APPLICATION NOTE

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The importance of correct UHPLC instrument setup for protein aggregate analysis by size-exclusion chromatography

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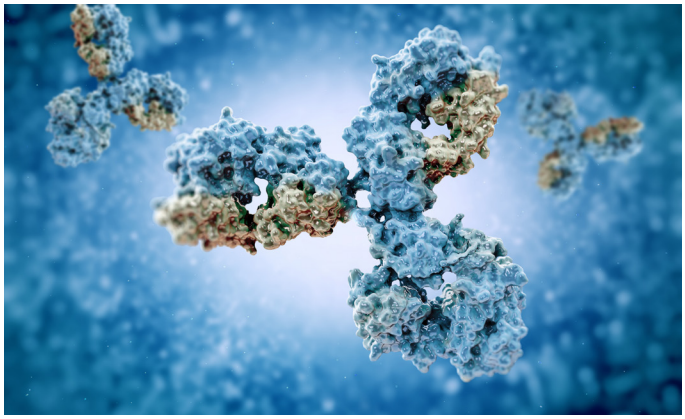
Key words
MAbPac SEC-1, monoclonal antibody, mAb, protein aggregation analysis, biotherapeutics, pre-column dispersion

Goal

Show the applicability of Thermo Scientific™ MabPac™ SEC-1 columns for monoclonal antibody aggregate analysis using the Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system.



Introduction

The industry-standard size-exclusion chromatography (SEC) column dimension for protein aggregate analysis is 7.8 mm internal diameter (i.d.), running at 1.0 mL/min. This is usually carried out using older HPLC systems, which perform well enough with these relatively high flow rates. The introduction of UHPLC, with much lower dispersion, has allowed the use of lower flow rate columns and higher resolution stationary phases. It is commonly believed that smaller dimension SEC columns are difficult to pack and this accounts for the reduction in performance seen with these columns compared to higher flow rate 7.8 mm i.d. columns. A more likely cause for apparent reduced performance is that the UHPLC system used for the comparison does not have the correct tubing or low dispersion flow path required to maintain peak integrity, an essential factor when using SEC at lower flowrates.[1,2]

A Vanquish Flex Quaternary UHPLC system was applied for the SEC analysis. This is a low dispersion, inert UHPLC, which can be used successfully for this type of application. For this analysis, pre-column dispersion was intentionally introduced to show the effects of dispersion in front of the column at different flow rates.

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The separation was performed on MabPac SEC-1 columns of differing internal diameters and flow rates.

The Vanquish UHPLC system was used to show the applicability of the MAbPac SEC-1 column for monoclonal antibody aggregate analysis. The MAbPac SEC-1 column is silica based and has been covalently modified with a proprietary diol hydrophilic layer to prevent secondary interactions which can hinder the chromatography of certain proteins.[2,3] SEC is one of the few chromatography methods that exhibits no 'on-column' focusing. Due to this, the pre-column dispersion on the system used is extremely important, especially at reduced flow rates on smaller i.d columns, as there will be no focusing of broad peak volumes at the head of the column. In adsorption chromatography, even under isocratic elution conditions, one would expect some focusing of the injection volume at the head of the column. In SEC, the volume in which the sample is presented to the column will only get larger in volume as it moves through the column. Therefore, many columns do not attain their expected resolution when using older HPLC systems with inherent dispersion on-injection. The effect of pre-column dispersion has been the subject of several reviews and can easily lead to up to 50% increase in peak widths on dispersive HPLC systems. The low dispersion Vanquish UHPLC system was applied for the SEC analysis to control and study the effects of dispersion. The separation was performed on MabPac SEC-1 columns with a commonly used high salt buffer 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 preparation

Bevacizumab was diluted to a 25 mg/mL solution with mobile phase containing 0, 5, 10, or 20% solvent as appropriate (see Figure 4).

