

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

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### Abstract

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, 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. A holistic solution to the problem of undesired secondary interactions in SEC is thereby achieved. The corresponding Waters XBridge and ACQUITY Premier Protein SEC 250 Å 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 250 Å columns require little to no salt or organic solvent additive to achieve excellent resolution and quantitation of protein aggregates and fragments. In turn, these columns are highly versatile and can be used with simple physiological buffers, like commercially available phosphate buffered saline (PBS).

## Benefits

- Reliable protein size variant analysis from MW ~10,000 to 650,000 Daltons
- Reduced undesired secondary interactions (ionic and hydrophobic) through the synergistic use of hydrophilic MaxPeak High Performance Surfaces Hardware with ACQUITY Protein SEC 250 Å, 1.7 µm and XBridge Protein SEC 250 Å, 2.5 µm particles
- Versatility for the use of physiological buffers, addressing desire for a platform SEC method
- “Plug-and-play” compatibility with existing methods
- Precise quantitation of protein aggregates, monomers, and fragments

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## Introduction

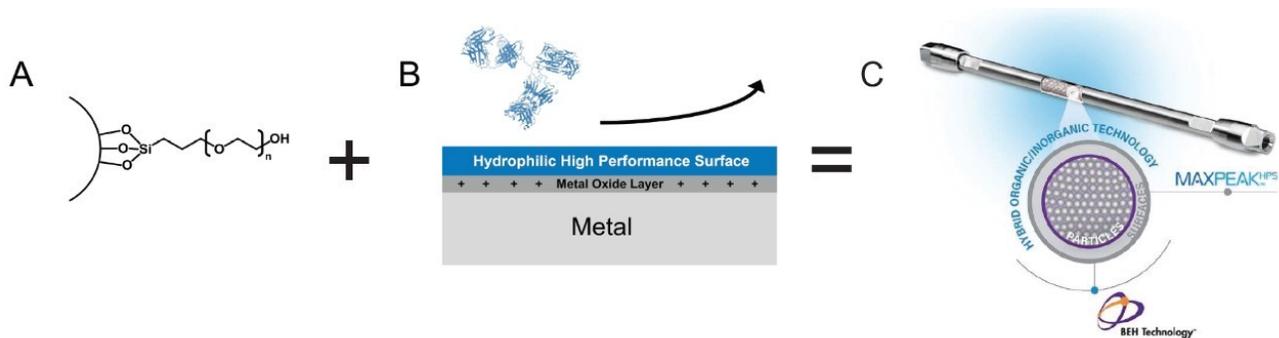
With the continued proliferation of biotherapeutic proteins, characterization and product monitoring assays have become increasingly important. These therapies are generally protein-based and thus susceptible to both aggregation<sup>1</sup> and fragmentation,<sup>2</sup> leading to reduced efficacy and potential adverse side-effects in patients. For this reason, accurate quantitation of protein size variants in biotherapeutic drug products is often mandated by regulatory agencies.<sup>3</sup> Size-exclusion chromatography (SEC) is a powerful tool for this purpose and is commonly employed for the analysis of monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), biosimilars, bi-specific mAbs, as well as other therapeutic proteins.<sup>4</sup>

In SEC, molecules will penetrate the internal porosity of the packing material to varying extents depending on

their hydrodynamic radii. Large molecules will be excluded and pass by the narrowest pores. As a result, larger molecules will elute from the column before smaller ones. Typically, the best analyte recoveries and highest resolution peaks, are produced when SEC separations are not influenced by adsorptive interactions along the chromatographic flow path. Preventing these interactions is extremely difficult because a protein's surface contains an array of chemical moieties. Having both negative and positive charges, as well as hydrophobic and hydrophilic sites, proteins have a strong propensity for secondary interactions.

Manufacturers of protein SEC columns are thus challenged to provide inert, yet robust columns. Metallic column hardware allows for the efficient packing of small particles, though it introduces electrostatically active surfaces and requires the use of high salt mobile phases to mitigate ionic secondary interactions. Polyether ether ketone (PEEK) column hardware presents a non-ionic alternative, but has pressure limitations and can also introduce hydrophobicity. In the area of packing materials, diol-bonded particles have a long history of use for protein SEC but sometimes show residual active sites. More recently, polyethylene oxide (PEO)-bonded particles were introduced to help address issues with hydrophobicity, though chromatographers have still had to contend with ionic interactions from column hardware.

In short, an ideal UPLC or HPLC SEC column has not addressed these concerns to date. With that in mind, Waters has developed the ACQUITY and XBridge Premier Protein SEC column family, which incorporates a novel 250 Å pore diameter, hydroxy-terminated PEO-bonded ethylene bridged-hybrid particle with a first of its kind, hydrophilic MaxPeak High Performance Surfaces (HPS) Column hardware. These columns mitigate undesired secondary interactions making it possible to more easily obtain reliable results for the quantitation of protein size variants.



*Figure 1. Use of MaxPeak 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) 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.*

It is shown here that the Waters XBridge and ACQUITY Premier Protein SEC 250 Å columns can be successfully applied to protein, mAb, and ADC analyses with fully aqueous phosphate buffered saline (PBS) mobile phases.

## Experimental

### Sample Description

Sample concentrations were 2 mg/mL for NISTmAb (RM 8671), and 2 or 5 mg/mL for ado-trastuzumab emtansine, as indicated per test. Both samples were made by dilution with Milli-Q water. Reagents used included acetonitrile LC-MS grade (Honeywell p/n: LC015-4), sodium phosphate dibasic anhydrous (Fisher Scientific p/n: S374-500), hydrochloric acid 36.5–38% (J.T. Baker p/n: 9535-02), and sodium chloride (Fisher Scientific p/n: S271-1). 200 mM Sodium Phosphate buffer (Mobile Phase A) was prepared by dissolving  $28.39 \pm 0.02$  g of sodium phosphate dibasic anhydrous salt into  $1000 \pm 0.02$  g 18.2 MΩ water, then adjusting pH to 6.8 by dropwise addition of hydrochloric acid (36.5–38%). 1.0 M NaCl (Mobile Phase B) was prepared by dissolving  $58.44 \pm 0.02$  g of sodium chloride salt into  $1000 \pm 0.02$  g 18.2 MΩ water. For robust SEC performance, it is

important to ensure mobile phase cleanliness. In the work outlined here, both Mobile Phases A and B were filtered prior to use through a sterile 0.2  $\mu\text{m}$  nylon filter (Thermo Scientific p/n: 1630020).

