APPLICATION NOTE

A universal chromatography method for aggregate analysis of monoclonal antibodies

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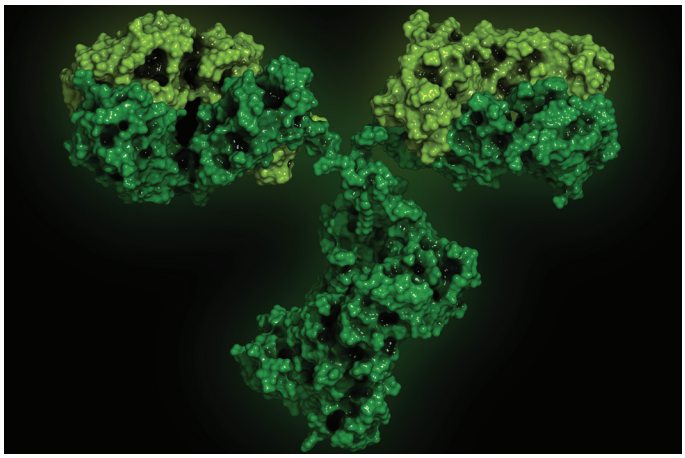
Key words
MAbPac SEC-1, monoclonal antibody, mAb, aggregation analysis, biotherapeutics, size-exclusion chromatography

Goal

Analysis of protein aggregation of five important biotherapeutic monoclonal antibodies (mAbs) by size-exclusion chromatography, showing the universal applicability of the Thermo Scientific™ MAbPac™ SEC-1 column for aggregate analysis of mAbs.

The aim of this study was threefold:

- **Produce a single globally applicable SEC chromatography method for the five mAb samples**
- **Show peak symmetry (implying limited secondary interaction with the column)**
- **Maintain the required monomer and dimer resolution**



Introduction

The biopharmaceutical industry continues to develop mAb-based biotherapeutics in increasing numbers. Due to the complexity of these biotherapeutics, there are several key quality attributes (CQAs) that need to be measured and controlled to guarantee their safety and efficacy. The presence of aggregates in a formulated drug product must be assessed to avoid potential issues with immunogenicity.

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Aggregates are typically dimers, trimers, or larger order structures of antibody molecules. They are formed at the following stages:

- Product expression during fermentation
- Product purification in downstream processing of the drug substance
- Storage or mishandling of the drug prior to patient administration.

Protein aggregation has been implicated as the cause of adverse immunological reactions that result in serious safety and efficacy issues. Aggregation must be monitored throughout the production process and during storage of the formulated biotherapeutic. MAb fragments that are smaller than the expected molecular weight elute after the parent peak and can also be determined.

Size-exclusion chromatography (SEC) is the standard method for this important analysis, but the compounds can show non-specific binding to the columns, which leads to retention time shifts, peak tailing, or even complete loss of protein peaks.[1,2] The MAbPac SEC-1 column is silica based with a proprietary, covalently bonded diol hydrophilic layer to prevent secondary interactions. Even so, the mobile phase eluents usually contain high salt concentrations to prevent ionic interactions, which can lead to corrosion of metal components. For this reason, an inert UHPLC system is recommended.[3] The MAbPac SEC-1 column separates by size and the pore size for this column (300 Å) was chosen to give a good separation in the molecular weight range of the monomer and dimers of a typical 150 kDa mAb. This column therefore serves as a good, broadly applicable column for mAb aggregate analysis.

Five important biotherapeutic mAbs (bevacizumab, cetuximab, infliximab, rituximab, and trastuzumab) were selected to investigate column performance. The monoclonal antibodies chosen are structurally diverse to investigate secondary interactions over a wide range of the physicochemical space. They cover a pI range between 7.6 and 8.7 and have widely different glycosylation patterns from very simple (bevacizumab) to highly complex (cetuximab). The MAbPac SEC-1 column and Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system were applied to the aggregate analysis of five important biotherapeutic mAbs using a common high salt buffer mobile phase at pH 6.8.

