Application Note 21206

HIC as a Complementary, Confirmatory Tool to SEC for the Analysis of mAb Aggregates

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Key Words
Size exclusion chromatography, SEC, hydrophobic interaction chromatography, HIC, mAb aggregate, MAbPac SEC-1, MAbPac HIC-10

Goal

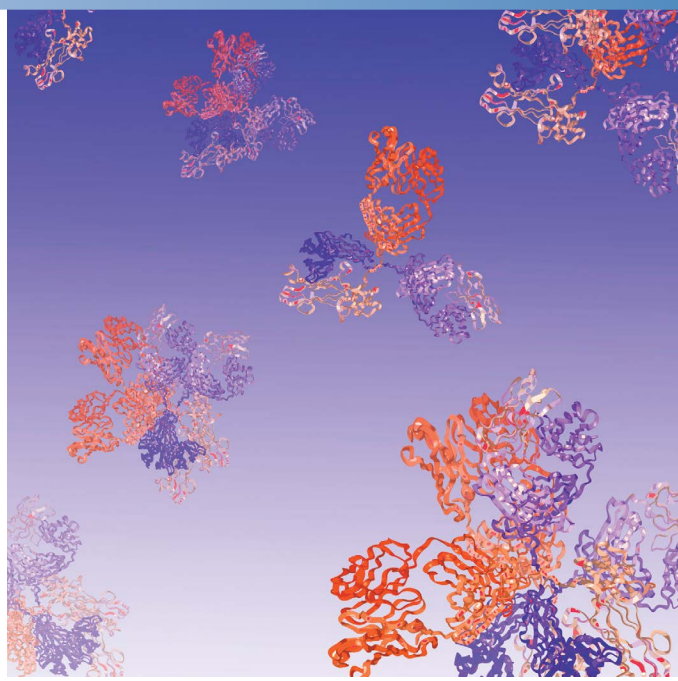
To demonstrate the analysis of monoclonal antibody (mAb) aggregates using the Thermo Scientific™ MAbPac™ SEC-1 and MAbPac™ HIC-10 columns. The MAbPac SEC-1 column provides fast and high-resolution separation of mAb aggregates from the monomer. The MAbPac HIC-10 column provides excellent separation of mAb aggregates from the monomer and the ability to resolve mAb variants.

Introduction

Monoclonal antibodies and related products are widely used as therapeutic agents to treat various cancers and autoimmune diseases. The production of mAb therapeutics can be highly challenging due to various chemical degradation processes and aggregation. MAb aggregates may form during protein expression, downstream processing, and storage, and may be caused by high concentration, elevated temperature, abrupt change in pH, shear strain, freeze-thaw cycle, or surface adsorption.

Aggregates in mAb therapeutics can result in an undesired immune response that affects the safety and the potency of the drug due to incorrect dosage.[1] Therefore, monitoring mAb aggregation is important for their production and quality assurance. Size-exclusion liquid chromatography (SEC) has been the method of choice for the detection and quantification of mAb aggregates. However, the use of both standard hydrophobic interaction chromatography (HIC) and mixed-mode HIC for separation of protein aggregates has been reported.[2-4] HIC separates proteins based on the hydrophobicity in the native state and can often detect changes in protein structure as well as aggregates.[5]

HIC can be used as a secondary assay to confirm aggregation or for removal of aggregates during purification. Here, we demonstrate the use of MAbPac SEC-1 and MAbPac HIC-10 columns for high-resolution mAb aggregate analysis.



The MAbPac SEC-1 column is based on high-purity, spherical, porous (300 Å), 5 µm silica particles covalently modified with a proprietary diol hydrophilic layer, designed for mAb aggregate analysis. The MAbPac HIC-10 column is a wide-pore (1000 Å), 5 µm silica-based HIC column with polyamide functionality, providing desired selectivity for intact mAb and mAb variants. Both columns can be used under non-denatured conditions to preserve the bioactivity of mAbs/proteins.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Fisher Scientific™ Isopropanol (P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$, ≥98.0%)
- Ammonium sulfate [$(NH_4)_2SO_4$, ≥99.0%]

Sample Handling Equipment

Thermo Scientific vial and closure kit (P/N 055428)

Sample Preparation

The mAb sample was provided by a customer.

HIC: The 8 mg/mL sample was diluted two-fold with mobile phase A.