Separation conditions

Instrumentation

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)

Columns:	MAbPac SEC-1, 7.8 x 300 mm (P/N 088460) MAbPac SEC-1, 4 x 300 mm (P/N 074696)

SEC buffer

Mobile phase:	0.2 M NaCl in 100 mM phosphate buffer pH 6.8
Flow rate:	1.0 mL/min for 7.8 mm i.d. column and 0.3 mL/min for 4 mm i.d. column
Column temperature:	30 °C
Injection volume:	1 µL, unless stated
UV:	214 nm

Data processing

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

Results and discussion

Pre-column dispersion

Columns of 4.0 and 7.8 mm i.d. were used to test the effects of pre-column dispersion. The optimal flow of these columns is 0.3 and 1.0 mL/min, respectively. The flow rate used has a profound effect on the setup of the system. Lower flow rates were much more prone to the effects of any pre-column dispersion. At the higher flow rate of 1.0 mL/min using the 7.8 mm i.d. column, there was no discernible difference when the pre-column tubing was changed from the standard 100 µm i.d. tubing to 75 µm. Peak width, asymmetry, and resolution were all the same in both analyses, as can be seen in the overlay in Figure 1. This column was flowing at 1.0 mL/min, which generated 30 bar backpressure on the pre-column 100 µm tubing alone. The backpressure generated by the 75 µm tubing was 90 bar, but the change showed no improvement, indicating the chromatography was already optimum with the standard configuration on the system.

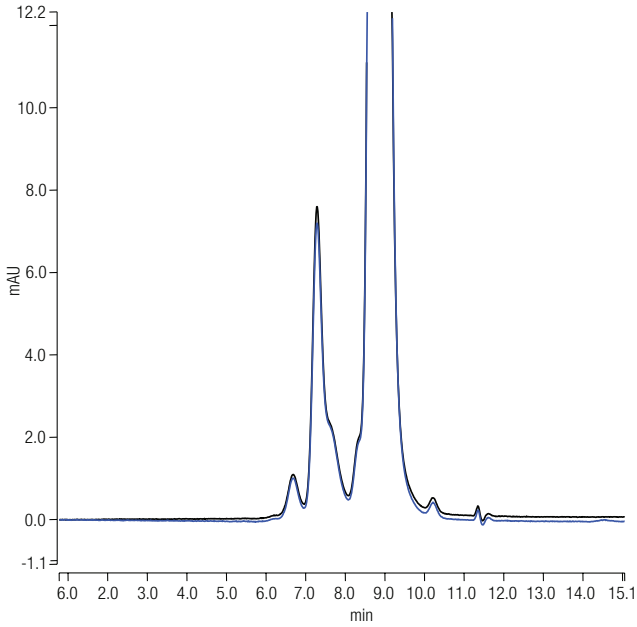
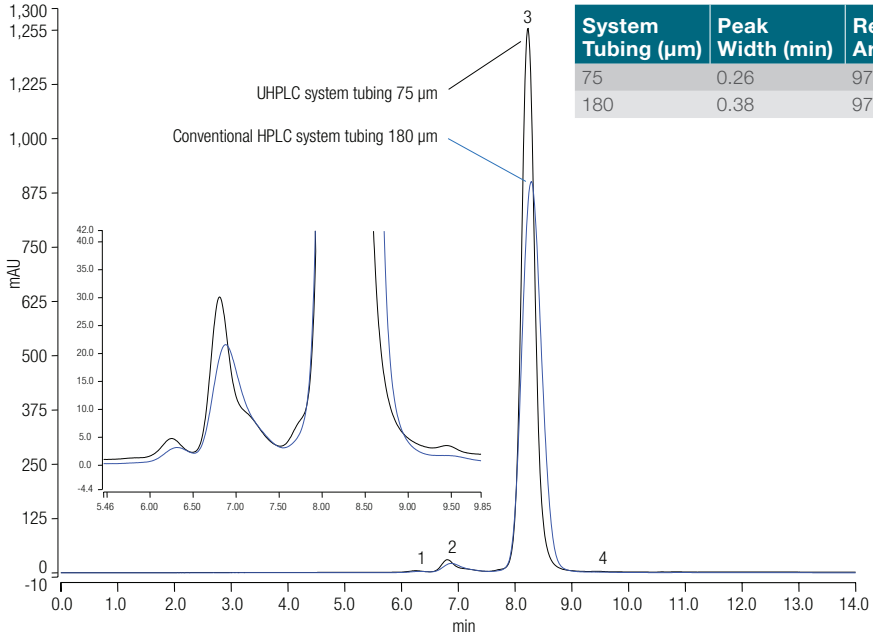


Figure 1. Overlay of bevacizumab SEC analysis with 100 µm and 75 µm i.d. tubing placed between the injection valve and the front of the column. Column i.d. was 7.8 mm.