## LC Conditions

LC system:	ACQUITY UPLC H-Class Bio
System volume:	<10 $\mu\text{L}$
Detection:	ACQUITY TUV Detector (Titanium Flow Cell, 5 mm, 1500 nL)
Wavelength:	280 nm
Data acquisition:	Empower 3
Vials:	Max Recovery Vials and Caps (Waters p/n: 186000327C) and Waters 300 $\mu\text{L}$ polypropylene screw neck vial (Waters p/n: 186004112)
Column(s):	XBridge Premier Protein SEC 250 $\text{\AA}$ , 2.5 $\mu\text{m}$ , 4.6 x 150 mm (Waters p/n: 186009959) BioResolve SEC mAb 200 $\text{\AA}$ , 2.5 $\mu\text{m}$ , 4.6 x 150 mm (Waters p/n: 186009435) Commercially available Diol-bonded Silica 250 $\text{\AA}$ , 2 $\mu\text{m}$ , 4.6 x 150 mm Commercially available MeO-PEO-bonded Silica 300 $\text{\AA}$ , 2.7 $\mu\text{m}$ , 4.6 x 150 mm
Column temp.:	30 $^{\circ}\text{C}$

Sample temp.:	8 °C
Injection volume:	NISTmAb RM 8671 (1 µL of 2 mg/mL); ADC (1 µL of 2 mg/mL when using MeCN modifier, and 1 µL of 5 mg/mL when using IPA modifier)
Flow rate:	0.350 mL/min
Mobile phase A:	200 mM Sodium Phosphate Buffer pH 6.8
Mobile phase B:	1.0 M NaCl
Mobile phase C:	50% Acetonitrile/50% Milli-Q Water or 50% Isopropanol/50% Milli-Water
Mobile phase D:	Milli-Q Water (18.2 MΩ)

## Gradient

- Isocratic (Ionic Secondary Interaction Test)
  - 0 mM NaCl – 100 mM Phosphate pH 6.8 (50% A/50% D)
  - 50 mM NaCl – 100 mM Phosphate pH 6.8 (50% A/5% B/45% D)
  - 100 mM NaCl – 100 mM Phosphate pH 6.8 (50% A/10% B/40% D)
  - 200 mM NaCl – 100 mM Phosphate pH 6.8 (50% A/20% B/30% D)
- Isocratic (Hydrophobic Secondary Interaction Test)
  - 0% MeCN – 100 mM Phosphate pH 6.8 + 200 mM NaCl (50% A/20% B/0% C/30% D)
  - 5% MeCN – 100 mM Phosphate pH 6.8 + 200 mM NaCl (50% A/20% B/10% C/20% D)
  - 10% MeCN – 100 mM Phosphate pH 6.8 + 200 mM NaCl (50% A/20% B/20% C/10% D)
  - 15% MeCN – 100 mM Phosphate pH 6.8 + 200 mM NaCl (50% A/20% B/30% C/0% D)
- In some experiments, MeCN was replaced with IPA (isopropanol).

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## Results and Discussion

Based on work previously published by Goyon, *et al*,<sup>5</sup> we devised a series of tests to demonstrate the degree to which columns exhibit secondary interactions. Separations of NISTmAb and ado-trastuzumab emtansine were applied along with mobile phase titrations involving the addition of NaCl or acetonitrile to explore ionic and hydrophobic secondary interactions. By incrementally increasing the concentration of NaCl or acetonitrile in the mobile phase, each type of secondary interaction is gradually suppressed. The degree of improvement in protein recovery and peak shape, from a low to a high concentration of NaCl or acetonitrile, can be used to gauge a column's dependence on modifiers. Figure 2 presents an example of this testing as applied to the analysis of a diol bonded particle packed into conventional stainless steel hardware.

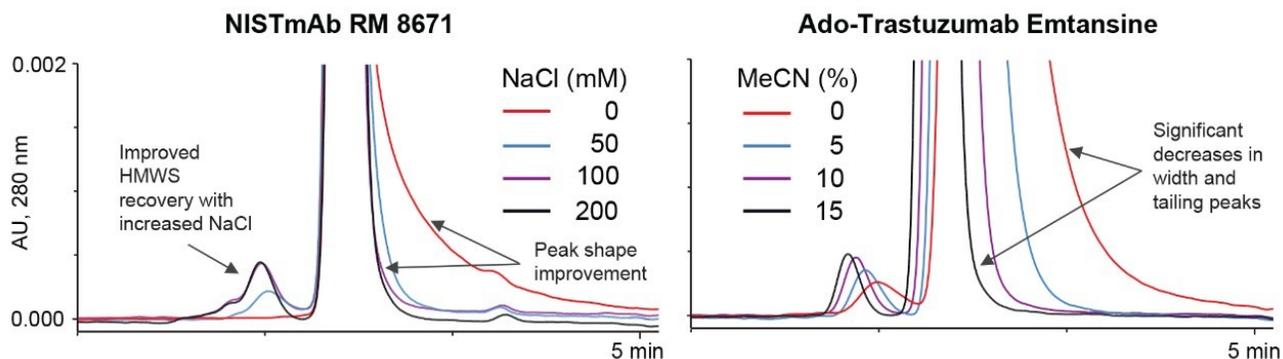


Figure 2. Example of ionic (left) and hydrophobic (right) secondary interaction testing using a commercially available diol stationary phase in stainless steel hardware with a 100 mM sodium phosphate pH 6.8 mobile phase containing varying amounts of salt and organic solvent additives. NaCl concentration is incrementally increased in the ionic test from 0 mM to 200 mM. The magnitude of improvement for NISTmAb monomer peak shape and HMWS recovery as NaCl is increased is an indication of ionic secondary interactions. Likewise, in the hydrophobic test, the observed improvement in peak shape and peak width for the ADC (ado-trastuzumab emtansine) as acetonitrile concentration is increased from 0 to 15%, is an indication of hydrophobic secondary interactions.

