotherapeutic molecules), have a propensity for interaction with metal oxide surfaces present in column hardware and both hydrophobic and electrostatically active sites present on silica and hybrid silica particles. These undesired secondary interactions create significant challenges for resolving protein aggregates, monomers, and fragments from one another, as well as for the accurate quantitation of these species. While there are several commercially available SEC columns that attempt to address these problems, either through column materials or protein pre-conditioning, most still require an appreciable amount of method development to achieve optimal results. Use of high ionic strength mobile phases and the addition of organic solvents are known to help suppress secondary interactions but are in many ways limiting and can make a chromatographer's work more challenging. Improved column inertness would reduce the need for such measures and provide better method flexibility and robustness.

To that end, Waters has developed a novel ethylene bridged-hybrid particle with a high coverage hydroxy-terminated polyethylene oxide (BEH-PEO) surface and coupled it with a first of its kind, hydrophilic high-performance surfaces (HPS) column hardware (Fig 2) . A holistic solution to the problem of undesired secondary interactions in SEC is thereby achieved. The corresponding Waters XBridge and ACQUITY Premier Protein SEC 250Å columns bring a new level of inertness to protein SEC. Through a series of chromatographic tests and comparisons to leading alternatives, we show that the XBridge and ACQUITY Premier Protein SEC 250Å columns require little to no salt or organic solvent additive to achieve excellent resolution and quantitation of protein aggregates and fragments. Figs. 3, 4, and 5) In turn, these columns are highly versatile and can be used with simple physiological buffers, like commercially available phosphate buffered saline (PBS) (Fig. 6).

Figure 2

Waters ACQUITY and XBridge Premier Protein SEC 250Å Columns: A New Benchmark in Inert SEC Column Design

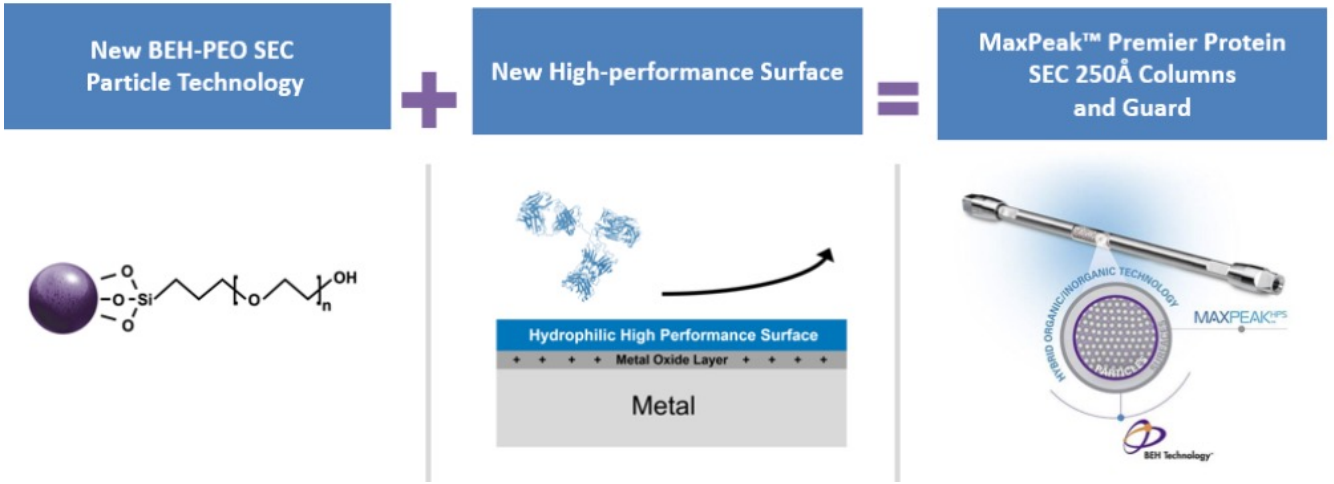


Figure 2. Use of MaxPeak Premier Protein SEC 250Å Column Technology to Reduce Undesired Secondary Interactions.
(A) Hydroxy-terminated PEO bonded BEH particles with low ionic and low hydrophobic secondary interactions.
(B) MaxPeak High Performance Surfaces with hydrophilic properties to minimize secondary interactions between biomolecules and column hardware.
(C) The combination of highly inert particle and hardware surfaces results in a holistic solution to the problem of undesired secondary interactions in protein SEC.

Figure 3

Waters ACQUITY and XBridge Premier Protein SEC 250Å Columns: A New Benchmark in Inert SEC Column Performance

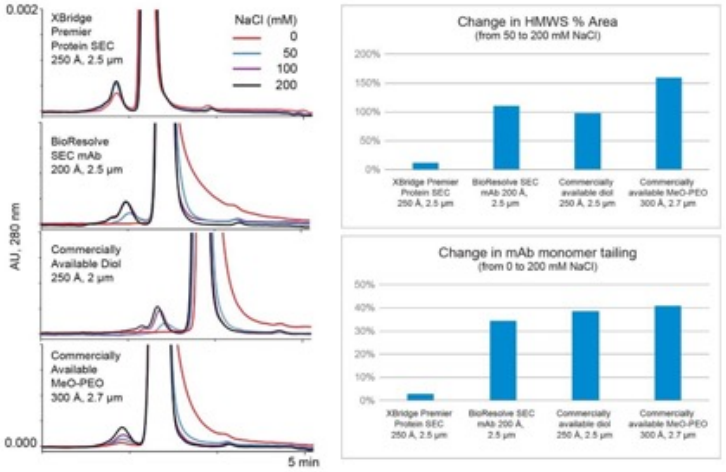


Figure 3. Comparison of ionic secondary interactions performance using NISTmAb (RM 8671) and a 100 mM sodium phosphate pH 6.8 mobile phase containing varying amounts of added NaCl salt. Upon an increase in NaCl concentration, the degree of change observed for the XBridge Premier Protein SEC 250Å, 2.5 µm Column is negligible, with outstanding peak shape and near complete recovery of aggregates with little to no added NaCl. Ionic secondary interactions from column hardware have been mitigated through use of hydrophilic MaxPeak High Performance Surfaces. (Note: As two columns in the comparison showed no recovery of HMWS at 0 mM NaCl, % change calculations for HMWS were made from 50 to 200 mM.)

Figure 4

Waters ACQUITY and XBridge Premier Protein SEC 250Å Columns: A New Benchmark in Inert SEC Column Performance

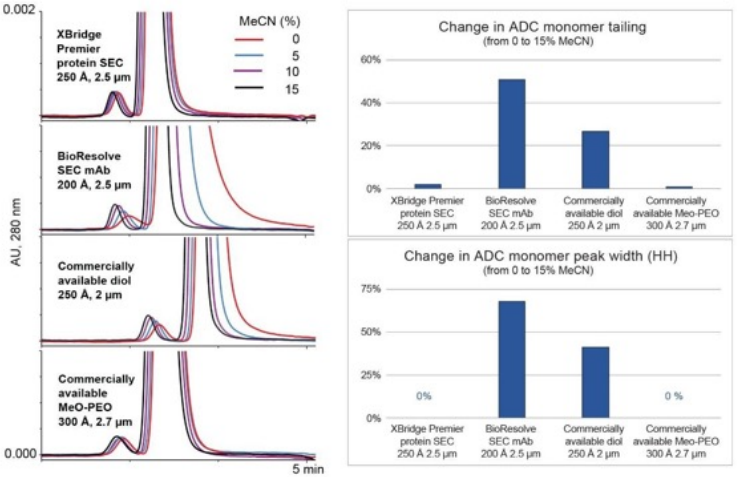


Figure 4. Comparison of hydrophobic secondary interactions performance using the ADC ado-trastuzumab emtansine. Upon increase of organic concentration, the degree of change observed for the XBridge Premier Protein SEC 250 Å, 2.5 µm Column is again negligible, with outstanding peak shape from 0 to 15% MeCN. Hydrophobic secondary interactions from have been largely mitigated through use high coverage hydroxy- terminated PEO packing material bonding.

Figure 5

Shorten SEC Method Development with MaxPeak Premier SEC Columns

Is a Platform SEC Eluent Method Possible?

mAb on Traditional, Diol-Bonded, SEC 200Å, 2.5 µm Column in Stainless Steel (SEC Method Development needed)

mAb on XBridge Premier Protein SEC 250Å, (BEH-PEO) 2.5 µm Column (Less Independent on pH and Salt)

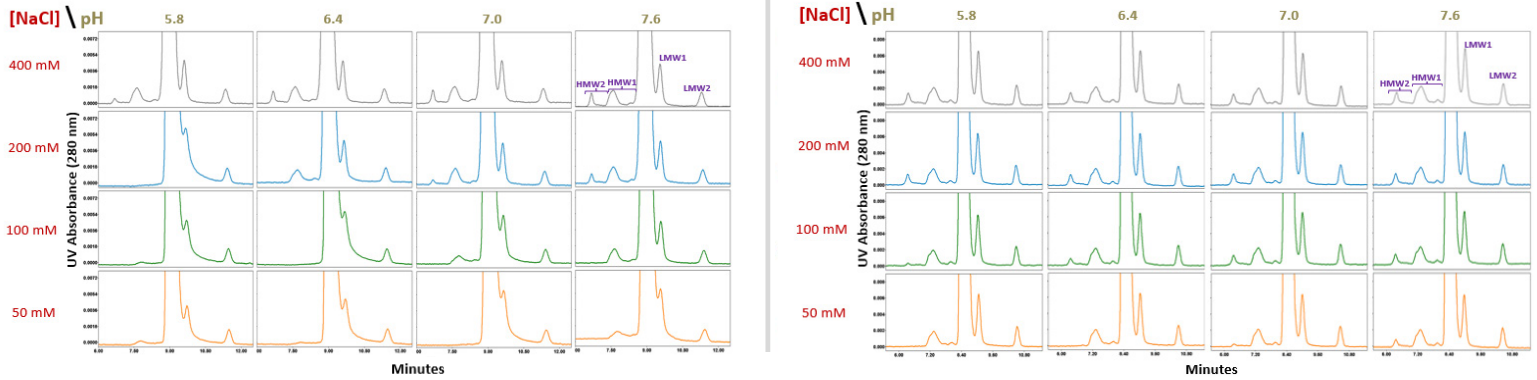


Figure 5. Protein separations using traditional SEC columns require SEC Eluent Method Optimization (Left) vs. results obtained using MaxPeak Premier SEC (Right).