SEC: The 8 mg/mL sample was directly injected onto the column.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRSLC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a micro flow cell
Columns	MAbPac HIC-10, 4.6 × 100 mm (P/N 088480)
	MAbPac SEC-1, 4 × 150 mm (P/N 075592)

SEC

Mobile phase	50 mM sodium phosphate, 300 mM NaCl, pH 6.8
Preparation of mobile phase	Dissolve 6.9 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \bullet H_2O$) and 17.5 g of sodium chloride in 900 mL of DI water, adjust the pH to 6.8 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.
Flow rate	0.2 mL/min
Column temperature	30 °C
UV detector wavelength	280 nm

HIC

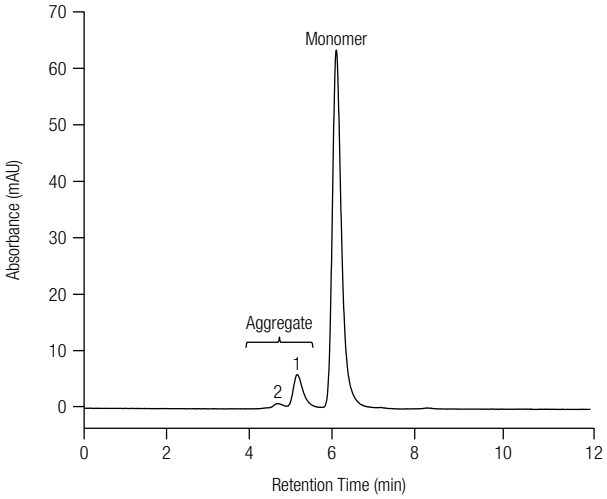
Mobile phase A	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile phase B	100 mM sodium phosphate, pH 7.0		
Preparation of mobile phase A	Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \bullet H_2O$) and 264.2 g of ammonium sulfate in 800 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.		
Preparation of mobile phase B	Dissolve 13.8 g of sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \bullet H_2O$) in 900 mL of DI water, adjust the pH to 7.0 with 50% NaOH solution and bring the volume to 1000 mL with DI water. Filter the mobile phase through a 0.22 µm filter.		
Gradient	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	29.0	0	100
	34.0	0	00
Flow rate	0.5 or 1.0 mL/min		
Column temperature	30 °C		
UV detector wavelength	280 nm		

Software

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

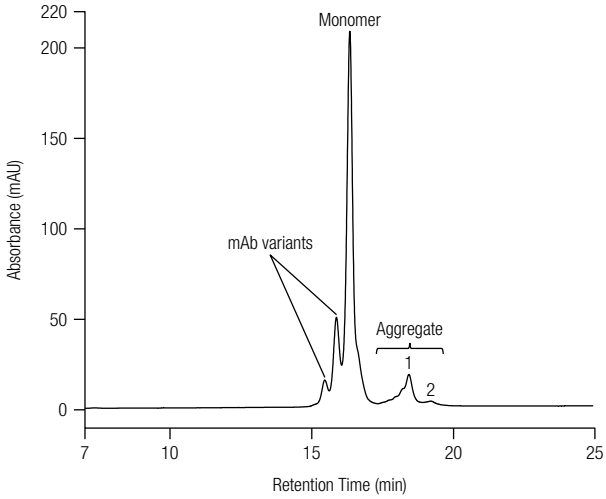
The mAb aggregates were separated within 8 minutes on a MAbPac SEC column (Figure 1); two aggregate peaks and a monomer peak were observed. The first aggregate peak was confirmed as a dimer using the Thermo Scientific™ Exactive™ Plus EMR mass spectrometer.[6]



Column:	**MAbPac SEC-1**, 5 µm
Format:	4.0 ×150 mm
Mobile Phase A:	50 mM sodium phosphate,300 mM NaCl, pH 6.8
Flow Rate:	0.2 mL/min
Inj. Volume:	1 µL (8 mg/mL)
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	mAb

Figure 1. Separation of mAb aggregates using MAbPac SEC-1 column.

Figure 2 shows the separation of two mAb aggregate peaks from the monomer form using the MAbPac HIC column. In HIC, aggregates typically elute later than the monomer due to the exposed hydrophobic groups in the denatured chains of aggregate.[7] The intensity ratios of the aggregate peaks to the monomer peak were consistent with the MAbPac SEC-1 result. The first aggregate peak was broad with obvious additional peaks, most likely due to different conformations of the mAb aggregate.