Applying this approach, secondary interactions testing was performed using XBridge Premier Protein SEC 250 Å, 2.5 µm and compared to Waters BioResolve SEC mAb 200 Å, 2.5 µm, as well as two commercially available SEC

columns. Each of these comparison columns are designed and marketed for the characterization and product monitoring of mAbs, ADC's, and other similarly sized biotherapeutic molecules. They are each known to perform well for this purpose after method development considerations are taken in account.

Ionic secondary interactions can have significant deleterious effects on chromatographic peak shape (tailing) and, importantly, the recovery of protein aggregates (HMWS). Aggregates are highly susceptible to ionic interactions because they are large and thus have a relatively high number of charged residues. A significant amount of their exposed surfaces will be highly charged too, as a result of non-covalent aggregates tending to form through intermolecular hydrophobic interactions. Aggregates are also the first analytes to migrate through an SEC column. They will therefore experience the strongest (least passivated) active sites as they traverse the length of the column. No matter, the accurate quantitation of aggregates, a recognized critical quality attribute (CQA), is an important function of protein SEC columns designed for protein size variant analysis. Robust performance for this measurement is essential.

To demonstrate the degree to which the columns in this study were susceptible to ionic secondary interactions, a base mobile phase of 100 mM sodium phosphate at pH 6.8 was used and the concentration of NaCl was incrementally increased from 0 mM to a maximum of 200 mM. 1  $\mu$ L of a 2 mg/mL dilution of NISTmAb (RM 8671) was injected in triplicate at each interval, with the third injection being reported. Representative chromatograms are provided in Figure 3. Remarkably, the XBridge Premier Protein SEC 250 Å, 2.5  $\mu$ m Column showed virtually no change in monomer peak shape across the entire range of NaCl concentrations, and aggregate recovery was equally impressive. By contrast, the conventional stainless steel columns exhibited significant ionic secondary interactions at the lower NaCl concentrations. The two diol-based columns showed reasonably good performance at 100 mM NaCl, though accurate quantitation of aggregates (HMWS) was not achieved until a increasing to a concentration of 200 mM NaCl. The MeO-PEO-based column underestimated the HMWS even at the highest NaCl concentration. Higher concentrations of NaCl were not explored.

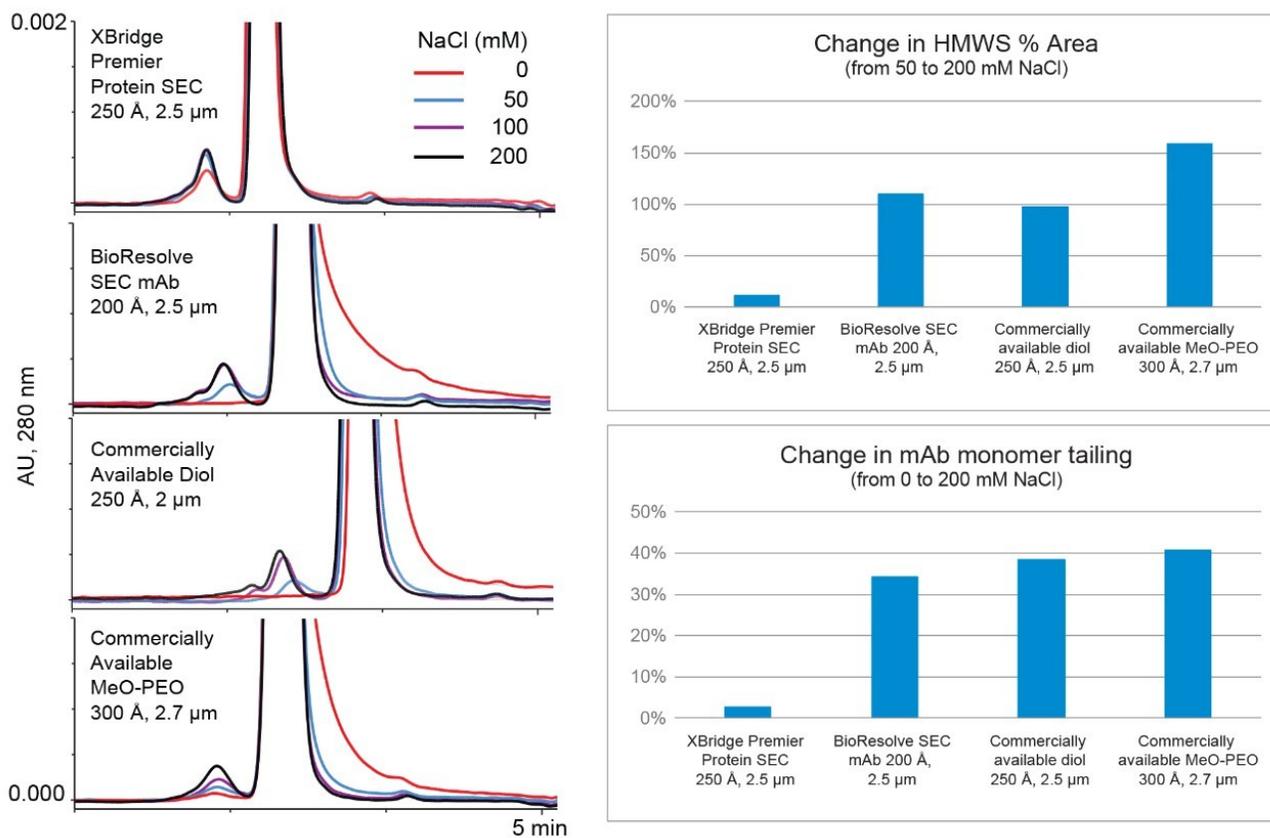
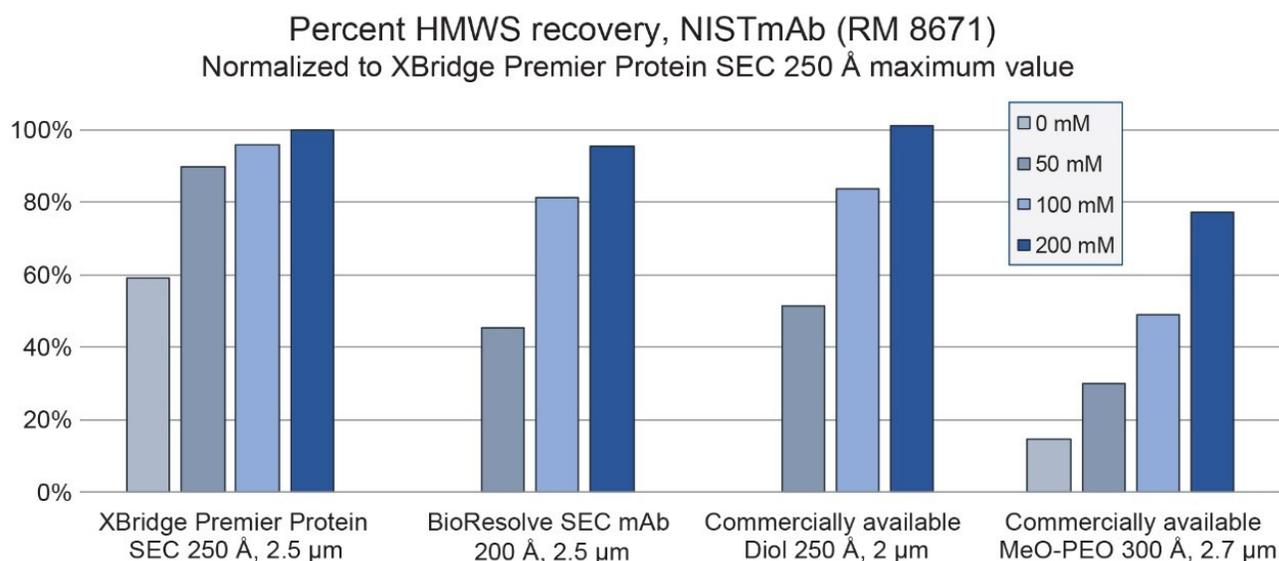


