EVALUATION OF SIZE EXCLUSION CHROMATOGRAPHY PACKING MATERIALS FOR THE ANALYSIS OF PROTEINS AND HIGHER ORDER AGGREGATES

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INTRODUCTION

Size exclusion chromatography (SEC) is typically used to measure protein aggregates and other size variants present in biopharmaceuticals. New advances in packing materials and instrumentation have allowed faster and more reproducible separations to be achieved. However, the recovery of proteins and higher order aggregates still remains a critical part of the success of an SEC method.

In this presentation, we will discuss the factors that can influence quantitation of biomolecules on size exclusion packing materials. Evaluation of an SEC method typically includes analysis of the resolution and aggregate quantitation. In the following discussion, we will outline the considerations in developing a SEC method. The effect of variables such as flow rate, mobile phase composition, and pH will be measured.

METHODS

UPLC-SEC Chromatographic Conditions:

Unless otherwise specified

LC System: ACQUITY UPLC® H-Class Bio System with
PDA detector with Titanium Flow Cell
Wavelength: 280 nm
Mobile Phase: 25 mM Sodium Phosphate, pH 6.8, 0.15 M NaCl

Wash and Purge Needle Washes: Mobile Phase
Seal Wash: 80/20 H_2O/MeOH
Temperature: 30°C
Flow rate: 0.4 mL/min

Columns, conditions (unless otherwise noted):
Waters: ACQUITY BEH200 SEC,1.7 µm, 4.6 x 300 mm
(p/n 186005226), 0.4 mL/min, 10 µL injection
Vendor A: Diol-coated silica 150Å, 3µm,
4.6 x 300mm, 0.4 mL/min, 10 µL injection
Vendor D: Diol-coated silica 250Å, 4µm,
4.6 x 300mm,0.4 mL/min, 10 µL injection

Protein Recovery

Colorimetric Assay: Pierce(R) BCA Assay Kit
Spectrometer: Perkin Elmer λ35

Samples were collected in 2mL volumetric flasks. Protocol for colorimetric assay followed enhanced test tube protocol as described in Pierce BCA Assay Kit instructions.

RESULTS AND DISCUSSION

PROTEIN RECOVERY

The recovery of proteins and higher order aggregates remains a critical part of the success of an SEC method. However, protein adsorption has been an area of concern. In order to ensure accurate quantitation, a variety of approaches have been used.[1] These techniques may include pre-treatment of the column with high mass loads of either the sample of interest or another protein, such as BSA. However these methods do not assess total protein recovery. In order to evaluate protein recovery of UPLC-SEC packing materials, protein samples were injected. The samples were subsequently collected and measured by a colorimetric assay for total protein quantitation. Mass load was based on sensitivity and colorimetric assay reproducibility.

Protein Recovery on an Unconditioned Column

To determine the effects of column conditioning on protein recovery, a new BEH200 SEC 1.7 µm column was tested. The first ten injections of a human IgG (2 mg/mL, 40 µL) were individually collected and measured for protein content. The protein collected was compared to a standard and to injected samples collected from the system with no column in line. The studies were repeated for diol-coated silica columns designed for monoclonal antibody analysis.

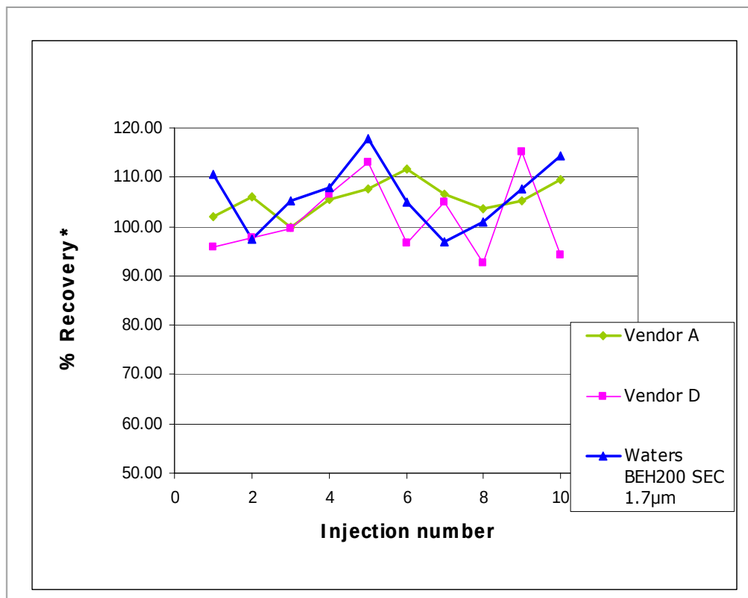

*Figure 1. Effect of column conditioning on protein recovery. A human IgG (2 mg/mL, 40 µL) was analyzed on a new BEH200 SEC 1.7 µm column. The first ten injections were collected and measured by colorimetric assay. Recoveries for the ten injections were within expected ranges of 94-114%, indicating no significant loss of protein due to adsorption onto the column. Analysis was also performed for silica diol-coated SEC columns. No significant trend was observed for any column tested. Based on variability of the colorimetric assay, no significant loss of protein was found to occur with any column tested. *Total protein collected is reported relative to the sample collected after injection onto the system, with no column in line.*