Experimental

Consumables

- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific Sodium phosphate dibasic anhydrous (P/N 10440481)
- Fisher Scientific Sodium phosphate monobasic anhydrous (P/N 10751135)
- Fisher Scientific Sodium chloride (P/N 11964051)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop Screw Thread Vial Convenience Kit (P/N 60180-VT405)

Sample pre-treatment

Samples were reconstituted in water for injection with gentle swirling to aid in mAb solubilization as directed from the manufacturer's product insert information.

The following formulated drug products were injected directly:

- Bevacizumab 25 mg/mL
- Cetuximab 5 mg/mL
- Infliximab 10 mg/mL
- Rituximab 10 mg/mL
- Trastuzumab 21 mg/mL

Separation conditions

Instrumentation

Vanquish Flex Quaternary UHPLC system, standard configuration, equipped with:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Diode Array Detector HL (P/N VH-D10-A)
- Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)

Column: MAbPac SEC-1, 7.8 × 300 mm (P/N 088460)

Mobile phase

Composition:	0.2 M sodium chloride in 100 mM phosphate buffer pH 6.8
Flow rate:	0.3 mL/min
Column temperature:	25 °C
Injection volume:	1 µL
UV:	214 nm

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.2 SR4, was used for data acquisition and analysis.

Results and discussion

The separation profiles obtained from each mAb sample are represented in Figures 1A to 1E and normalized in an overlay in Figure 2.

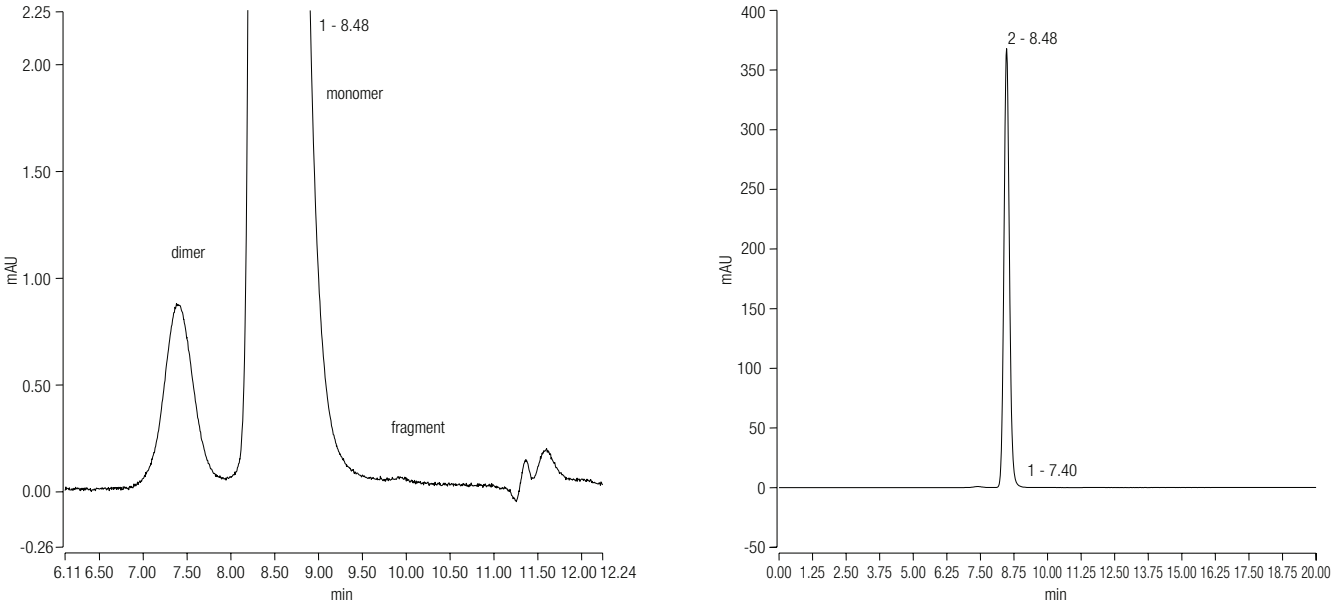

Figure 1A. Trastuzumab SEC separation, expanded view (left), full range chromatogram (right).