Figure 3. Comparison of ionic secondary interactions performance using NISTmAb (RM 8671) and a 100 mM sodium phosphate pH 6.8 mobile phase containing varying amounts of added NaCl salt. Upon an increase in NaCl concentration, the degree of change observed for the XBridge Premier Protein SEC 250 Å, 2.5 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. Percent recovery for NISTmAb (RM 8671) HMWS relative to the maximum value observed for XBridge Premier Protein SEC 250 Å, 2.5 µm Column at 200 mM NaCl concentration. The XBridge Column, with its hydrophilic MaxPeak High Performance Surfaces hardware yields robust HMWS recovery with 0 mM, and is near maximum recovery at only 50 mM NaCl concentration. The diol-based columns with conventional stainless steel required 200 mM NaCl to reach optimal recovery, while the MeO-PEO column severely underestimated the % HMWS even at the high concentration.*

In sum, it would appear that the reduced ionic secondary interactions of the XBridge Premier Protein SEC 250 Å, 2.5 µm Column can be attributed to the inertness of the hydrophilic MaxPeak HPS Column stainless steel hardware. To confirm this, SEC 250 Å, 2.5 µm columns were prepared with both MaxPeak HPS and conventional hardware. Indeed, testing on these columns showed the sizable benefits conferred by the hybrid surface column hardware (Figure 5).

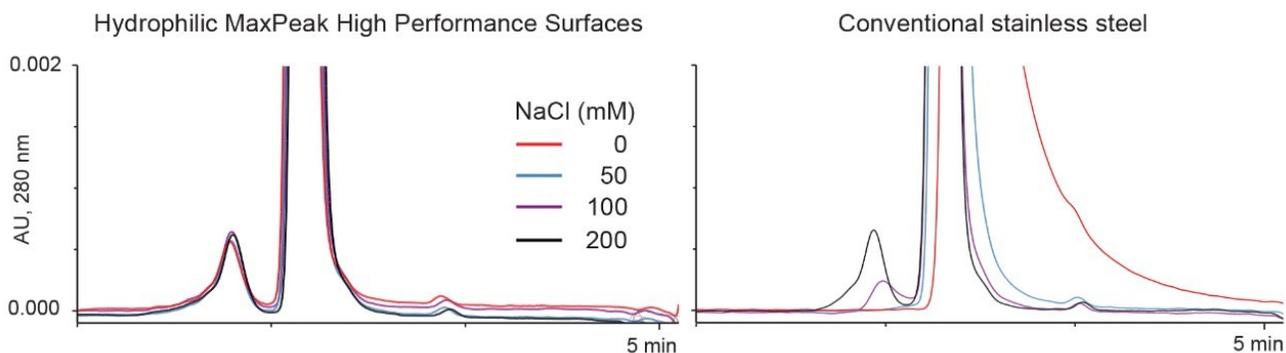


Figure 5. XBridge Protein SEC 250 Å, 2.5 µm particles were packed into both hydrophilic MaxPeak High Performance Surfaces hardware (left) and conventional stainless steel hardware (right) to clearly demonstrate the benefit afforded by the HPS hardware. Excellent monomer peak shape and HMWS recovery for NISTmAb (RM 8671) was achieved with the hydrophilic MaxPeak HPS Column, even at low NaCl concentrations

