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Optimizing protein aggregate analysis by size exclusion chromatography

Authors

Rowan Moore,[1] Amy Farrell,[2] Jonathan Bones,[2] and Ken Cook[1]

[1]Thermo Fisher Scientific, Hemel Hempstead, UK
[2]NIBRT - The National Institute for Bioprocessing Research & Training, Dublin, Ireland

Keywords

MAbPac SEC-1, monoclonal antibody, mAb, aggregation analysis, biotherapeutics, size-exclusion chromatography, pre-column dispersion

ABSTRACT

Purpose: Monoclonal antibody (mAb)-based therapeutics are structurally complex. A number of critical quality attributes (CQAs) must be monitored during development, bioproduction and drug product manufacture to meet standards for clinical use. Protein aggregation can occur during manufacture and storage and could seriously affect product safety and efficacy. Robust analytical methods capable of quantifying the extent of protein aggregation are essential to maintain product performance and patient safety. Size exclusion chromatography (SEC) is commonly used for this purpose, but without proper system optimization and conditions, problems that affect analytical performance can arise. In this study a universal SEC method for monitoring and quantifying the aggregation of mAbs was developed and an optimized ultra high performance liquid chromatography (UHPLC) system setup for aggregate analysis by SEC was determined.

Methods: MAb samples (bevacizumab, cetuximab, infliximab, rituximab and trastuzumab) were analysed on a bio-inert quaternary UHPLC system with a diode-array detector (DAD). The column employed was a 7.8 × 300 mm silica - based column with a proprietary, covalently bonded diol hydrophilic layer. System optimization was conducted using the mAb bevacizumab analysed on both 7.8 and 4 mm i.d. variants of the silica-based column. Dispersion effects were studied through variation of pre-column tubing internal diameter (i.d.) and the addition of solvent to the mobile phase.

Results: The mAbs studied did not display any non-specific interactions with the stationary phase of the selected column, allowing a single, globally applicable SEC chromatography method to be developed. It was determined that poor peak resolution associated with pre-column dispersion at low flow rates can be minimized through the use of pre-column transfer tubing of narrower i.d. and smaller injection volumes.

INTRODUCTION

MAbs are a dominant class of protein biotherapeutics which have achieved outstanding success in treating many life-threatening and chronic diseases. Over 20 mAb targeted therapy products reached ‘blockbuster’ status in 2015 (1). With many commercially successful biologic patents now expired, or nearing expiry over the next few years, there exists a great opportunity for mAb-based biosimilars to enter the global market. MAbs are known to form aggregates in the course of product expression during fermentation, product purification in downstream processing, or in storage or through mishandling of the product prior to patient administration.

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Aggregation of mAb monomers to dimers, trimers and other higher order structures is undesirable for two key reasons:

1. Aggregates may cause a decrease in product efficiency by lowering the effective concentration of the product.
2. Aggregation can expose normally unexposed epitopes leading to increased immunogenicity.

In order to demonstrate the safety and efficacy of the mAb and gain regulatory approval, it is essential to monitor the formation of aggregation products throughout the production process.

SEC is the standard method for protein aggregate analysis. The technique involves passing molecules through a column containing porous polymer or silica beads. The choice of pore size is related to the size of the molecule to be separated. For the separation of mAbs and their aggregates this is around 300 Å. Molecules are separated based on their hydrodynamic volume. Smaller molecules can penetrate fully into the pores of the stationary phase, while larger molecules cannot get totally inside the porous bead and therefore have less distance to travel and so elute through the column more quickly.

One requirement of the technique is that the analyte does not interact with the surface of the stationary phase. Ideally, differences in elution time are based solely on a protein's hydrodynamic volume, rather than its chemical or electrostatic interactions with the stationary phase.

However, mAbs are structurally diverse and can exhibit unwanted secondary interactions with residual groups on the column during analysis, affecting analytical data quality. Non-specific hydrophobic binding of proteins to the columns can, for example, lead to retention time shifts, peak tailing, or even a complete loss of protein peaks (2,3). A general SEC method applicable to a wide range of mAbs is therefore highly sought after.