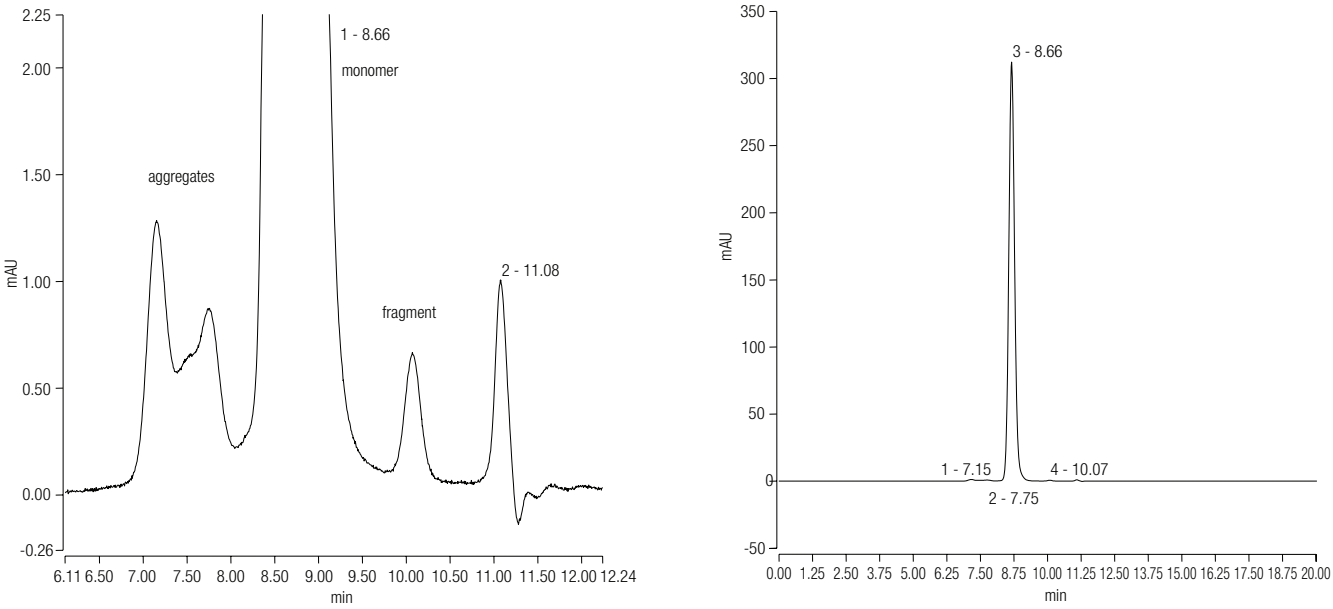

Figure 1B. Rituximab SEC separation, expanded view (left), full range chromatogram (right).