In addition to controlling mobile phase ionic strength, pH can also be leveraged as a means to mitigate ionic secondary interactions. One reason why this effect is encountered is because the electrostatic potential of metallic hardware is reduced with increasingly higher pH. In general, a higher pH mobile phase can therefore mitigate ionic secondary interactions, but it can come at a compromise to the stability of the sample and the accuracy of the assay. With their MaxPeak HPS hardware, the XBridge and ACQUITY Premier Protein SEC 250 Å columns exhibit less dependence on mobile phase pH. This means that it can be easier to implement buffers prepared with a physiological pH. Figure 6 provides chromatograms from ionic interaction testing with the previously employed pH 6.8 mobile phase as well as a pH 9 buffer.

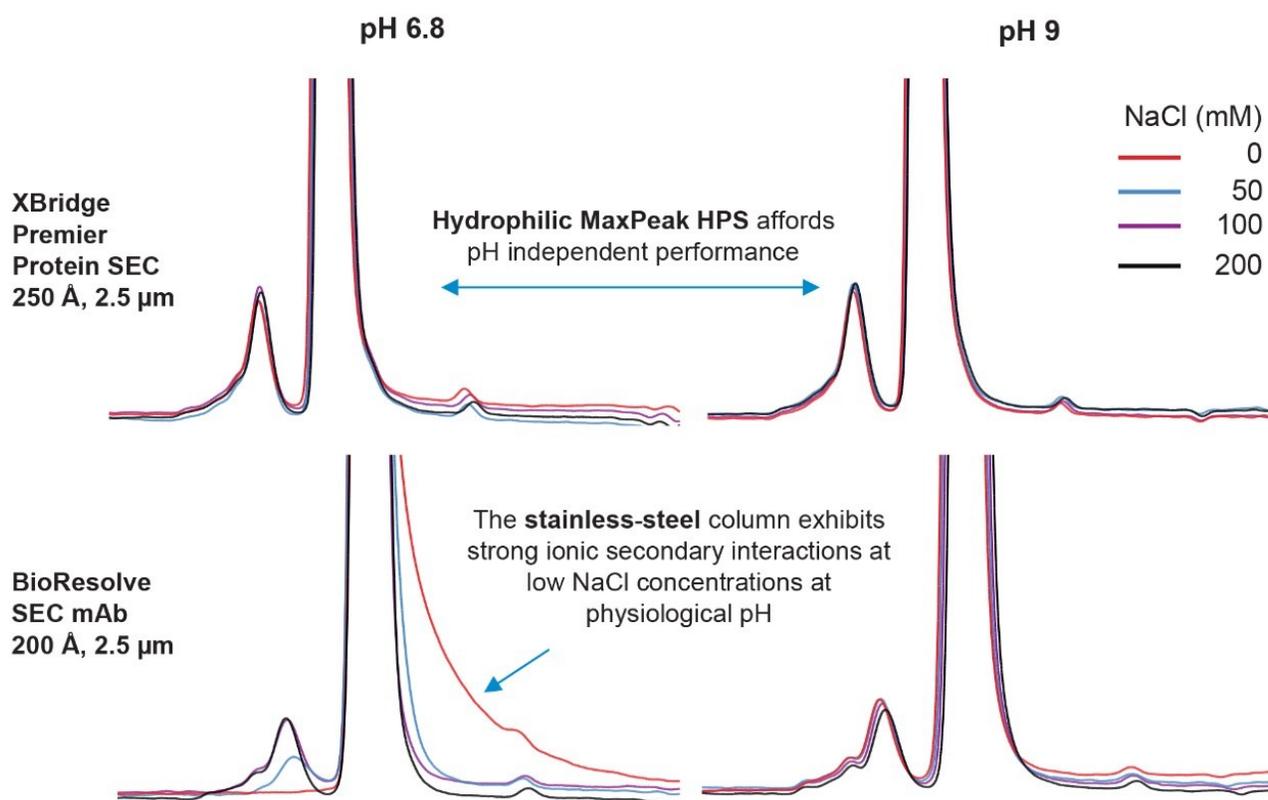


Figure 6. SEC separations can show a pH dependence as with NISTmAb (RM 8671) ( $pI = 9.18 \pm 0.01$ )<sub>6</sub> separated with mobile phases made with different pH values and ionic strengths XBridge or ACQUITY Premier Protein SEC 250 Å columns do not show significant pH dependence as are result of their hydrophilic MaxPeak High Performance Surfaces.

Hydrophobic secondary interactions primarily occur between protein molecules and hydrophobic sites on the SEC packing material itself. This interaction compromises monomer peak shape, particularly for extremely hydrophobic molecules like ADCs. Excessive tailing of the monomer greatly affects peak efficiency and can inhibit the ability to resolve or quantify fragment species. The dependence of SEC columns on organic solvent was thus studied and example results are shown in Figure 7. Organic mobile phase modifiers, such as acetonitrile, are known to be effective for suppressing hydrophobic secondary interactions in protein SEC.<sup>7</sup> However, PEO-based columns are not particularly sensitive to the addition of acetonitrile and perform similarly with or without it. By contrast, diol-bonded silica or hybrid particle columns tend to exhibit higher levels of hydrophobicity and can require significant amounts of acetonitrile to improve peak shape sufficiently for the

analysis of a very hydrophobic ADC, like ado-trastuzumab emtansine. The XBridge and ACQUITY Protein SEC 250 Å particles, with their high coverage hydroxy-terminated PEO bonding, are significantly more hydrophilic than alternative SEC packing materials. This yields highly efficient peaks in 100% aqueous mobile phase.