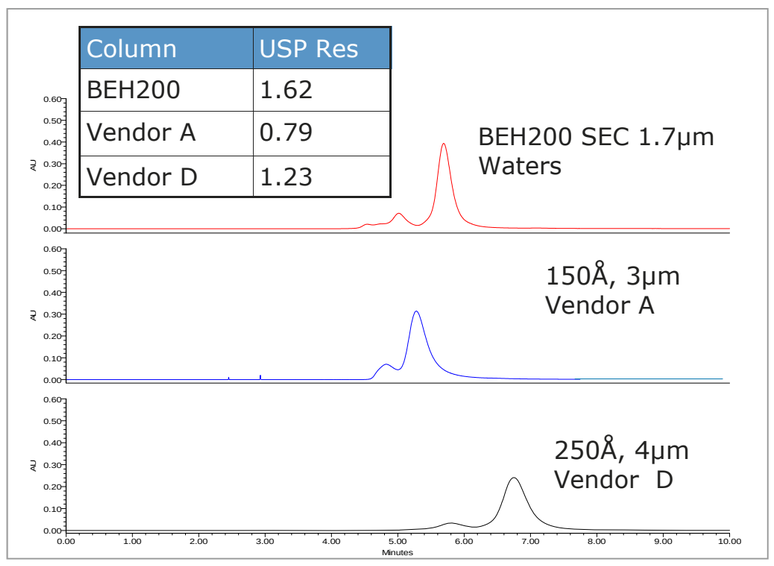

Figure 2. Effect of packing material on monomer/dimer resolution. A pooled polyclonal human IgG antibody (2 mg/mL, 40 µL) was analyzed on UPLC BEH200 SEC 1.7 µm column and traditional silica diol-coated columns. Flow rates and injection volumes were scaled based on column configurations. USP monomer/dimer resolution was more than 30% greater on BEH200 1.7µm SEC column as compared to traditional silica diol-coated columns. .

METHOD DEVELOPMENT STRATEGIES

Size exclusion chromatography of biomolecules is typically performed under aqueous, native conditions. These conditions can result in undesired interactions between the packing material and the proteins: charged sites on the matrix can interact with the proteins in an "ion- exchange effect." Secondary interactions can be affected by varying the ionic strength and/or pH of the mobile phase.[2] In order to fully determine the effect of SEC method conditions on protein aggregation, orthogonal analysis may be required.

Effect of Mobile Phase Salt Concentration

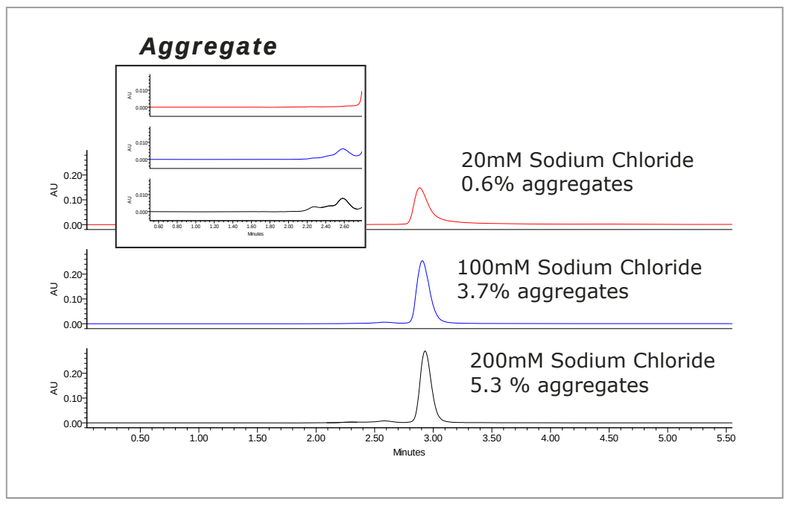

Figure 3. Effect of salt concentration on murine mAb peak shape and aggregate recovery. At low buffer concentration, secondary interactions affect peak shape, i.e. tailing. Higher buffer concentration improves peak shape and resolution as well as recovery of mAb (inset). Note: Orthogonal analysis may be required to determine effect of salt concentration on protein aggregation. Buffer: XXM Sodium Chloride, pH 6. 8.