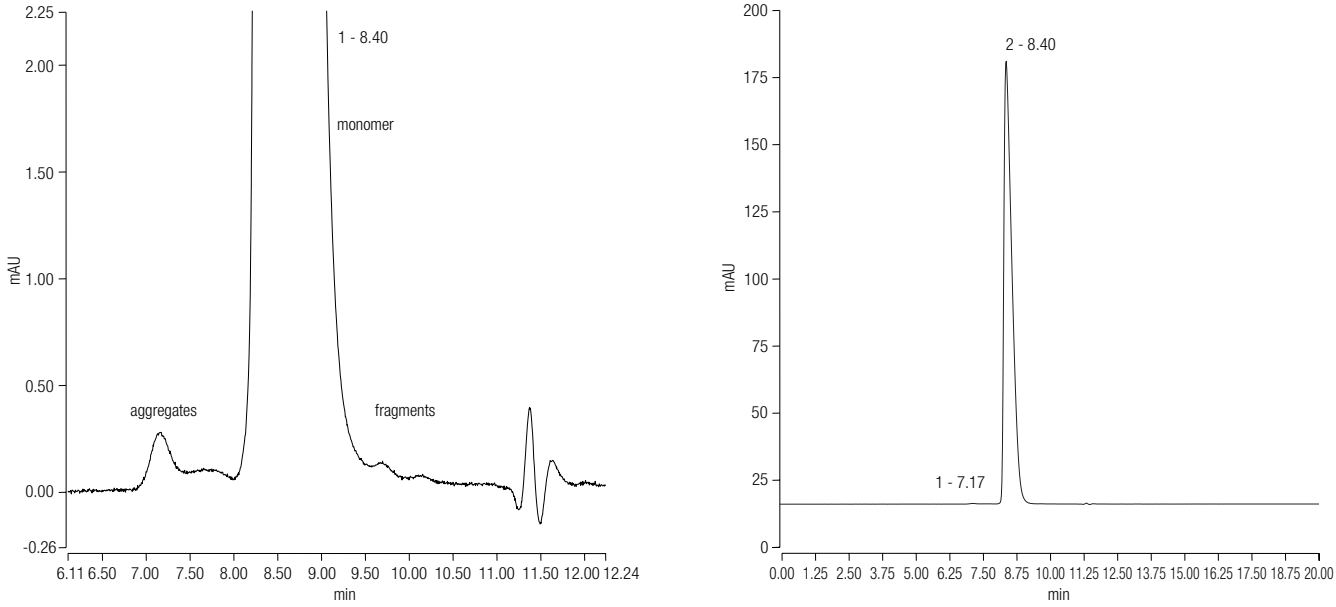

Figure 1C. Infliximab SEC separation, expanded view (left), full range chromatogram (right).