MATERIALS AND METHODS

Sample Preparation

Universal MAb Aggregate SEC Method Development:

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

Optimized Instrument Setup Determination:

Bevacizumab was diluted 1:1 with mobile phase (see Table 1).

Columns

Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 4.0 × 300 mm (P/N 074696)
Thermo Scientific™ MAbPac™ SEC-1, 5 µm, 7.8 × 300 mm (P/N 088460)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system equipped with:

- System Base Vanquish Flex (VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment (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)

Test Methods

Pre-Column Dispersion

180 µm ,100 µm, 75 µm, and 50 µm i.d. tubing was fitted pre-column to test dispersion.

Solvent Addition

0.2 M sodium chloride (NaCl) in 100 mM phosphate buffer, pH 6.8 containing 0, 5, 10 and 20 % (v/v) ACN was used as mobile phase to test the effect of solvent on peak shape and retention time (see Figure 6).

Chromatography Conditions

Table 1. Chromatography conditions applied when developing the universal mAb method and testing the various SEC conditions.

Test	Mobile Phase	Flow Rate (mL/min)	Column Temperature (° C)	Injection Volume (µL)	UV Wavelength (nm)
Universal Method – 7.8 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	1.0	25	1	214
Dispersion Test – 7.8 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	1.0	30	1	214
Dispersion Test – 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	0.3	30	1	214
Injection Volume Test – 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8	0.3	30	1 & 10	214
Solvent Addition Test - 4 mm i.d. column	0.2 M NaCl in 100 mM phosphate buffer, pH 6.8 containing 0, 5, 10 or 20 % (v/v) ACN	0.3	30	1	214

Data Analysis

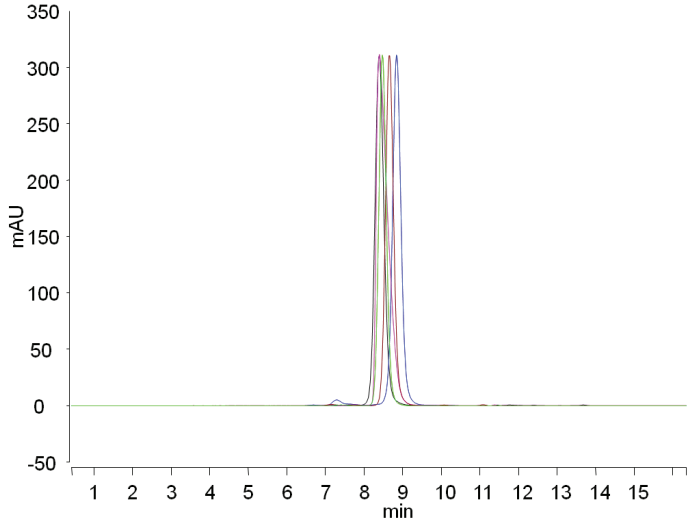
Thermo Scientific™ Chromeleon™ 7.2 SR2 Chromatography Data System was used for data acquisition and analysis.