The change when using the 4 mm id. column at 0.3 mL/min is quite dramatic. To mimic the configuration of a standard HPLC system, 180 µm tubing was used as well as 100 µm and 75 µm. These configurations are commonly used in SEC separations. In the overlay shown in Figure 2 there is a marked reduction in performance using 180 µm tubing in front of the column. This is what can be expected using a standard HPLC system at this flow rate on 4 mm i.d. SEC columns. This effect is compounded by the addition of the dispersion in the injection valves of older HPLC systems.

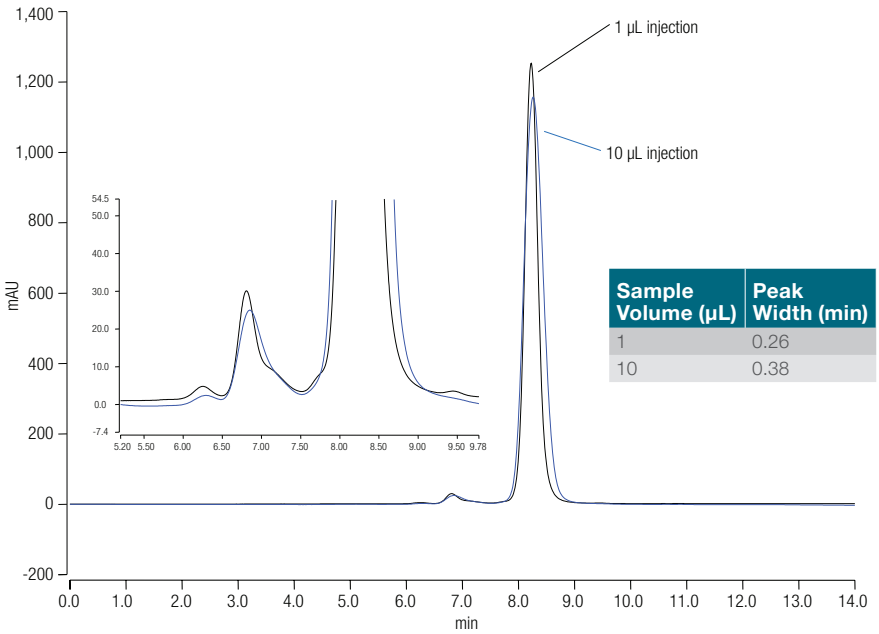


System Tubing (µm)	Peak Width (min)	Relative Area (%)
75	0.26	97.48
180	0.38	97.77

Figure 2. Overlay of bevacizumab using 180 µm and 75 µm i.d. tubing in front of the column. Column i.d. was 4.0 mm.

The relative area of the main monomer peak remains consistent. However, the peak shows marked dispersion with an increase in peak width at half height from 0.26 to 0.38 min and reduced sensitivity due to peak height reduction. There is also a loss of resolution and definition in the aggregate peaks, which leads to difficulty in identification of the aggregate forms. Peak dispersion and broader peak shape also influence the accuracy of integration with lower abundant variants. The 100 µm tubing showed some signs of dispersion (not shown for clarity). However, reducing the tubing inner diameter to 50 µm showed no further improvement over the 75 µm tubing, indicating that the optimum tubing to eliminate peak dispersion was 75 µm.

The volume of sample injected also plays an important part in peak dispersion in SEC, as it is essentially a form of inherent dispersion in the method. First, 1 µL of bevacizumab was injected onto the 4 mm i.d. column at a flow rate of 300 µL/min. The next injection was a 10× dilution using the same amount of sample in a 10 µL injection. The dispersion is evident in the increase in peak width and the loss in resolution and definition of the smaller aggregate peaks (Figure 3). There is a shoulder in front of the main peak that disappears along with the resolution of a second dimer peak. This confirms the importance of keeping injection volume as low as possible for SEC. Maintaining a high concentration of sample allows less volume to be injected onto the column and therefore lowers dispersion.