Figure 6

Out of Box Performance of Size Based Protein Variant Separations Using PBS as a Platform SEC Eluent

(Approx. 10,000 to 650,000 Daltons)

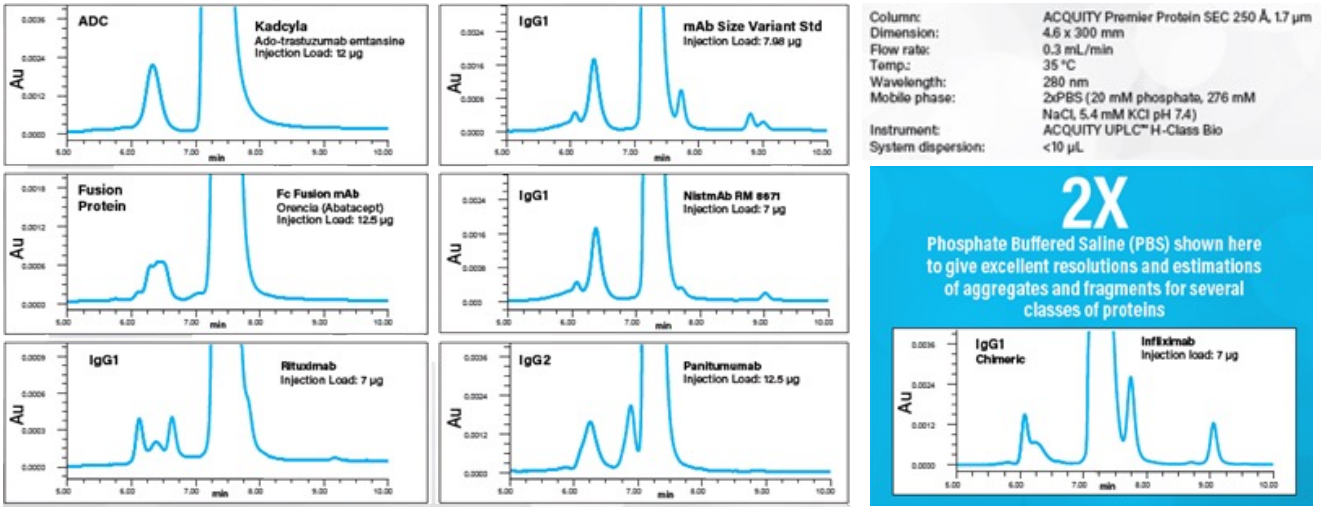
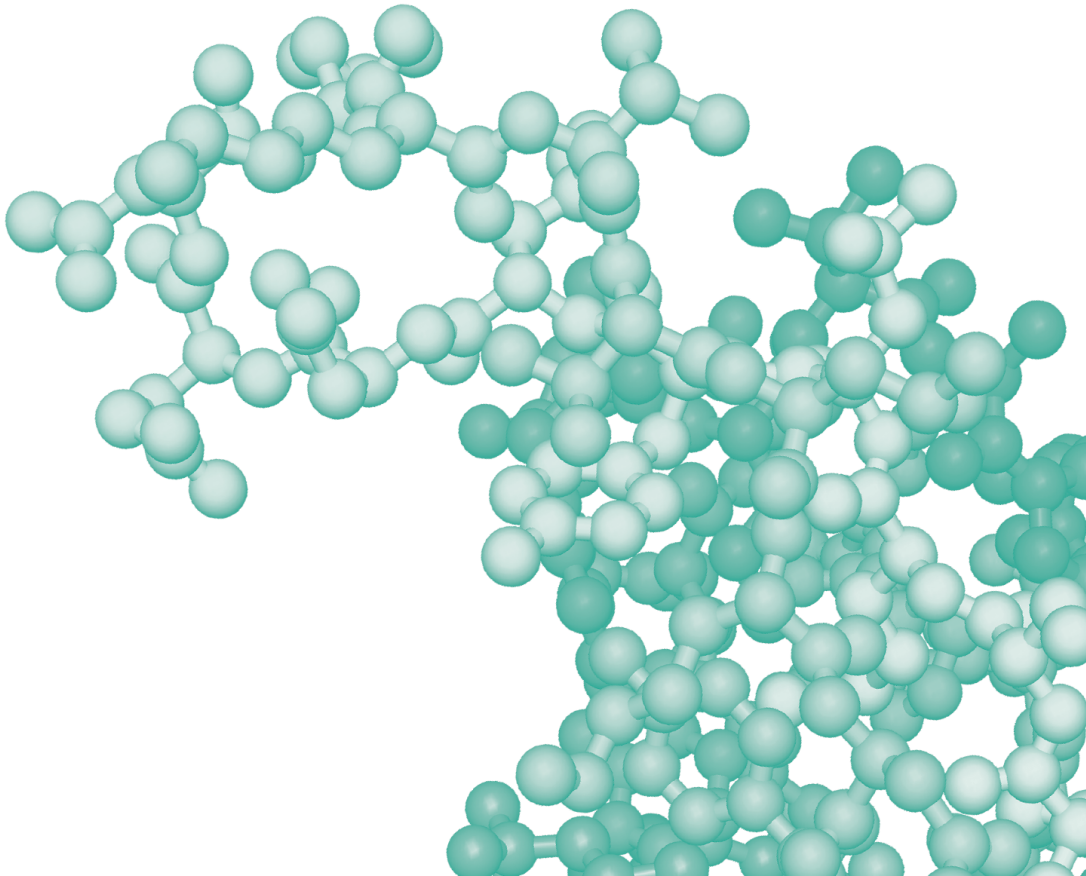
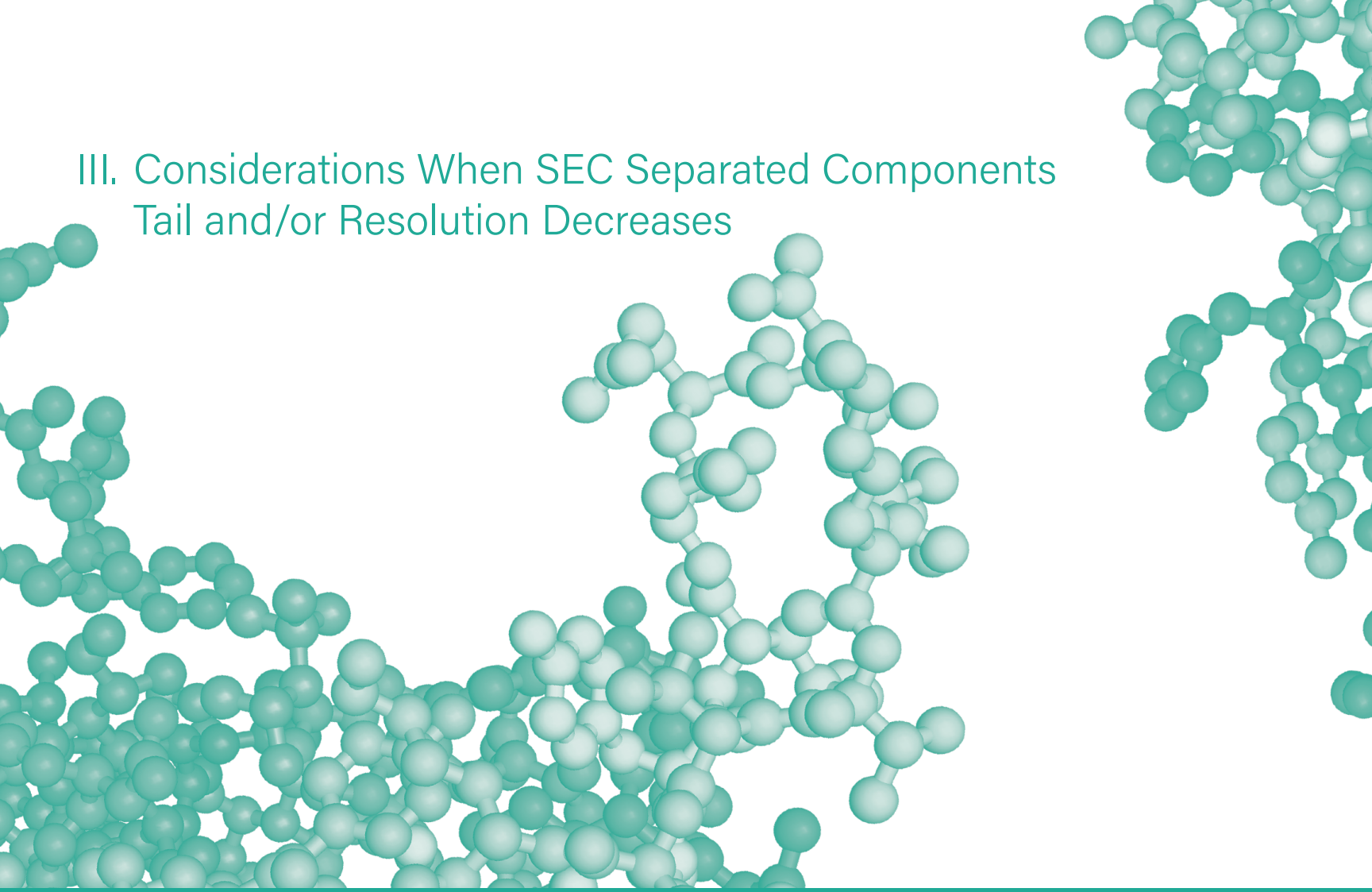


Figure 6. A variety of biotherapeutic proteins can be successfully separated on MaxPeak Premier Protein SEC 250Å Columns using 2X Phosphate Buffered Saline as a Platform SEC Eluent.