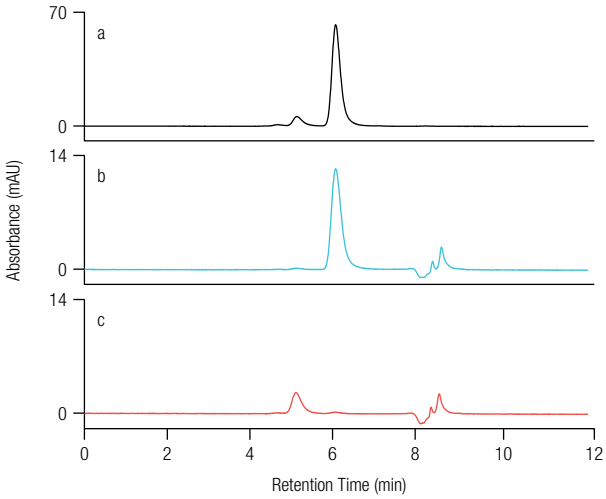


Column:	**MAbPac HIC-10**, 5 µm		
Format:	4.6 ×100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient:	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	29.0	0	100
	34.0	0	100
Flow Rate:	0.5 mL/min		
Inj. Volume:	10 µL (4 mg/mL)		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb		

Figure 2. Separation of mAb aggregates and hydrophilic variants using MAbPac HIC-10 column.

The SEC column separates by size and gives one peak for a mAb dimer, whereas the HIC column separates by hydrophobicity and resolves the different isomeric forms of the dimer. This gives additional information of the aggregate forms present which you cannot get from SEC.

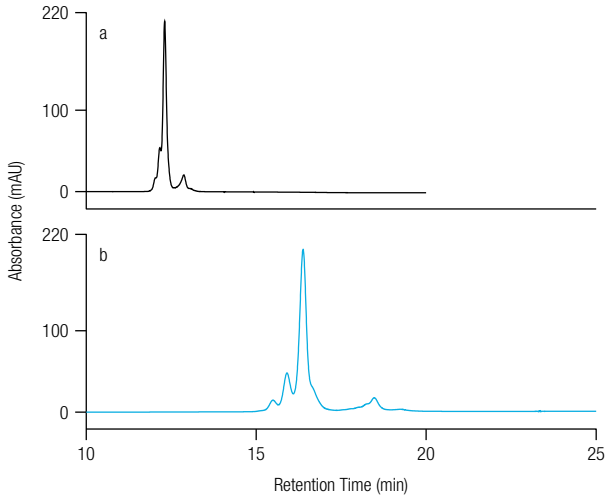
Aggregates observed on the MAbPac HIC column were collected and re-injected onto the MAbPac SEC-1 column (Figure 3). The retention time of the collected fraction was compared to the retention time of the mAb aggregate peak. The retention times of the monomer and the first aggregate peak fractions matched the retention time of the chromatogram obtained by directly injecting the mAb sample onto the MAbPac SEC-1 column.



Column:	**MAbPac SEC-1**, 5 µm
Format:	4.0 × 150 mm
Mobile Phase A:	50 mM sodium phosphate,300 mM NaCl, pH 6.8
Flow Rate:	0.2 mL/min
Inj. Volume:	(a) 1 µL (8 mg/mL) (b) 20 µL (c) 20 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	(a) mAb (b) Monomer fraction from MAbPac HIC-10 run (c) Aggregate fraction from MAbPac HIC-10 run

Figure 3. Separation of mAb fractions obtained from MAbPac HIC-10 column on the MAbPac SEC-1 column.

In addition to the aggregate peaks, the MAbPac HIC-10 column was able to separate two hydrophilic mAb variant peaks. Several chromatographic conditions were investigated including mobile phase, temperature, and flow rate. Separation of both mAb aggregate and hydrophilic variants was best achieved using a 1.2 M to 0 M ammonium sulfate gradient in 28 minutes at 0.5 mL/min flow rate. Addition of organic solvent to the mobile phases caused the hydrophilic variant peaks to disappear while the aggregate peaks were still separated. Lower flow rates (0.5 mL/min) with longer gradient time gave better resolution of the aggregates and hydrophilic variants compared to 1 mL/min with shorter gradient time (Figure 4).



Column:	**MAbPac HIC-10**, 5 µm		
Format:	4.6 ×100 mm		
Mobile Phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0		
Mobile Phase B:	100 mM sodium phosphate, pH 7.0		
Gradient (a)	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	15.0	0	100
	20.0	0	100
Gradient (b)	Time (min)	%A	%B
	-5.0	60	40
	0.0	60	40
	1.0	60	40
	29.0	0	100
	34.0	0	100
Flow Rate:	(a) 1.0 mL/min (b) 0.5 mL/min		
Inj. Volume:	10 µL (4 mg/mL)		
Temp.:	30 °C		
Detection:	UV (280 nm)		
Sample:	mAb		

Figure 4. Separation of mAb aggregates using MAbPac HIC-10 column with different flow rates.

Conclusion

- MAbPac SEC-1 column provides fast and high-resolution separation of mAb and its aggregates.
- MAbPac HIC-10 column provides excellent separation of mAb and its aggregates and can be used as a secondary method for mAb aggregate analysis.
- MAbPac HIC-10 column can resolve mAb variants and mAb aggregates within the same analysis.

References

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