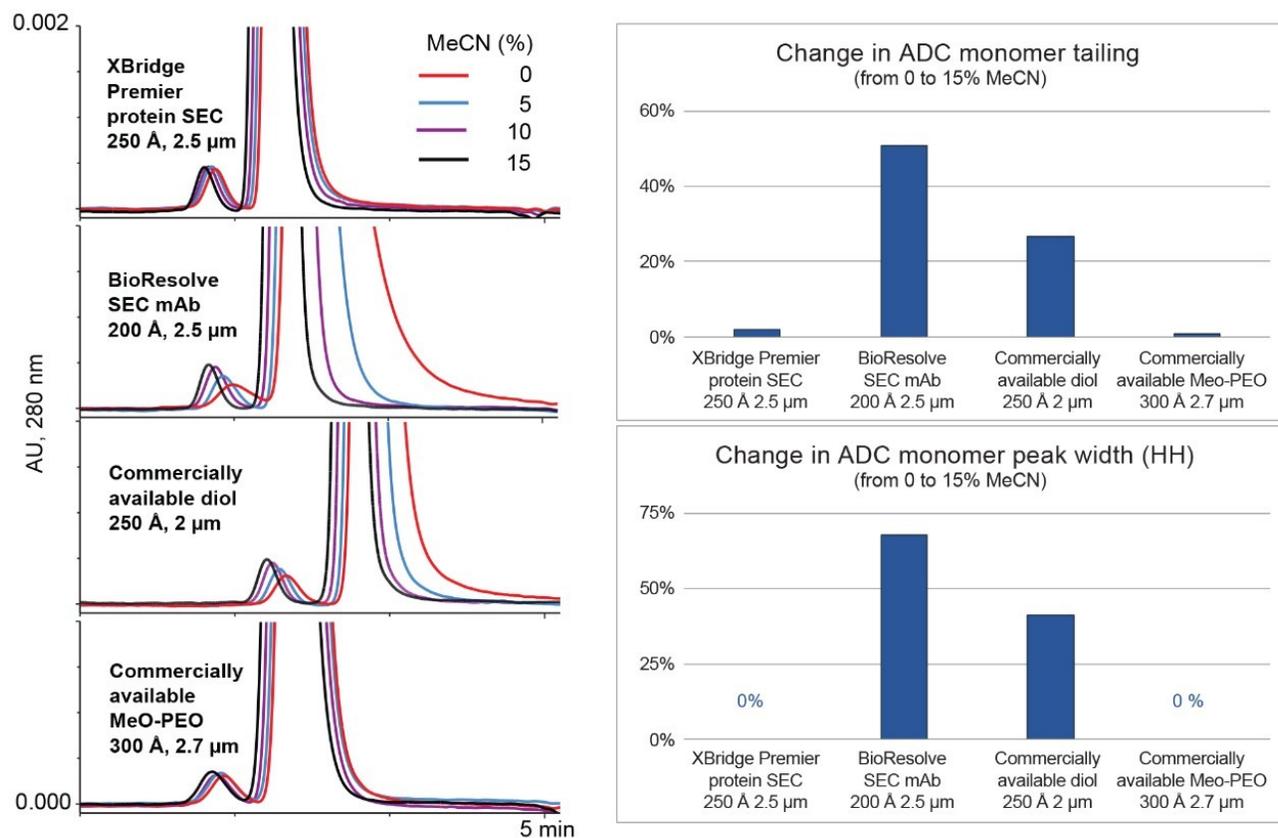


Figure 7. Comparison of hydrophobic secondary interactions performance using the ADC ado-trastuzumab emtansine. Upon increase of organic concentration, the degree of change observed for the XBridge Premier Protein SEC 250 Å, 2.5 µm Column is again negligible, with outstanding peak shape from 0 to 15% MeCN. Hydrophobic secondary interactions from have been largely mitigated through use high coverage hydroxy-terminated PEO packing material bonding.

While PEO-based particle columns are not particularly sensitive to the addition of acetonitrile, due to their already low levels of hydrophobicity, there might be scenarios in which analytes might still benefit from the addition of an organic additive. We have accordingly studied the addition of isopropanol (IPA) to mobile phases. Titration of mobile phase with IPA instead of acetonitrile is represented by the data provided in Figure 8. These

results show that enhanced performance for a ado-trastuzumab emtansine separation can be realized through the use of small amounts of IPA. The majority of performance improvement occurs with the use of only 5% IPA. Keeping organic concentration low also means fewer concerns with protein denaturation. The use of 5% IPA can be reserved as a method development option for the most challenging separations.