III. Considerations When SEC Separated Components Tail and/or Resolution Decreases



III. Considerations When SEC Separated Components Tail and/or Resolution Decreases

a. Connecting MaxPeak Premier Protein SEC 250Å Column to an LC System

It is critical that the MaxPeak Premier Protein SEC 250Å Column be correctly installed onto the LC system. Figure 7 details the effects of proper vs. improper column connections on chromatographic performance. It is strongly recommended that new column inlet and outlet connections be made whenever a new MaxPeak Premier Protein SEC 250Å Column is installed onto the LC system.

⊳► For more information on proper column connections, reference these videos on waters.com:

How to Install a UPLC Column onto an ACQUITY UPLC System with an Active Column Preheater

How to Connect an ACQUITY UPLC Column to an ACQUITY UPLC System

Figure 7

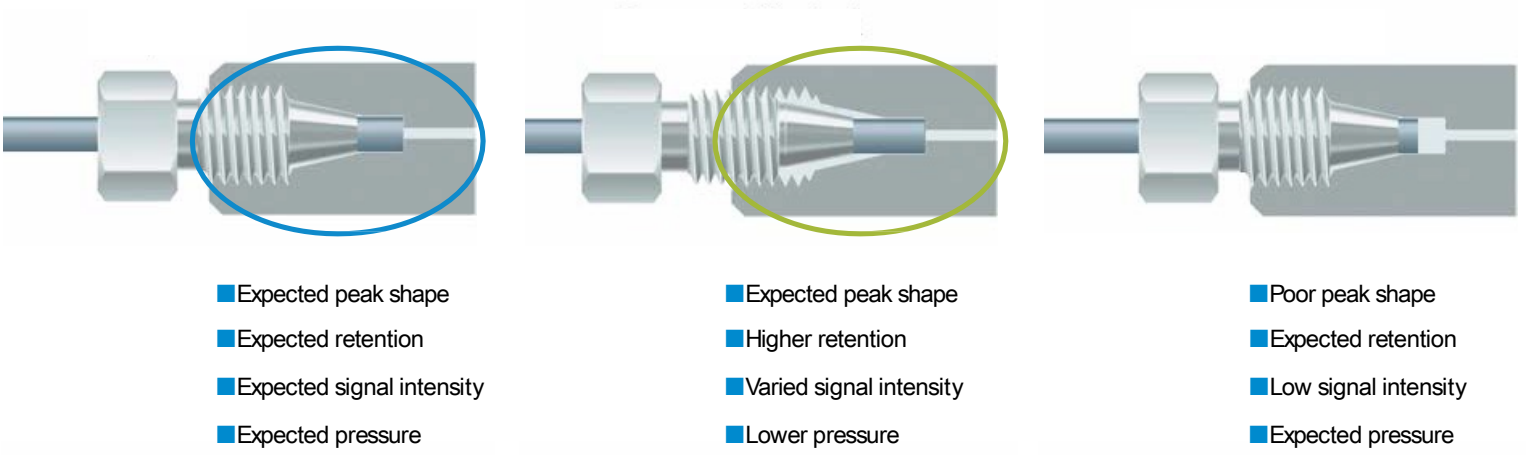


Figure 7. Effects of column-to-LC-system connections on chromatographic performance.

b. Effect of LC System Dispersion on an ACQUITY Premier Protein SEC 250Å, 1.7µm Separation

In SEC, analytes elute within a single column volume during the isocratic separation. It is therefore critical to appreciate that the total LC system volume, including the injector, tubing, and detector volumes, affect the obtained separation. In general, and as shown in Figure 8, the lower the total LC system dispersion volume relative to the column volume, the narrower the peaks, and the more dramatic effect of LC system dispersion volume on the ability to resolve challenging or closely eluting peaks (e.g., mAb monomer from LMW mAb clip 1). For demonstrative purposes, Figure 8 compares the separation of the same mAb on the same ACQUITY Premier Protein SEC 250Å, 1.7 µm 4.6 x 150mm Column with LC Systems that have different dispersions.

Note: Use of the MaxPeak Premier Protein SEC 250Å, 2.5um, 7.8 x 30mm column is recommended for the reliable separation and quantitation of a mAb aggregate, monomer, and fragment on an LC System that may have has a LC System Dispersion greater than approx. 25 µLs.

Figure 8

Effect of LC system dispersion on an ACQUITY UPLC Protein BEH SEC, 200Å, separation of infliximab, mAb IgG1.

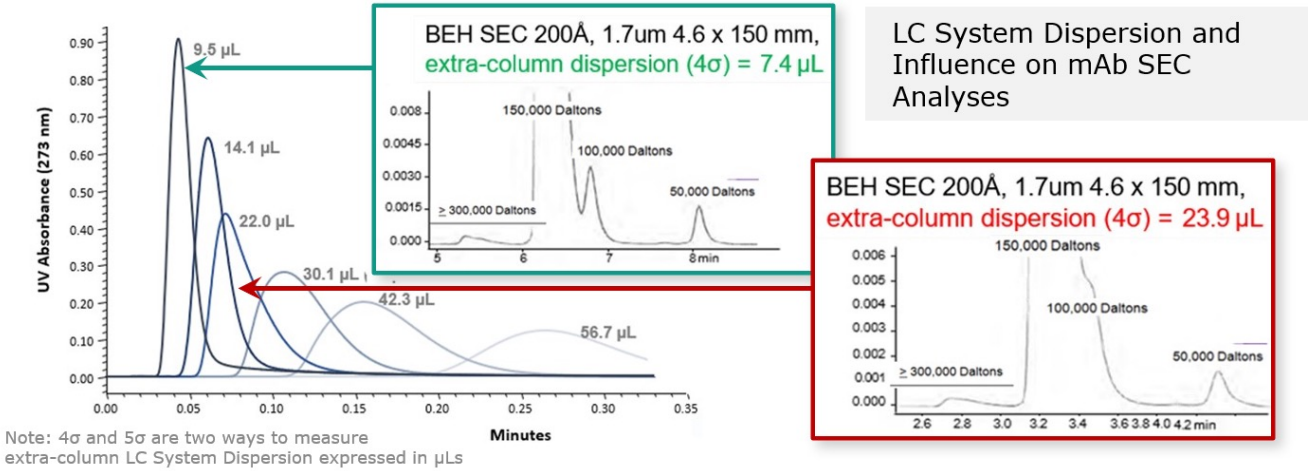


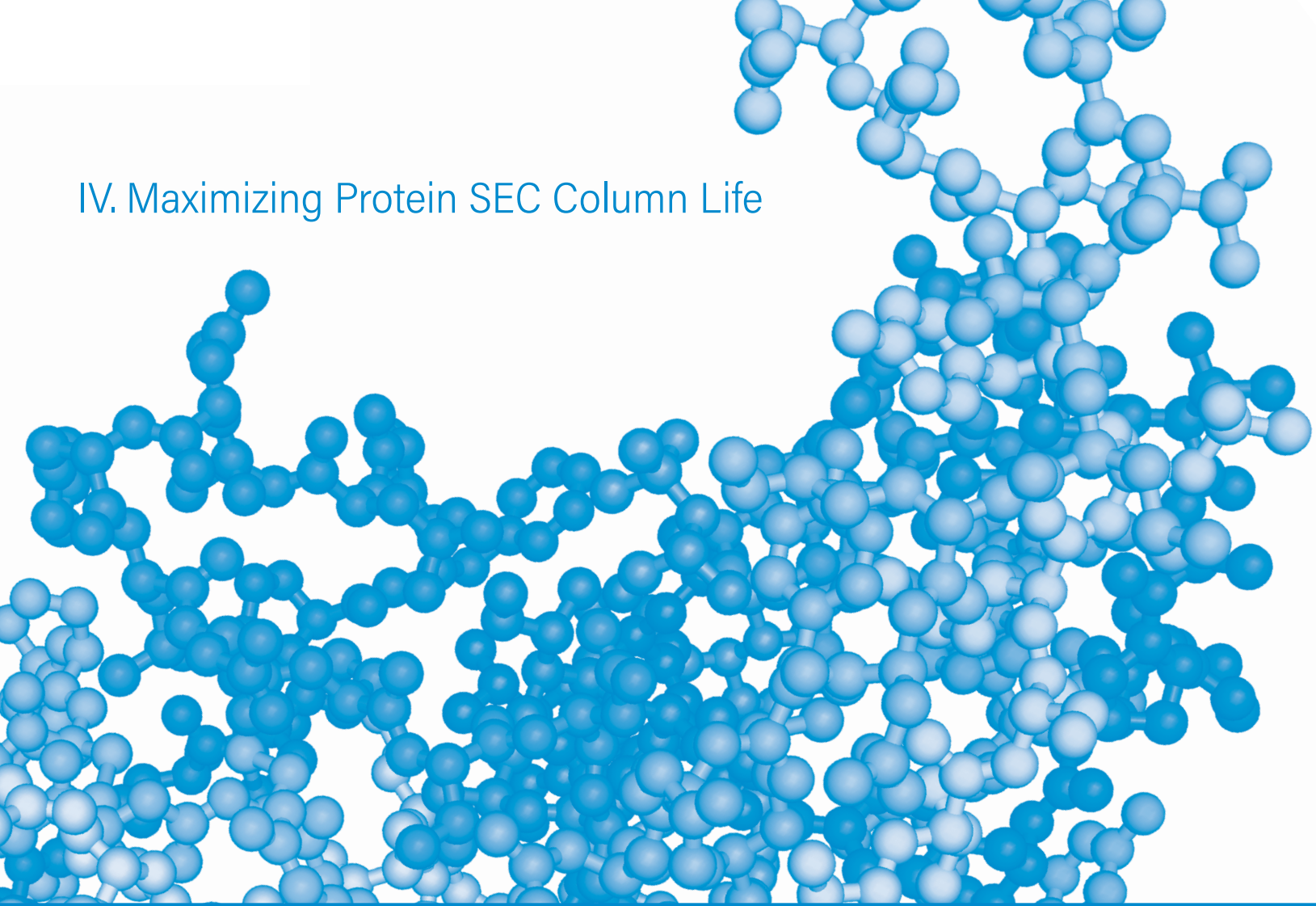
Figure 8. Figure clearly shows how acceptable mAb monomer (150K Da) from fragment (100K Da) results obtained using the 4.6 x 300mm column on a low dispersion LC (i.e., 7.5 µL) with the same SEC column yield poor results on the LC System with 23.9 µL of measure system. Note that the ability to resolve the mAb Aggregate from Monomer (> 2X different in molecular weight / size in solution) is minimally affected by LC System Dispersion