RESULTS

Universal MAb Aggregate SEC Method Development

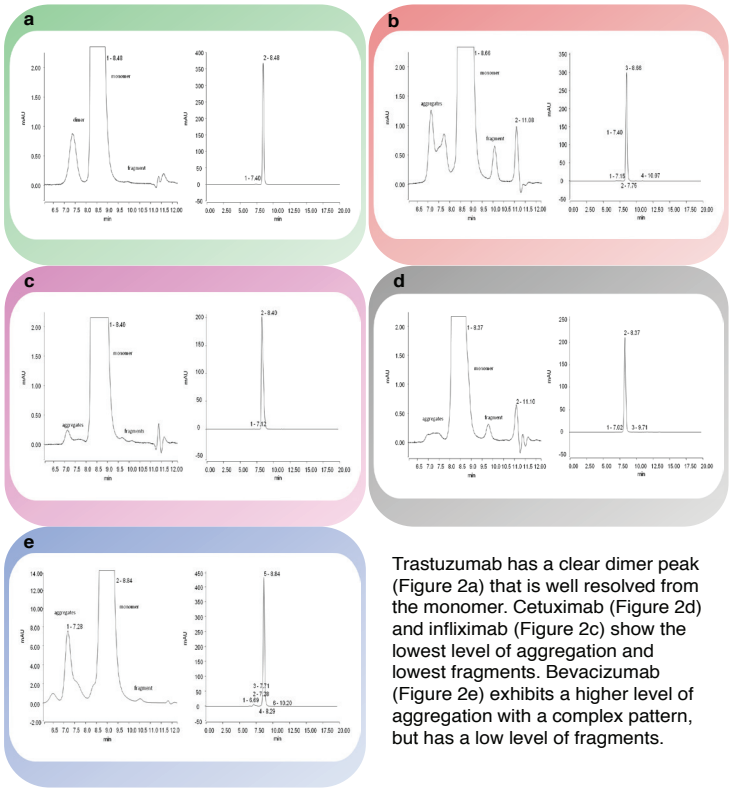
Using the MAbPac SEC-1 column, suitable resolution of aggregates and fragments from the monomer was achieved for all five mAbs (Figure 1), permitting determination of the percentage aggregation in each case (Table 2).

Figure 1. All 5 mAb samples overlaid showing relative retention time and peak shape (traztuzumab, rituximab, infliximab, cetuximab, bevicuzumab).



Cetuximab and infliximab showed the lowest levels of aggregation and fragmentation. Bevacizumab exhibited a higher level of aggregation and a complex aggregation pattern, but had a lower level of fragmentation (Figure 2).

Figure 2. SEC mAb separations. Expanded view (left) and full range chromatogram (right). Traztuzumab (a), rituximab (b), infliximab (c), cetuximab (d), bevicuzumab (e).



Trastuzumab has a clear dimer peak (Figure 2a) that is well resolved from the monomer. Cetuximab (Figure 2d) and infliximab (Figure 2c) show the lowest level of aggregation and lowest fragments. Bevacizumab (Figure 2e) exhibits a higher level of aggregation with a complex pattern, but has a low level of fragments.

Given the narrow range of molecular weights of the proteins studied, the similar retention times obtained for the samples, shown in Table 2, indicates a lack of secondary interactions with the column. This conclusion is further supported by the good symmetry of the chromatogram peaks.

Table 2. Retention time comparison of mAbs, associated molecular weights, asymmetry and percentage of aggregates in each mAb.

mAb Sample	Molecular Weight (kDa)	Retention Time (min)	Asymmetry	% Aggregates
Rituximab	145	8.66	1.10	0.92
Trastusumab	148	8.48	1.10	0.39
Bevacizumab	149	8.84	1.07	2.99
Infliximab	149	8.40	2.20	0.10
Cetuximab	152	8.37	1.13	0.29

Optimized Instrument Setup Determination

SEC is one of the few chromatography methods that exhibits no on-column focusing. Therefore, careful control of pre-column dispersion is essential to achieve optimum separation results, especially at reduced flow rates on smaller i.d. columns as broad peak volumes are not focused at the column head. To investigate the effect of pre-column dispersion on analytical performance, pre-column tubing of various diameters were used in combination with 4 and 7.8 mm i.d. columns for the analysis of bevacizumab.

Using the 7.8 mm i.d. column at the higher flow rate of 1.0 mL/min, there was essentially no difference in the produced chromatograms when the pre-column tubing was changed from the standard i.d. of 100 µm to 75 µm. Peak width, asymmetry and resolution were the same in both analytical runs, as shown in Figure 3.

Figure 3. Overlay of bevacizumab SEC analysis with 100 µm and 75 µm i.d. tubing placed between injection valve and column. Column i.d. was 7.8 mm.