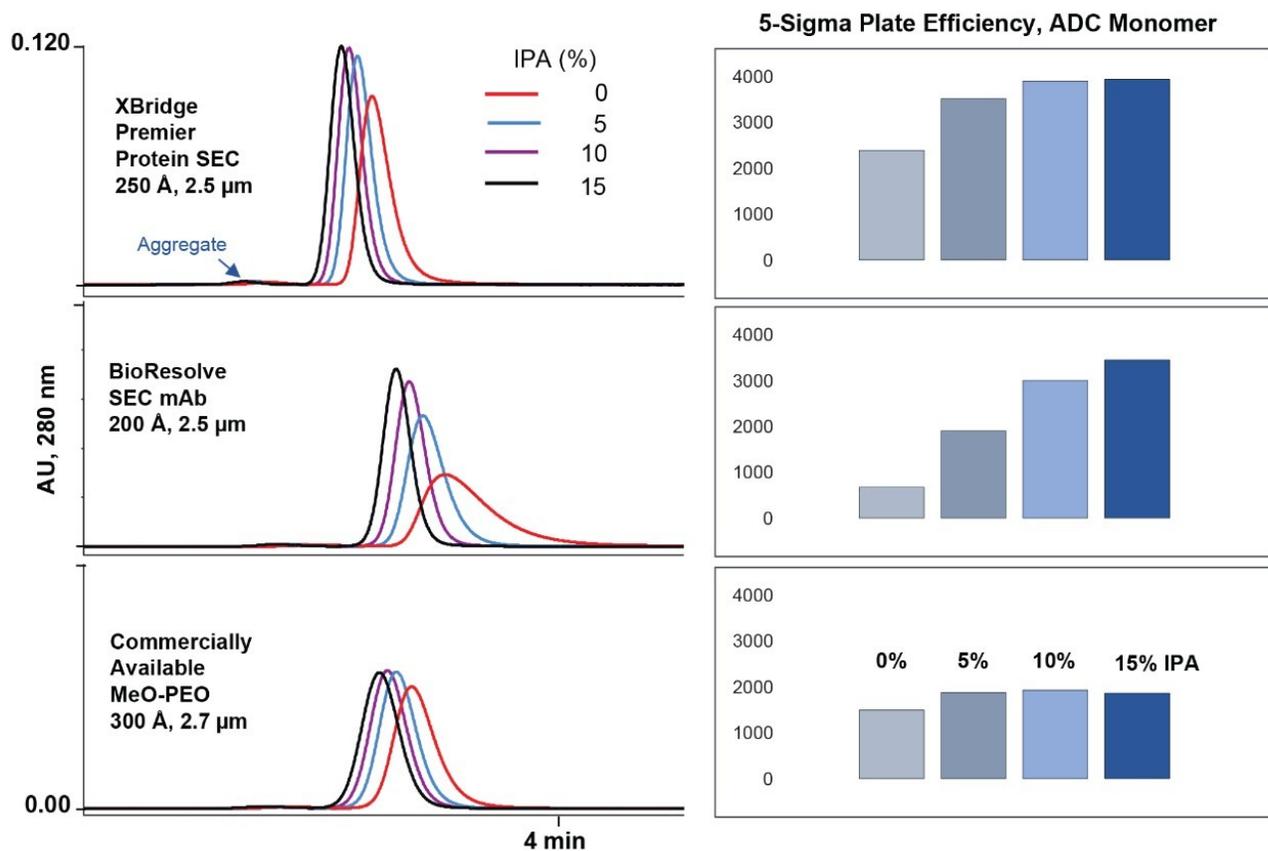


Figure 8. Full view of monomer peak with isopropanol (IPA) as the organic modifier for ado-trastuzumab emtansine separations. Peak shape and efficiency were quite good for the XBridge Premier Protein SEC 250 Å, 2.5 μm Column without any organic modifier. However, a slight enhancement in efficiency was achieved with the use of 5% IPA. The increase was also observed for the MeO-PEO column, though efficiency was roughly half that of the XBridge Premier Protein Column. The diol-based column required significantly more organic to reach optimal efficiency for the ADC monomer.

These benefits of minimized secondary interactions and method versatility are only truly impactful if column performance can be reliably and reproducibly achieved across the life of the method. The lot-to-lot

reproducibility of XBridge and ACQUITY Premier Protein SEC 250 Å columns was thus studied. Seven different columns were prepared with 7 different lots of XBridge Protein SEC 250 Å, 2.5 µm packing material and intermixed with different lots of hydrophilic MaxPeak HPS hardware. These columns were then tested for secondary interactions, and the resulting chromatograms are shown in Figure 9. Outstanding reproducibility was observed from one lot of columns to the next. For the ionic interaction test, NISTmAb aggregate percent area change, measured as the difference in results obtained at 50 mM versus 200 mM NaCl, was less than 20% for all seven column lots. NISTmAb monomer tailing change was less than 5% for all lots, measured from 0 mM to 200 mM NaCl. Results were equally compelling in the hydrophobic interaction test, where the ADC (ado-trastuzumab emtansine) aggregate percent area was shown to change less than 10%, and ADC monomer tailing less than 5% across acetonitrile concentrations ranging from 0% to 15%. As shown in Figure 10, lot-to-lot reproducibility was also very strong at each single interval within the tests