c. Determining the LC System Dispersion Volume

1. Replace the column with a Zero-Volume Union (p/n: 700002636). The LC tubing should be 0.005" I.D. or smaller.
2. Purge all LC solvent, wash, and purge lines with water, then 50/50 water/acetonitrile.
3. Set the detector to 273 nm and collect data at >40 points per second.
4. Flow rate: 0.5 mL/min.
5. Run time: 1 min.
6. Sample: 0.16 mg/mL caffeine in 50/50 water/acetonitrile.
7. Injection volume: 0.5 µL.
8. Inject three mobile-phase blanks followed by five caffeine sample injections.
9. To calculate the LC system volume:
   - Measure the caffeine peak width (in minutes) at 4.4% peak height.
   - Multiply the peak width by the flow rate to determine the peak volume width in mL.
   - Multiply the peak volume width in mL by 1000 to determine the peak volume width in µL.

Note: The average ACQUITY UPLC H-Class System volume dispersion, when measured using 4-Sigma Method, should be = 10.0 µL when used with CH-A, = 12 µL for CM-A, and <22.0 µL for 30CH-A column heater. If your value is greater, determine the source(s) of the deleterious extra peak dispersion volume and correct.

IV. Maximizing Protein SEC Column Life



IV. Maximizing Protein SEC Column Life

With proper care in preparing and handling the SEC mobile phase and samples, Waters SEC columns can deliver stable performance for >1000 sample injections (Figure 9). However, the performance and lifetime of a Waters SEC column will deteriorate if the samples and/or mobile phase introduced into the column contain particulates.

To maximize column lifetime, high quality filtered water (e.g., Milli-Q® Millipak 0.22 µm filtered water) should be used, and mobile-phase buffers should be passed through a 0.2 µm filter. It is recommended that mobile-phase buffers be filtered using a sterile disposable filter. It is further recommended that sintered glass filter supports be avoided when filtering mobile phases due to the potential of introducing silicates which could alter column performance. Use of sterile 0.2 µm filter units is recommended.

Figure 9

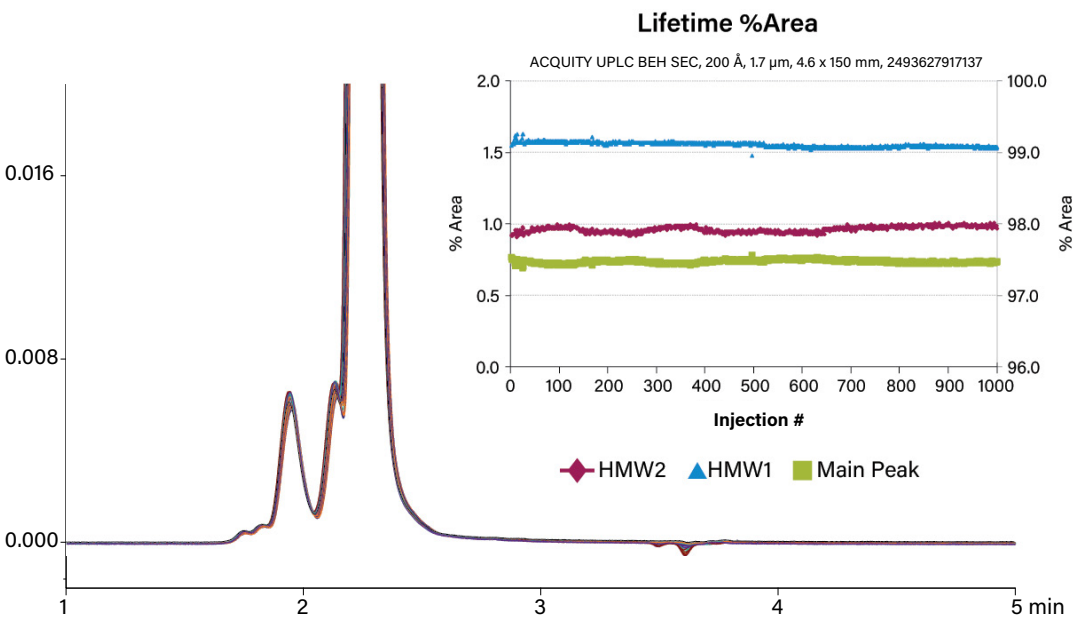


Figure 9. One hundred overlays from 1000 repetitive injections of commercial Vectibix® mAb formulation on an ACQUITY UPLC Protein BEH SEC, 200Å, 1.7 µm, 4.6 x 150 mm Column.

Conditions

System:	ACQUITY UPLC H-Class Bio with Tunable UV (TUV) detector
Column:	ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186005225)
Sample:	Vectibix, 10 mg/mL diluted 1:1 with water
Injection:	3.5 µL
Mobile phase:	0.1 M sodium phosphate with 0.1 M sodium chloride, pH 7.0
Flow rate:	0.5 mL/min
Temp.:	30 °C
UV detection:	280 nm

a. Mobile Phase Preparation and Use

Mobile phases with pH values in the physiological range that do not contain organic solvent can support microbial growth. Implementation of the following recommendations will help minimize microbial contamination, thus avoiding premature column failure.

- Clean laboratory glassware properly and dry at >170 °C for at least 1 hour to avoid potential microbial growth in remaining water droplets. The use of sterile containers is advised.
- Use only high purity water (18.2 MΩ cm) when preparing SEC mobile phases. If bottled water is used, it should be opened the day of use.
- Always filter prepared mobile phase through compatible 0.22 µm or smaller membrane filters. The use of sterile filters and containers is also recommended.
- Never "top-off" mobile-phase bottles. Always change bottles when replacing the mobile phase.
- Replace low-ionic-strength mobile phases (<150 mM) every 2–3 days.
- High-ionic-strength mobile phases (>150 mM) should be replaced every two weeks. This longer interval is acceptable because high salt concentrations will inhibit microbial growth.
- All mobile-phase bottles should be visually inspected daily for microbial growth and/or particulates. Microbial growth may form a film on the bottle surface and may be observed by swirling the bottle.
- **It is highly recommended to remove and not use the Waters Mobile-Phase Line Sinkers when performing SEC with 100% aqueous mobile phases since they may become contaminated with microbes, thus contaminating freshly prepared solutions of filtered mobile phase.**

Note: Waters Corporation will honor an existing service contract at sites that have removed the Mobile-Phase Line Sinkers provided that the account's SOP states that all SEC mobile phases must be filtered and contained in sterile glassware.

- If microbial growth is observed, immediately discontinue SEC analysis and perform the LC cleaning procedure detailed later in this document.

Additional Considerations:

- To prevent potential precipitation of the buffer from the mobile phase in the LC system, good laboratory practice suggests maintaining a low flow rate (e.g., 0.1 mL/min) through the system after the SEC column has been removed and stored as recommended.
- If the LC system will be idle for more than two days, and after the SEC column has been removed and stored as recommended, prime each mobile-phase line for 15 minutes at 1 mL/min with high purity water followed by flushing and storage in 70/30 isopropanol (IPA)/water to prevent microbial growth.

b. Avoiding Microbial Contamination of the Solvent Delivery System

Microbial contamination of the LC system may cause BEH SEC column fouling, resulting in an unacceptable separation (Figure 10). The column pressure may also increase significantly. Note that injection of particulates (e.g., microbes) will result in premature failure of any HPLC or UPLC column. However, failure may occur sooner on columns containing <3 µm particles. If the mobile-phase reservoir becomes contaminated with microbes, the microbes will enter the solvent delivery flow path and may contaminate fresh mobile phase. The mobile-phase line sinkers are one location that commonly harbors microbes (Figs. 11 and 12).