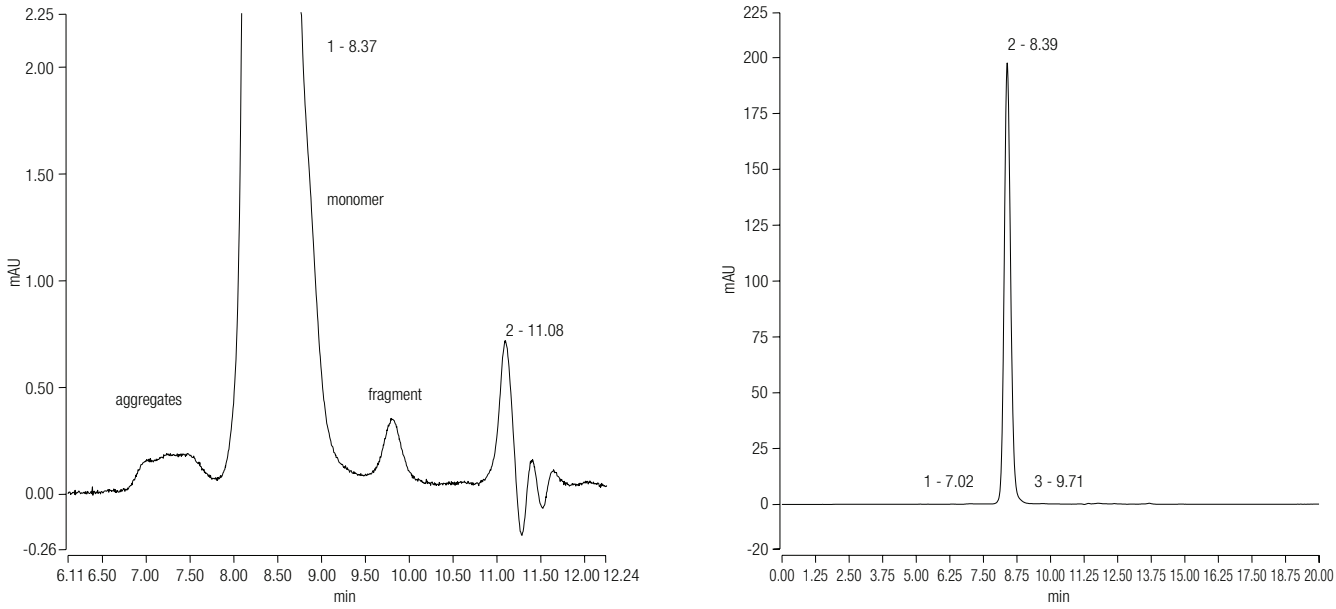

Figure 1D. Cetuximab SEC separation, expanded view (left), full range chromatogram (right).

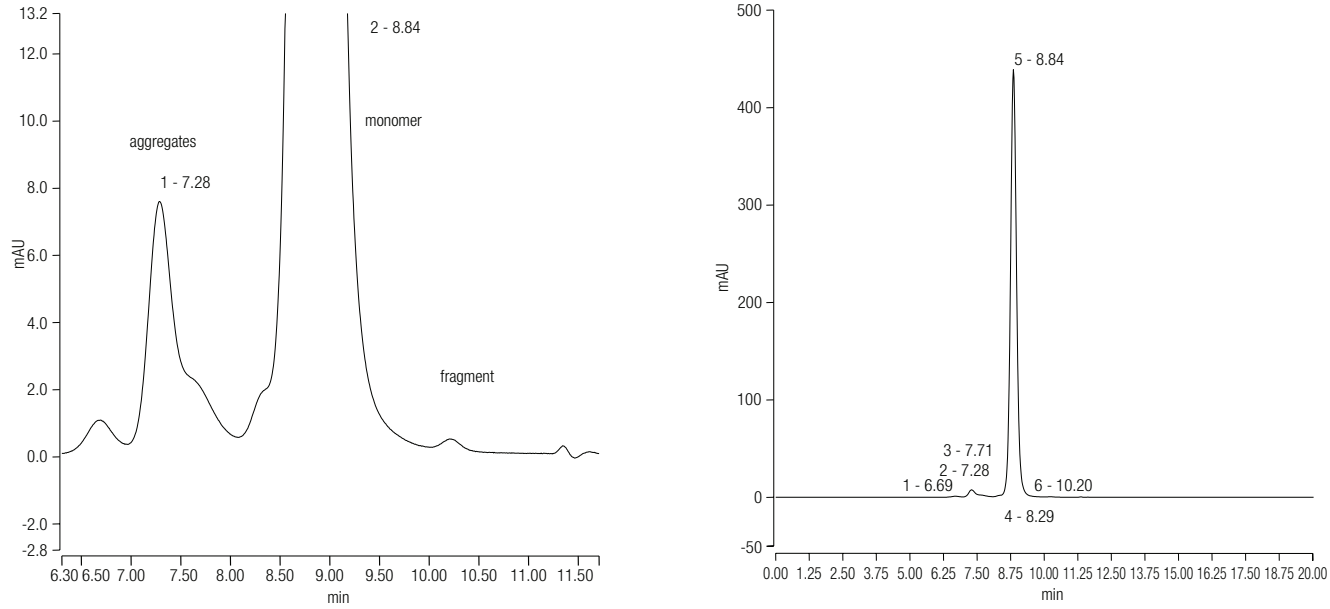

Figure 1E. Bevacizumab SEC separation, expanded view (left), full range chromatogram (right).