Effect of pH

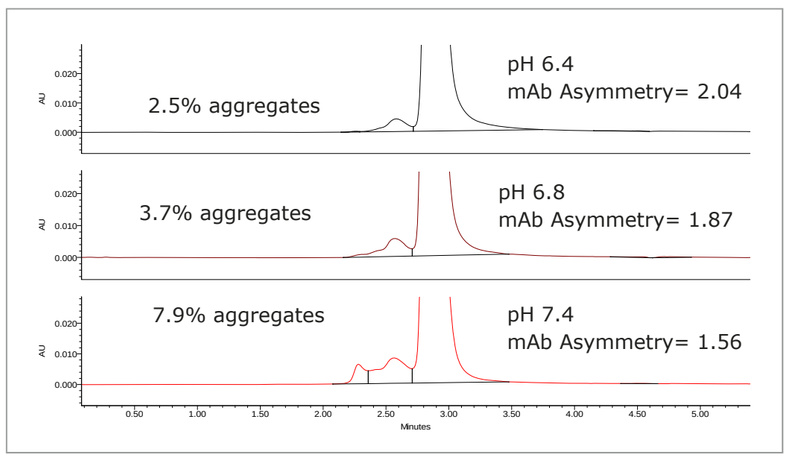

Figure 4. Effect of pH on murine mAb peak shape and aggregate recovery. For this sample, at pH 6.4 secondary interactions affect peak shape (asymmetry). Increasing the pH improves peak shape and affects aggregate recovery. Note: Orthogonal analysis may be required to determine effect of pH on protein aggregation. Buffer: 100M Sodium Phosphate, pH listed above.

Effect of Flow Rate

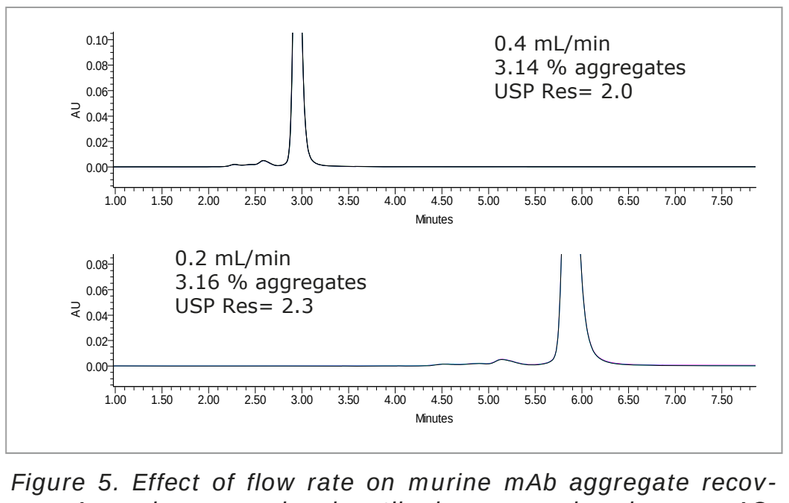

Figure 5. Effect of flow rate on murine mAb aggregate recovery. A murine monoclonal antibody was analyzed on an ACQUITY BEH200 SEC column at varying flow rates. Triplicate injections of the mAb were analyzed at flow rates of 0.2 and 0.4 mL/min. Analysis of the separations shows no significant change in aggregate quantitation with flow rate. However, decreasing flow rate increases the monomer/dimer resolution.

CONCLUSION

- **UPLC-SEC technology does not result in measurable loss of proteins either due to the UPLC H Class Bio system or BEH200 SEC 1.7 µm column**
- **UPLC-SEC can provide improved resolution of monoclonal antibody aggregates as compared to traditional silica diol-coated SEC columns**
- **SEC method development strategies (mobile phase concentration, pH and flow rate) can be used to evaluate and/or minimize secondary interactions**

References

1. . Arakawa, D. Ejima, T. Li, J.S. Philo, "The Critical Role of Mobile Phase Composition in Size Exclusion Chromatography of Protein Pharmaceuticals," *J. Pharm. Sci.*, *99* (2010), 1674-1692
2. Golovchenko, N. P., Kataeva, I.A., Akimenko, V. K., "Analysis of pH- dependent protein interactions with gel filtration medium," *J. of Chrom.*, 591 (1992) 121.

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