Figure 10

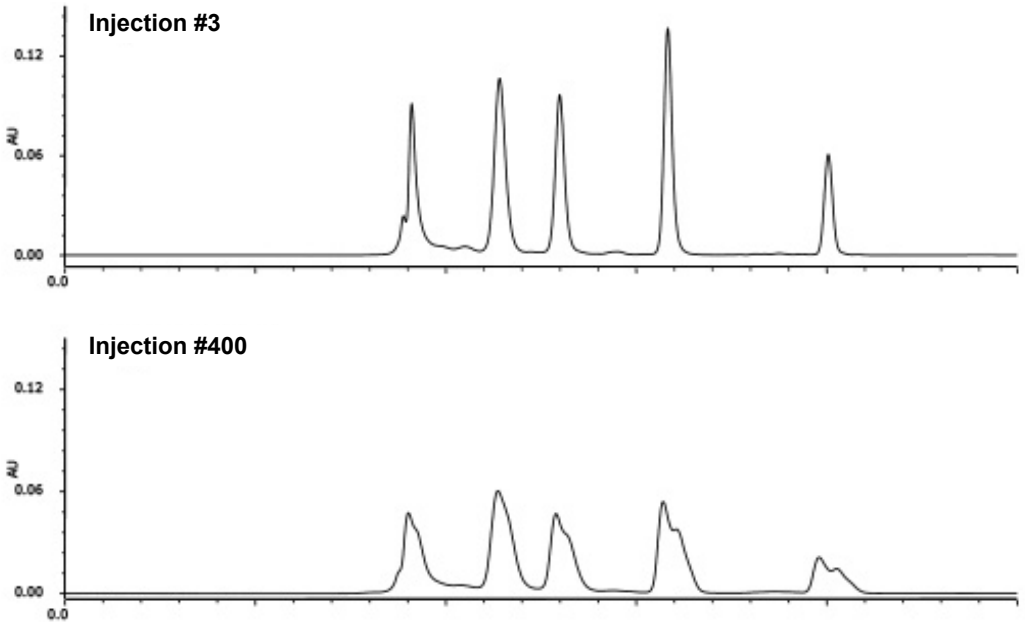


Figure 10. Effect of microbial growth on an ACQUITY UPLC Protein SEC separation of the BEH200 SEC Protein Standard Mix (p/n: 186006519). Contamination was confirmed by analysis of the column inlet frit (Figures 11 and 12).

▻► For sample and separation conditions, reference "Waters ACQUITY UPLC Protein BEH SEC Columns and Standards Care and Use Manual", p/n: 720003385EN on waters.com.

Figures 11 and 12

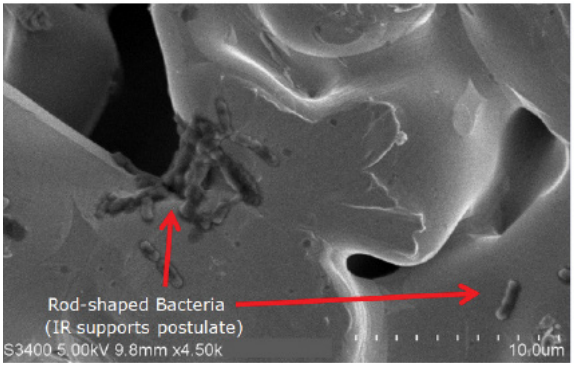
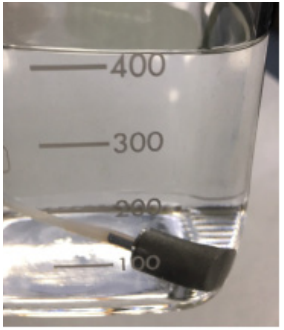


Figure 11. Scanning electron micrograph of the inlet frit removed from an ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm Column contaminated with bacteria.

a)



b)

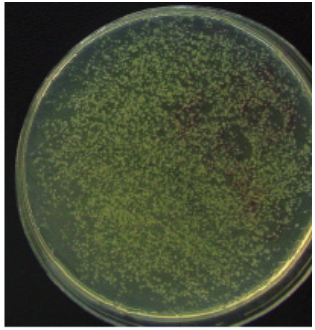


Figure 12. Mobile-phase line sinker (Figure 11) from a microbe-contaminated SEC mobile-phase bottle. The sinker was aseptically removed, suspended in sterile PBS, then 500 µL was applied to a microbe supporting agar plate. Figure 12 confirms the presence of microbes contained in the removed SEC mobile-phase line sinker.

c. Cleaning System for SEC Analyses

To clean an LC system used in 100% aqueous SEC methods, the use of 100% IPA, as used in the standard UPLC cleaning protocols, should be replaced by using 70% isopropanol (IPA) or 70% ethyl alcohol (EtOH) due to their ability to kill bacteria. One hundred percent concentration of either IPA or EtOH does not penetrate the cell wall of bacteria and is therefore not an effective bactericide nor are methanol-containing solutions (Reference 3). Waters has experienced situations where "microbe-contaminated-mobile-phase filters" (a.k.a. sinkers) have resulted in rapid particulate fouling of a new ACQUITY UPLC Protein BEH SEC Column even when freshly prepared and filtered mobile phase is used. This is why Waters recommends that mobile phase sinkers NOT be used for this SEC application as previously detailed.

Note: The suggested LC system cleaning protocols below (e.g., flow rates and times) were developed for the ACQUITY UPLC System. Adjustments in flow rates and flow duration may be necessary for those using different LC systems.

There are three different protocols for cleaning/purging ACQUITY UPLC Systems that can be used under different circumstances.

1. **For a system that is in continuous use with sterile filtered mobile phase:**
   The best practice for the daily use of buffers starts with fresh mobile phase in a new bottle (no topping off). After installing fresh mobile phases, wet prime (a function of Empower™ Software) the buffer lines for 4 minutes if the system was used the day before. If the system was not used the day before, wet prime all lines for 6–10 minutes. This may be accomplished by using the system startup function and changing the prime time as needed. This function also primes the seal wash, purge, and needle wash solvent lines. Keep in mind that when the ACQUITY UPLC System is wet primed, under the control of the ACQUITY UPLC console, the column can remain on the system connected to the pump and detectors.

   The Empower solvent priming function diverts the priming solvents directly to waste. This makes it very easy to purge your lines with mobile phase or the other solvent sequences described below. It does require the movement of your solvent lines, but if you catch a bubble during the process, the wet priming command will eliminate it.

2. **For a system that has been left unused for more than 2–3 days:**
   If your system is left in buffer with or without flow for more than 2–3 days, the lifetime of your column may benefit by wet priming all buffer lines with **water for 4 minutes**, then with **70% IPA for 6–10 minutes** before going **back to water for 4 minutes** and **then your buffer for 4 minutes** before starting another analysis. It is further recommended to leave your system in 70% IPA (after priming the buffer lines with water) when not in use. When restarting, wet prime your system with 70% IPA for 3–4 minutes then water for 4 minutes before wet priming your system with your desired buffer. Before attaching the SEC column to the LC system, allow the freshly prepared SEC buffer to flow through the ACQUITY UPLC System for approximately 5 minutes at a flow of 0.4 mL/min to clear out any previously used mobile phase.

3. **For routine periodic cleaning for preventive maintenance or when the system is contaminated:**
   Table 1 is a summary of the cleaning protocol and reagents required to execute this procedure, which can be used at regular intervals (every 3-6 months) for preventive maintenance.
   a. Disconnect the column and attach a V-Detail Zero Volume Union (p/n: 700002636) to the column inlet and outlet lines.
   b. For each step of the cleaning protocol, monitor the system pressure to keep it <1000 psi to prevent damage to the detector flow cell. If necessary, replace the backpressure device with large bore waste tubing.
   c. If the system has recently been used with mobile-phase buffers or salts, it should be flushed thoroughly with 100% high-purity water prior to the introduction of organic solvents.
   d. Place A, B, C, D, seal wash, sample wash, and purge lines into a clean bottle containing 70% isopropanol as the cleaning solvent.

Note: If there is an incompatibility between the mobile phase or wash solvents with 70% isopropanol, flush first with the appropriate intermediate solutions to ensure compatibility.

e. Prime solvent lines A, B, C, and D for 5 minutes each. The Empower Prime command vigorously forces solvents through the lines and system at 4 mL/min to assist in the cleaning process.

f. Prime the seal wash.

g. Prime the wash solvent for 200 seconds and the purge solvent for 40 cycles.

h. Purge the system at 0.2 mL/min for 10 minutes using 25% A, 25% B, 25% C, and 25% D.

i. Repeat Steps d through h using **100% methanol** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

j. Repeat Steps d through h using **100% high-purity water** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

Caution: If a nebulization-based detector is connected (MS or ELSD), take it offline before performing Step k. Direct the flow from the union outlet to waste.

k. Repeat Steps d through h using **10% aqueous phosphoric acid** as the cleaning solvent, using a flow rate of 0.2 mL/min for Step h.

Caution: To avoid damage, do not place the seal wash line in this solution. Place the seal wash line in high purity water.

l. Repeat Steps d through h using **100% high-purity water** as the cleaning solvent, using a flow rate of 1.0 mL/min for Step h.

m. If applicable, reconnect the MS or ELS detector.

n. Repeat Steps d through h using **100% methanol** with a flow rate of 1.0 mL/min for Step h.