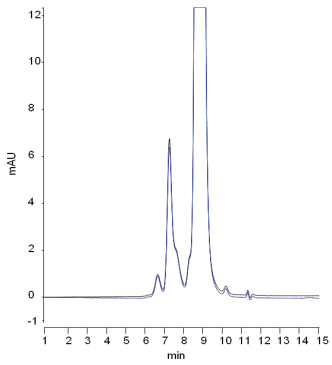
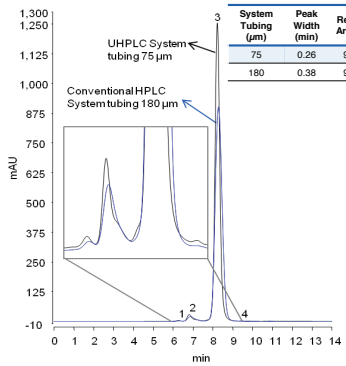


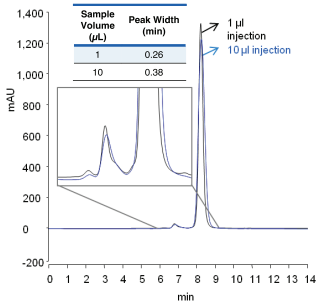
Figure 4. Overlay of bevacizumab using 180 µm and 75 µm i.d. tubing in front of the column. Column i.d. was 4.0 mm.



System Tubing (µm)	Peak Width (min)	Relative Area (%)
75	0.26	97.48
180	0.38	97.77

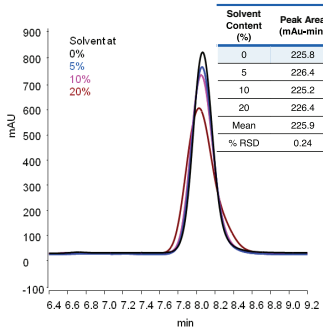
Using the 4 mm i.d. column at the lower flow rate of 0.3 mL/min, pre-column dispersion had a significant impact on analytical performance. Using 180 µm i.d. tubing markedly reduced peak shape quality (Figure 4), this effect would be compounded by the dispersion associated with the injection valves of older HPLC systems. Reduced tubing i.d. provided a significant improvement in peak resolution, the optimum tubing diameter was determined to be 75 µm.

Figure 5. Effect of injection volume on peak dispersion with 4 mm i.d. SEC column.



Sample Volume (µL)	Peak Width (min)
1	0.26
10	0.38

Figure 6. Inclusion of solvent in the SEC buffer conditions.



Solvent Content (%)	Peak Area (mAu·min)	Peak Area (mAu)
0	225.8	825.3
5	226.4	769.3
10	225.2	731.6
20	226.4	595.5
Mean	225.9	730.4
% RSD	0.24	13.39

The effect of injection volume on analytical performance was also studied (Figure 5). The 10 µL injection volume resulted in greater peak width and a loss in resolution and definition of the smaller aggregate peak.

The use of solvent changes the properties of the surrounding matrix and affects the structural folding of the protein. Peak retention time and asymmetry do not change with the addition of solvent (Figure 6). This would be expected if there were no secondary hydrophobic interactions between column and protein. At 5 % solvent, the peak height reduces and the peak width increases. At 20 % solvent peak shape is compromised (Figure 6).

CONCLUSIONS

- Robust and accurate protein aggregation analysis was achieved for five structurally diverse mAbs using a SEC method that employed a MAbPac SEC-1 silica-based column.
- The column's hydrophilic diol layer eliminates non-specific protein–column interactions.
- Poor peak resolution, associated with pre-column dispersion at lower flow rates, could be minimized through the use of pre-column transfer tubing of narrower i.d. (75 μm) and smaller injection volumes (1 μL).
- The addition of solvent is not required or recommended as peak asymmetry is acceptable without it.
- These findings highlight the importance of correct instrument set-up when using UHPLC systems, operating at lower flow rates.
- Through correct instrument optimization and the appropriate choice of chromatography columns, protein aggregation analysis by SEC can be a useful technique to ensure the safety and efficacy of mAb-based biotherapeutic products.

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