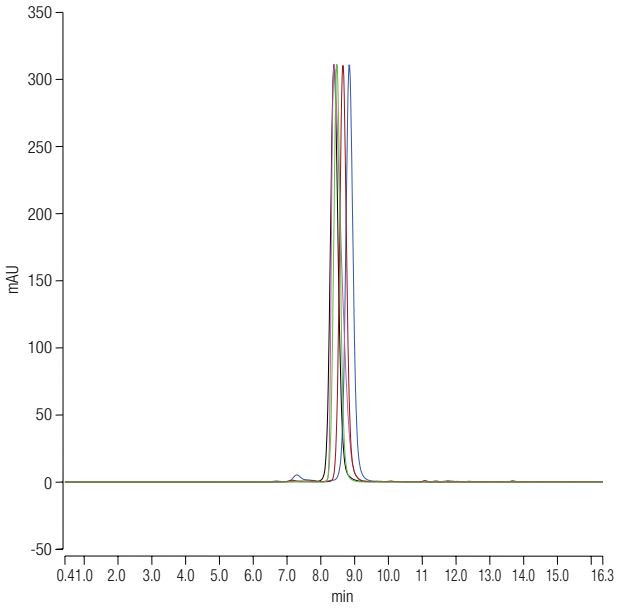

Figure 2. All 5 mAb samples overlaid to show relative retention time and peak shape (cetuximab, infliximab, trastuzumab, rituximab, bevacizumab).

Each pharmaceutically available mAb drug product shows a different aggregation pattern and different levels of aggregation (Figures 1A to 1E). Table 1 shows the percentage of aggregates in each mAb. Trastuzumab has a clear dimer peak that is well resolved from the monomer. Cetuximab and infliximab show the lowest level of aggregation and lowest fragment peaks. In contrast, bevacizumab exhibits a higher level of aggregation with a complex aggregation pattern, but has a low level of fragments.

Table 1. Percentage of aggregates in each mAb.

mAb Sample	% Aggregates
Trastuzumab	0.39
Rituximab	0.92
Infliximab	0.10
Cetuximab	0.29
Bevacizumab	2.99

In terms of performance, despite structural differences in the mAb samples, the MAbPac SEC-1 column gives resolution of the aggregates and fragments from the main monomer peak for all the samples tested. This resolution between monomer and dimer peaks was high enough to allow a good determination of the percentage aggregation. This was best demonstrated with trastuzumab (Figure 1A) where the calculated resolution was 2.21, which compares favorably with other SEC methods.

Figure 2 shows the overlaid chromatograms for the five mAbs. Molecular weights for these proteins are all relatively close to each other, which is reflected by their similar retention times. Given the retention times and the symmetrical peaks observed (Table 2), this demonstrates a lack of secondary interactions with the column packing material and hardware. Infliximab exhibits the worst asymmetry, which does not improve upon addition of solvent.[4]

In general, the relationship between retention time and mass is conserved with the largest mAb, cetuximab, eluting first and the smaller mAbs eluting in reverse molecular weight order as would be expected in SEC. The only anomaly is bevacizumab, which elutes last but is not the smallest protein. This may be a result of protein folding or differences in the N-glycans structures present on this molecule relative to the other mAbs studied. These subtle effects are magnified due to the narrow molecular weight range of the analytes.

The results demonstrate the general applicability of the MAbPac SEC-1 column for aggregate analysis for a range of monoclonal antibodies. The data also shows that this SEC column delivers high-resolution power for the separation of monomers and dimers, allowing easy quantitative analysis of any aggregates present in the samples. The method described here can be applied to the characterization of these top-selling mAbs and mAbs in general.

Table 2. Retention time comparison of mAbs and associated molecular weights.

mAb Sample	Molecular Weight (kD)	Retention Time (min)	Asymmetry
Rituximab	145	8.66	1.10
Trastuzumab	148	8.48	1.10
Bevacizumab	149	8.84	1.07
Infliximab	149	8.40	2.20
Cetuximab	152	8.37	1.13

Conclusions

Non-specific interactions with the column resin that occur during SEC analysis have been shown to be eliminated when using the MAbPac SEC-1 column, and the column exhibits the required resolution for aggregate analysis. Using the MAbPac SEC-1 column allows a single globally applicable SEC chromatography method for biotherapeutic monoclonal antibodies.

References

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