Table 1. ACQUITY UPLC System Cleaning Protocol Summary. *Check (✓) when step is completed.*

Eluent	Flow Rate	Prime Lines A, B, C, D (5 min each)	Prime Wash Solvent (200 sec)	Prime Purge Solvent (40 cycles)	Prime Seal Wash	Purge A/B/C/D 25/25/25/25 (10 min)
IPA	0.2 mL/min					
Methanol	1 mL/min					
High Purity Water	1 mL/min					
Leave seal wash line in high purity water. If used, remove nebulization-based detectors (MS or ELSD) from flow path.						
10% Aqueous Phosphoric Acid	0.2 mL/min					
High Purity Water	1 mL/min					
If used, return nebulization-based detectors to flow path.						
Methanol	1 mL/min					

Required Cleaning Solvents	
100% high purity water	Milli-Q water or equivalent
100% methanol	Fisher Methanol, Optima™ #A454-4 or equivalent
70% isopropanol (IPA) in high purity water (v,v)	Fisher 2-Propanol, Optima #A464-4 or equivalent
10% phosphoric acid (aqueous)	Prepare 10% (v/v) using HPLC-grade 85% phosphoric acid and high-purity water

d. Minimizing Sample Particulate Contamination of a Waters SEC Column

The performance and lifetime of a Waters SEC Column can deteriorate due to the accumulation of particulates. These include insoluble protein aggregates contained in the injected sample. As a result, it is recommended to centrifuge all protein-containing samples so that any insoluble particulates form a solid pellet at the bottom of the vial (Fig 13) being sure to inject ONLY the clear supernatant.

Note: Be sure to adjust the ACQUITY UPLC needle depth to prevent sampling any particulates located at the bottom of the centrifuged vial. For more information on setting the needle depth and minimum volume in vials, reference "Sample Vials and Accessories", p/n: 720001818EN on waters.com.

Note: Some scientists have expressed a concern that protein sample centrifugation will compromise need to obtain accurate quantitation of the injected protein aggregates. However, injection of insoluble protein aggregates results in their accumulation at the head of the SEC column resulting in premature column failure. It does not compromise the generation of accurate solution protein aggregate vs protein monomer quantitation. (Data not shown)

Figure 13

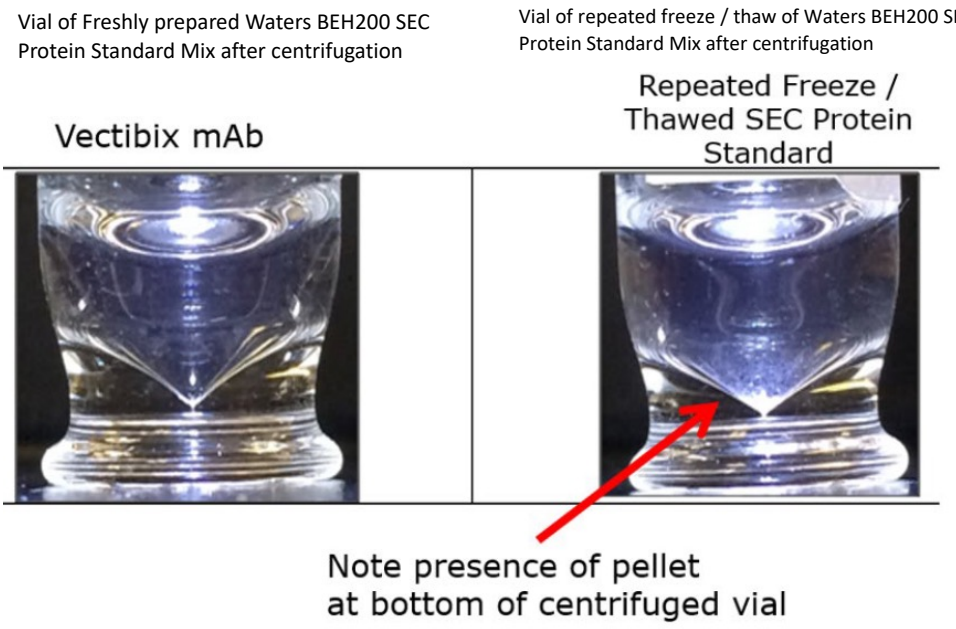


Figure 13. Centrifugation of a freshly prepared Waters BEH200 SEC Protein Standard Mix at 15,000 for 15 min does not show presence of a protein pellet (left) while a pellet is clearly visible (right) after repeated freeze / thawed BEH200 SEC Protein Standard Mix that was used in the previously shown column stability study

e. Minimizing SEC Eluent or LC System Related Particulate Injection onto a Waters SEC Column

Injection of particulates as well as excipients contained in the mAb sample matrix onto any SEC column can shorten its useful life . Use of an appropriate Waters SEC Guard can effectively prolong analytical column lifetime without compromising the desired SEC separation (Figure 14). The useful lifetime of the guard column can be influenced by many factors including:

- Mobile phase cleanliness/microbial contamination
- Sample precipitates/aggregation
- Excipients in sample formulations
- Working at extremes of pressure, pH, and/or temperature

A Waters SEC Guard Column may need replacement, if the following are observed:

- Significant increase in measure SEC column pressure
- Tailing or split peaks (Fig 10).

Figure 14

LC system:	ACQUITY UPLC H-Class Bio
Column:	XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm; MaxPeak Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 30 mm Guard; and Waters mAb Size Variant Standard (p/n: 176004795)
Column temp.:	Ambient
Mobile phase:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Flow rate:	0.200 mL/min
Sample manager temp.:	10 °C
UV detection:	280 nm, 10 Hz, fast filter
Seal wash:	10% HPLC-grade methanol/ 90% 18.2 MΩ water v/v
Sample manager washes:	18.2 MΩ water
Reconstitution:	70 µL of 18.2 MΩ water
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	3.5 µL of Waters mAb Size Variant Standard

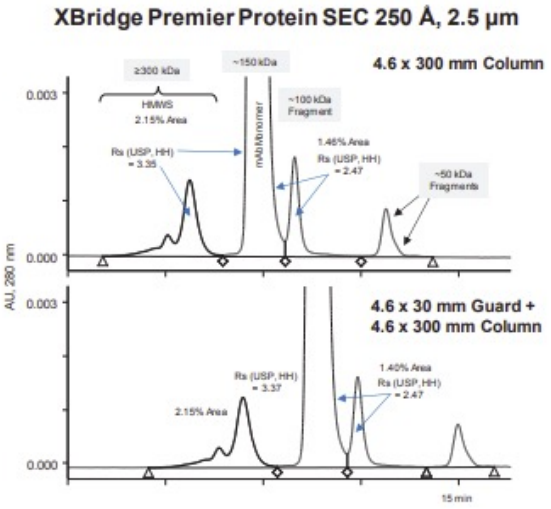
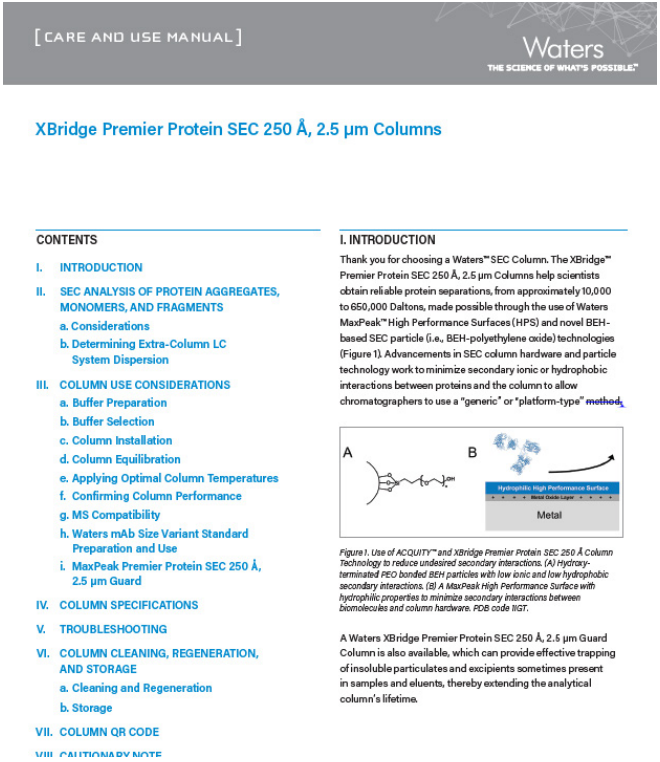


Figure 14. Separation of Waters mAb Size Variant Standard on an XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm Column with (bottom) and without (top) a MaxPeak Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 30 mm Guard. The % area and resolution values are virtually unchanged when running with the guard.

f. Column Storage



[CARE AND USE MANUAL]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

XBridge Premier Protein SEC 250 Å, 2.5 µm Columns

CONTENTS

I. INTRODUCTION

Thank you for choosing a Waters™ SEC Column. The XBridge™ Premier Protein SEC 250 Å, 2.5 µm Columns help scientists obtain reliable protein separations, from approximately 10,000 to 650,000 Daltons, made possible through the use of Waters MaxPeak™ High Performance Surfaces (HPS) and novel BEH-based SEC particle (i.e., BEH-polyethylene oxide) technologies (Figure 1). Advancements in SEC column hardware and particle technology work to minimize secondary ionic or hydrophobic interactions between proteins and the column to allow chromatographers to use a "generic" or "platform-type" method.