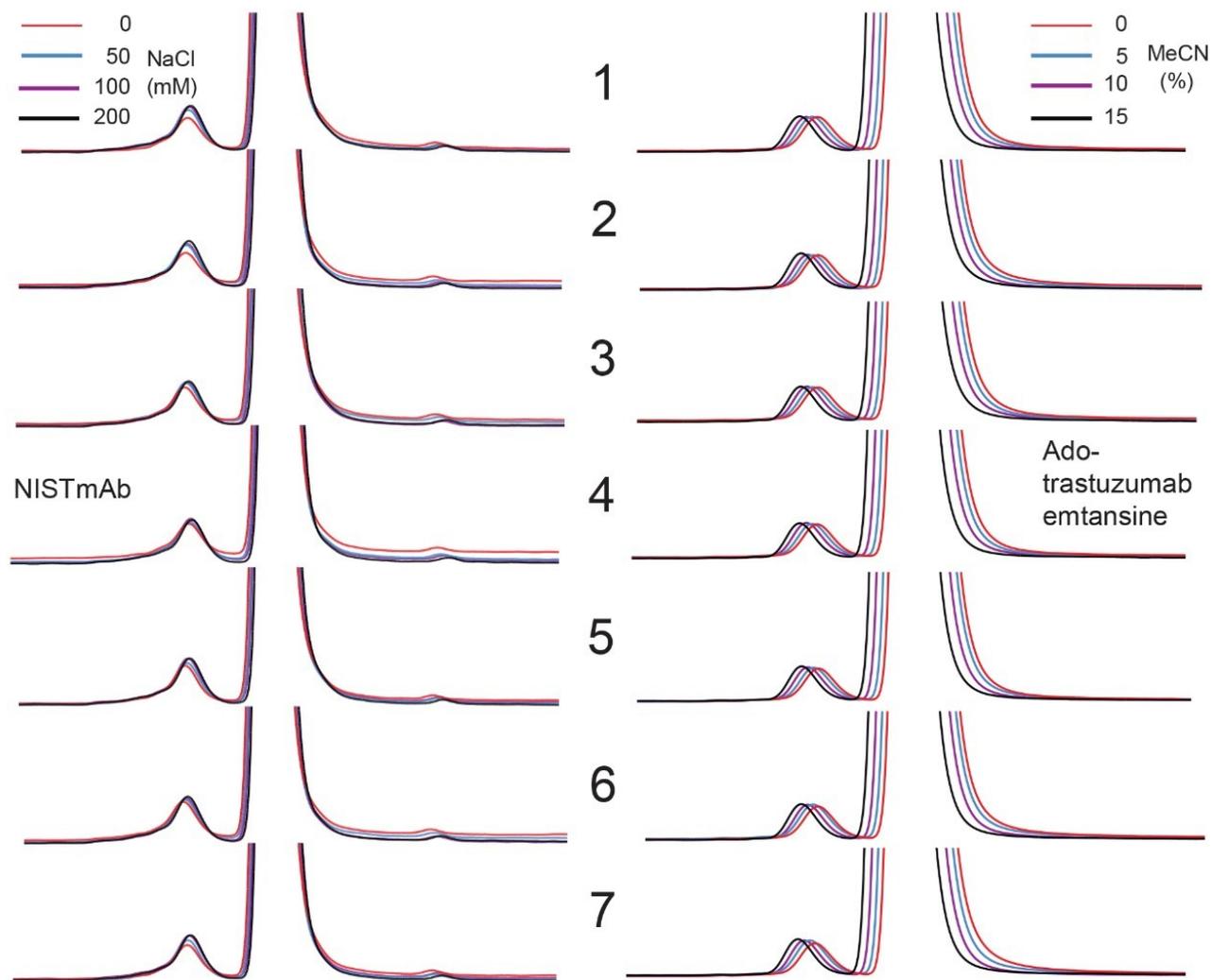


Figure 9. Lot-to-lot reproducibility as observed through secondary interaction testing. A total of seven (7) unique lots of XBridge Protein SEC 250 Å, 2.5 µm particles were packed into varying lots of 4.6 x 150 mm hydrophilic MaxPeak High Performance Surfaces hardware and tested for ionic (left) and hydrophobic (right) secondary interactions. Results across the entire titrated ranges show excellent recovery of aggregates, and outstanding peak shape for the monomer, for both NISTmAb and the ADC.

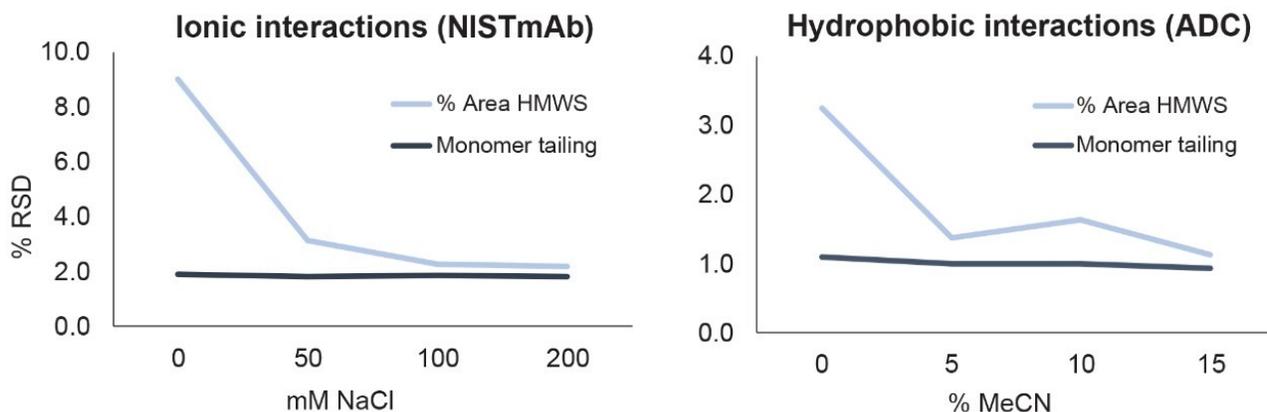


Figure 10. Percent relative standard deviation across 7 lots of XBridge Premier Protein SEC 250 Å, 2.5 µm Columns for each interval in secondary interaction testing. Although slightly higher lot-to-lot variation was observed at 0 mM NaCl for aggregate (HMWS) percent area in the ionic interaction test (left), overall %RSD's were extremely low for monomer tailing and aggregate percent area in both tests regardless of NaCl or acetonitrile concentration.

## Conclusion

SEC can be a powerful tool for the characterization and product monitoring of mAbs, ADC's, and other similarly sized biotherapeutics. Ideally, these separations are entropically driven, and protein aggregates, monomers, and fragments are separated based solely on their hydrodynamic radii. However, secondary interactions stemming from a lack of column inertness are often present and create significant challenges. Undesired adsorptive interactions between proteins and electrostatic or hydrophobic active sites on column hardware and particles contribute to low recoveries, distorted peak shapes, and thus inaccurate quantitation of critical quality attributes of drug products. To overcome these problems, a great deal of time and effort is often required in method development. Often times, a compromise must be made to use higher than desired salt or organic solvent concentrations.

Here, we have shown the performance of a new column technology designed to provide a holistic solution to the problems of undesired secondary interactions in SEC. By combining a high coverage hydroxy-terminated PEO

surface particle with hydrophilic MaxPeak High Performance Surfaces (HPS) Column hardware, excellent recovery, peak shape, and resolution of aggregates, monomers, and fragments has been achieved using low concentrations of salt or organic solvent in the mobile phase. With this performance, the ACQUITY and XBridge Premier Protein SEC 250 Å columns have shown themselves to be extremely versatile and to confer performance attributes that will improve the robustness of any present or future SEC assay.

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