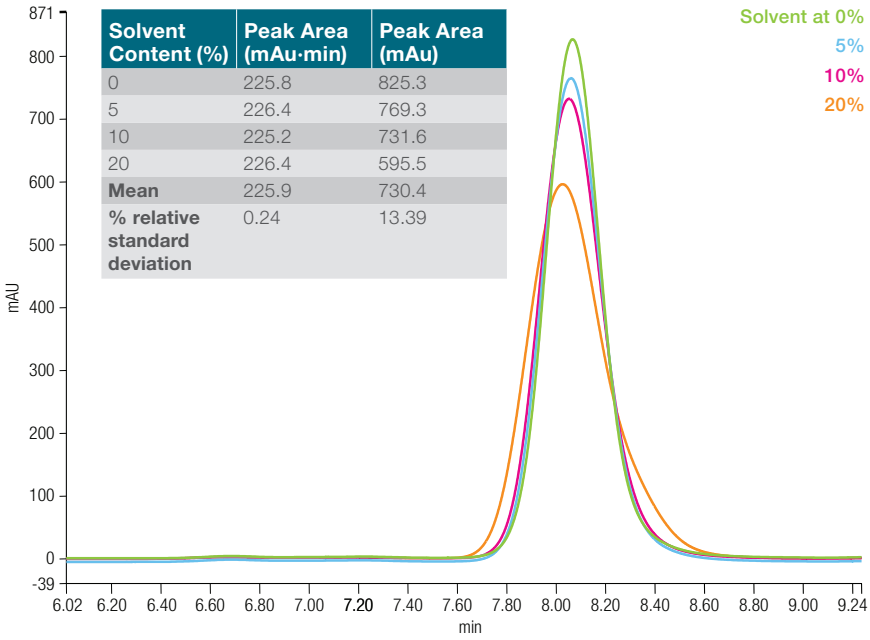


Sample Volume (µL)	Peak Width (min)
1	0.26
10	0.38

Figure 3. Effect of injection volume on peak dispersion with 4 mm SEC columns.

Solvent addition

There are reports of the use of solvents to improve peak shape on SEC columns that show secondary hydrophobic interactions. The use of any solvent with native protein samples can lead to unfolding of the protein, and the severity of the effect will be protein dependent. Proteins fold in a way that keeps the hydrophobic amino acids in the center of the folded protein with the hydrophilic amino acids on the outside in contact with the hydrophilic environment. The use of solvent changes the properties of the surrounding matrix and affects the structural folding of the protein. The MAbPac SEC-1 column has a proprietary hydrophilic boundary layer to eliminate secondary interactions with the silica resin. The effects of solvent addition can be seen in Figure 4.



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
% relative standard deviation	0.24	13.39

Figure 4. Inclusion of solvent in the SEC buffer conditions.

The peak retention time and asymmetry do not change with the addition of solvent, in this case acetonitrile, to the mobile phase. This would be expected if there were no secondary hydrophobic interactions between the column and the protein sample. However, even at 5% solvent, the peak height reduces and the peak width increases. This is possibly the effects of partial unfolding of the protein and a loss of structural stability. The shape and folded size of the protein has a greater distribution and creates a wider peak on size exclusion analysis. At 20% solvent, the effect is quite dramatic and the peak shape is compromised. This result has been confirmed with different mAb samples [not shown]. The level of the effect varies with the protein but all show some peak disruption with solvent. The addition of solvent is not required or recommended as peak asymmetry is acceptable without it.

Conclusions

SEC is prone to the effects of pre-column dispersion. Effects can be minimized by using higher flow rates on wider bore column formats. After the flow rates are reduced to 300 µL/min, or column formats are less than 4 mm i.d., the use of low dispersion UHPLC systems becomes essential for optimum performance. These systems may even need further optimization to include the use of 75 µm i.d. transfer tubing from the injection valve to the column.

Issues from non-specific interactions with the column resin during SEC analysis appear to have been eliminated with the measures taken to produce the MAbPac SEC-1 column. It proves to have more than satisfactory resolution for protein aggregate analysis and, in particular, for monoclonal antibodies. The use of solvent is not required to improve peak shape.

The addition of solvent to improve peak shape on columns that exhibit hydrophobic secondary interactions may help reduce the unwanted interaction but thought must be given to the additional effect of the solvent on the protein itself.

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