Figure 1. Use of ACQUITY™ and XBridge Premier Protein SEC 250 Å Column Technology to reduce undesired secondary interactions. (A) Hydroxy-terminated PEO bonded BEH particles with low ionic and low hydrophobic secondary interactions. (B) A MaxPeak High Performance Surface with hydrophilic properties to minimize secondary interactions between biomolecules and column hardware. PDB code 1IGT.

A Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Guard Column is also available, which can provide effective trapping of insoluble particulates and excipients sometimes present in samples and eluents, thereby extending the analytical column's lifetime.

Example of Waters very comprehensive SEC care and use manuals to help ensure satisfactory SEC Column performance

Note: Storage of column in 100% water or 100% buffer is not recommended since this may compromise column performance and allow microbial growth

1. Recommended storage solution is to purge the column with the shipping solvent
   - ► 10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM KCl
   - ► A minimum of five-column volumes should be used
   - ► When storing the column in the shipping solvent listed above, it is preferable to keep the column at ambient (room) temperature
2. Alternative buffer combinations may also be used
   - ► Buffers should contain 50–100 mM salt (KCl or NaCl) and a maximum phosphate concentration of 50 mM
   - ► Addition of 10% acetonitrile, 20% methanol, or 0.05% sodium azide should be added to eliminate microbial growth
   - ► Do NOT store in 100% water
   - ► Refrigeration is not recommended as buffer salts can precipitate and lead to shortercolumn lifetimes

V. Summary and References

V. Summary and References

Summary

With the practices described, reliable protein aggregate, monomer, and fragment separations, with accurate quantitation can be obtained on Water MaxPeak Premier Protein SEC 250Å Columns, offering faster and higher resolution separations than those obtained using a previous generation of LC-based SEC methods. To maximize the lifetime of a Water MaxPeak Premier Protein SEC 250Å Columns, it is important to avoid the injection of sample, SEC Eluent, or LC System related particulates. The use a MaxPeak Premier Protein SEC 250Å Guard Column is an effective way to protecting your analytical SEC column from deterioration due to these types of SEC separation failure mechanisms.

V. Summary and References

References

1. Hong, P.; Koza, S.M.; Bouvier, E.S.P. A Review: Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. J. Liq. Chrom. Rel. Tech. 2012, 35(20), 2923-2950.
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5. Kizekai, L.; Shiner, S.; Lauber, M. Waters ACQUITY and XBridge Premier Protein SEC 250 Å Columns: A New Benchmark in Inert SEC Column Design. Waters Application Note, 720007493EN (2022).
6. Berthelette, K.D.; Nguyen, Jennifer.; Kizekai, L.; Warren, J.; Shiner, S. Improved Separation Performance of Monoclonal Antibody and Antibody Drug Conjugates Using Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Column Waters Application Note, 720007528EN (2022).
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10. Koza, S. M.; Jiang, A.; Yu, Y.Q. Rapid Size Variant Analysis of Monoclonal Antibodies Using UPLC™ and HPLC Compatible MaxPeak™ Premier Protein SEC Columns. Waters Application Note, 720007584EN (2022).
11. Koza, S. M.; Yu, Y.Q. MaxPeak Premier Protein SEC 250 Å Column Lifetimes at Physiological pH for Polysorbate (Tween) Formulated Biosimilar Monoclonal Antibodies. Waters Application Note, 720007523EN (2022).
12. Yang, H.; Koza, S. M., Yu, Y.Q. USP Monograph mAb SEC Method Robustness on the XBridge Premier Protein SEC Column. Waters Application Note, 720007481EN, (2021).
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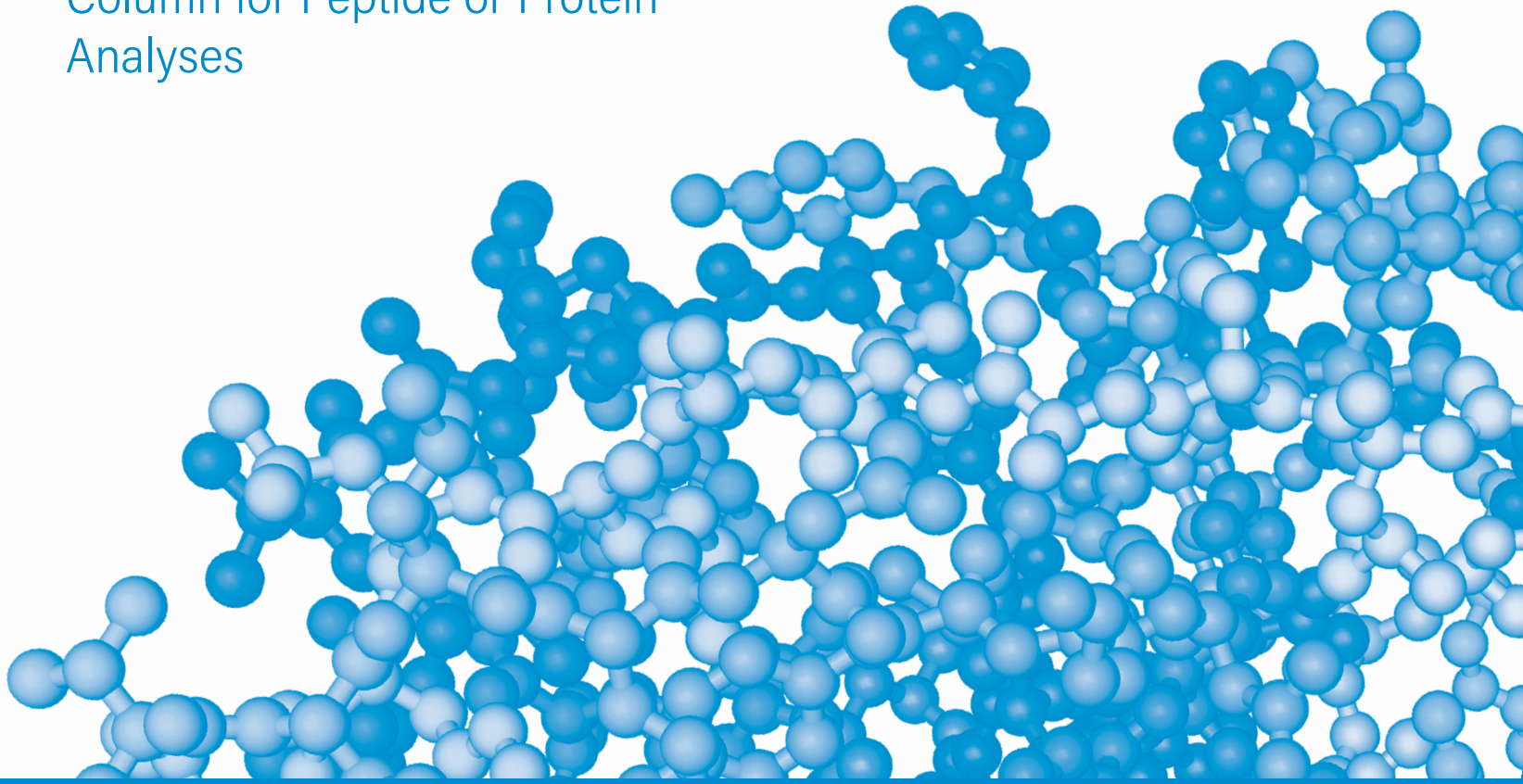
VI. Selecting an Appropriate SEC Column for Peptide or Protein Analyses



VI. Selecting an Appropriate SEC Column for Peptide or Protein Analyses

As previously discussed, SEC is the analytical "gold standard" for the separation and accurate quantitation of aggregates contained in biotherapeutic peptides and proteins.
The principle of SEC chromatography involves the ability of an appropriately selected column to separate molecules based on differences in the molecules' "size in solution" that loosely correlates to their molecular weight.
The following four factors provide guidance for selecting an appropriate SEC column that matches your application and laboratory needs.

1) Molecular Weight vs. SEC Particle Pore Size

The SEC particle's pore size and sample molecular weight (MW) go in-hand when selecting an SEC column. The pore size of the column media, generally expressed in angstroms (Å), determines both how quickly a sample will travel through the column and how well the sample will be retained in relation to the sample's molecular weight. The inclusion of "in relation to your samples molecular weight" is an important distinction to make here. Without it, it might be assumed that smaller pore size equals better results, however, that is not the case. For example, if the pore size is too small, based on the sample's MW, larger molecules will not move as freely, reducing retention and column efficiency.

Therefore, the MW of the substance being tested would influence, if not determine, what column pore size to choose. A sample with a molecular weight between 1000–8000 Da would be best suited for a 125 Å column. This selection will provide better retention characteristics in separating small compounds compared to a similar column with a pore size of 200 or 450 Å. If the sample's molecular weight is between 10,000–450,000 Da, then a column pore size of 200, 250 or 450 Å should be chosen. Any sample with a MW over 450,000 Da should be analyzed with a 450 Å column.

2) LC System Dispersion

LC system dispersion can also significantly affect SEC column choice. In SEC, analytes elute within a single column volume during the isocratic separation. This makes it important to consider the total LC system volume, including the injector, tubing, and detector volumes of the obtained separation. In general, the lower the total LC system dispersion volume relative to the column volume, the narrower the peaks.

Examples of LC system dispersion specifications, without column heater, and SEC column recommendation:
LC system dispersions <20 µL (UPLC) = 4.6mm ID column containing 1.7 µm SEC particles
LC system dispersions >20 – <35 µL (UHPLC) = 7.8mm ID column containing 2.5 µm SEC particles
LC system dispersions >35 µL (HPLC) = 7.8mm ID column containing 2.5 or 3.5 µm SEC particles

3) Resolving Components That Are Less Than Two-Fold Different in Molecular Weight

The ability to adequately resolve compounds that differ by two-fold in molecular weight (e.g., 300K Dalton, mAb IgG dimer from 150K Dalton monomer) can be relatively easy to accomplish when using an appropriate SEC column. However, a far more challenging scenario involves the species separation that differs by less than 2x molecular weight (e.g., 150K Dalton, mAb IgG monomer from 100K Dalton "Clip"). In addition, the ability to obtain reliable quantitation is challenged when the minor components exist at <0.5% compared to the major peak of interest.

4) Speed of Separation

The final factor to consider when selecting an appropriate SEC column is the desired speed for the separation. Generally, there is a trade-off between resolution and speed when implementing size-exclusion chromatography. However, a balance can be achieved by selecting the appropriate column. When an SEC column containing comparatively smaller particles (e.g., 1.7 µm) is used on an appropriate LC system, quicker results are obtained which differs from separations performed on larger particle-sized (2.5 or 3.5 µm) SEC columns. For example, an SEC 1.7 µm, 4.6 × 300 mm column can provide excellent resolution in under nine minutes. Meanwhile a separation on an SEC 2.5 µm, 7.8 × 300 mm column will generally take approximately 12 minutes; and, on an SEC 3.5 µm, 7.8 × 300 mm column it will take 18 minutes.

An appropriate SEC column selection, that is based on the separation needs and the LC system being used, can generate reproducible separations and accurate component quantitation for various protein and peptide samples. To get the best resolution, reproducibility, and speed, keep in mind the four factors outlined above and how they relate to your specific samples. This will help ensure you select the best possible column for your application.

Four-Step Guide for Successful SEC Column Selection

What is the molecular weight of what you are trying to separate?			
NEED:	**MW 1–8K Da**	**MW 10–450K / 650K Da**	**MW 100–1500K Da**
Recommended column specifications	125 Å	200 A / 250 A	450 Å

What type of LC system dispersion* are you using?			
NEED:	**<20 µL (UPLC)**	**>20–<35 µL (UHPLC)**	**>35 µL (HPLC)**
Recommended column specifications	1.7 µm or 2.5 µm	2.5 µm	2.5 µm or 3.5 µm

Do you need to resolve something that is less than 2-fold difference in MW?**			
NEED:	**2.5 µm**	**2.5 µm**	**2.5 µm or 3.5 µm**
REC. Recommended column specifications SPEC:	4.6 × 300 mm or 7.8 × 300 mm	7.8 × 300 mm	7.8 × 300 mm

Do you need maximum speed on a MW greater than two-fold?			
NEED:	**<9 min**	**<12 min**	**<18 min**
REC. Recommended column specifications SPEC:	1.7 µm 4.6 × 150 mm	2.5 µm 4.6 × 150 mm	2.5 µm 7.8 × 150 mm

**For guidance on measuring system dispersion, download the SEC Optimization Guide (720006067EN) on waters.com.*
*** To understand the "why" behind these recommendations, read the Application Note (720006336EN) on waters.com.*

VII. Educational Webinars on Principles and Practice of SEC

Online LC Tutorials

Size-Exclusion Chromatography: An Overview of the Principles and Techniques to Obtain Reliable Protein Size-Based Separations

The available tutorials in this course include:

- [Part 1] Analytical and Use of SEC for mAbs
- [Part 2] Principles and Practice of SEC for Reliable Monoclonal Antibody Analyses
- [Part 3] Selecting Appropriate SEC Pore Size for Reliable Protein / Peptide Separations
- [Part 4] Choosing Appropriate SEC Column Size based on LC System and Application Goals
- [Part 5] Considerations to Maximize SEC Column Life
- [Part 6] Developing Robust SEC Methods for Protein Size Variants

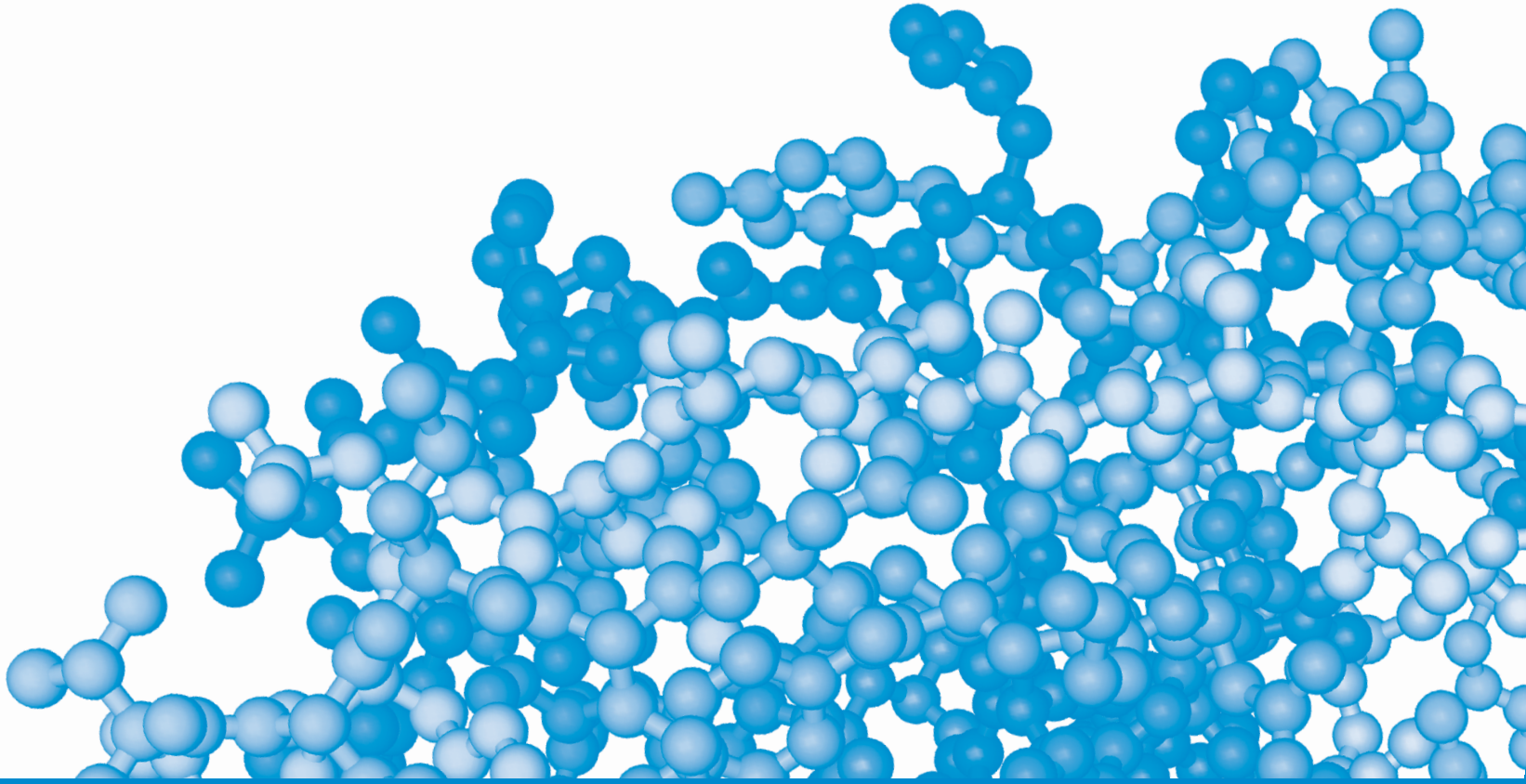
CLICK HERE TO LEARN MORE

On-Demand Webcast

Principles of Size-Exclusion Chromatography: Optimizing Particle Size, Pore Size, and Column Dimensions

CLICK HERE TO REGISTER

VIII. Ordering Information



Ordering Information

MaxPeak Premier SEC 1.7 and 2.5 µm

			P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N	P/N
			4.6 mm ID × Column Length								
Pore Size	MW Range	Particle Size	30 mm Guard	150 mm No Std	300 mm No Std	150 mm w/Std	300 mm w/Std	150 mm w/Guard	300 mm w/Guard	150 mm Guard w/Std	300 mm Guard w/Std
250 Å	10 K–650 K	1.7 µm	—	186009963	186009964	176005071	176005072	176004783	176004784	176004794	176004795
250 Å	10 K–650 K	2.5 µm	186009969	186009959	186009960	176005067	176005068	176004779	176004780	176004790	176004791

			7.8 mm ID × Column Length								
Pore Size	MW Range	Particle Size	30 mm Guard	150 mm No Std	300 mm No Std	150 mm w/Std	300 mm w/Std	150 mm Guard w/Std	300 mm Guard w/Std	150 mm Guard w/Std	300 mm Guard w/Std
250 Å	10 K–650 K	2.5 µm	—	186009961	186009962	176005069	176005070	176004781	176004782	176004792	176004793

mAb Size Variant Standard, 160 g	186009429
XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 × 150 mm Column MVK	1860011140
XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 × 300 mm Column MVK	1860011141
XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 × 150 mm Column MVK	1860011142
XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 × 300 mm Column MVK	1860011143
ACQUITY™ Premier Protein SEC 250 Å, 1.7 µm, 4.6 × 150 mm Column MVK	1860011144
ACQUITY Premier Protein SEC 250 Å, 1.7 µm, 4.6 × 300 mm Column MVK	1860011145
Straight Connection Tubing and End-fittings	WAT022681
U-Bend Connection Tubing and End-fittings	WAT084080

** Method Validation Kit (MVK) contains three columns from three different batches.

Waters™