APPLICATION NOTE

AN1611: High-Speed Process Monitoring with UHP-SEC-MALS

Sophia Kenrick, Wyatt Technology Corp.

Summary

Size exclusion chromatography (SEC) is widely used for detecting aggregates, fragments, and other impurities in a biotherapeutic sample. When coupled with multi-angle light scattering (MALS), SEC enables researchers to quantify the true solution molar mass of each species in a sample, in addition to the mass fraction of each species.

In many applications, ultra-high performance liquid chromatography (UHP-SEC) is preferable to traditional HPLC thanks to faster run times, lower solvent consumption, and enhanced resolution. A particularly important application of UHP-SEC is at-line process monitoring (ALPM), made feasible by the five-minute runs typical of UHP-SEC. ALPM minimizes tedious and costly off-line analyses performed by collecting and transferring aliquots to the analytical lab. ALPM is particularly valuable in production because it can minimize the losses encountered when a process begins to fail.

MALS is widely used in conjunction with standard HPLC-SEC, but MALS detectors for HPLC conditions are not suitable for UHPLC. The µDAWN® three-angle MALS detector and Optilab® UT-rEX™ differential refractive index (dRI) detector provide the low dispersion and high data rate required for ultra-high-performance liquid chromatography (UHPLC), making UHP-SEC-MALS attractive for in-process monitoring as well as analysis of the final product.

In this application note, we perform rapid UHP-SEC-MALS analysis on two antibodies, mAb1 and mAb2, under various conditions mimicking different manufacturing and purification processing steps. MALS, RI, and dynamic light scattering (DLS) data were collected in each of the five-minute analyses, providing robust, reproducible, and reliable quantification of molar mass, size, and amount of each species in a given sample.

Introduction

At-line process monitoring (ALPM) of biological drugs is of major benefit in reducing the time and effort involved in process analytics. While conventional HPLC can provide good analytical capabilities, it is too slow to be considered useful in ALPM. Ultra-high-performance liquid chromatography (UHPLC) provides improved resolution and greatly reduced analysis time compared to conventional HPLC, making feasible the quantification of aggregate and fragment samples, at-line, in process environments and in the course of accelerated stability tests.

This study evaluated the utility of UHP-SEC-MALS—combining UHPLC size-exclusion chromatography (UHP-SEC), multi-angle light scattering (MALS) and inline dynamic light scattering (DLS)—as an effective tool for characterizing monoclonal antibodies and impurities or degradants encountered at line, under processing and accelerated stability conditions. The µDAWN and UT-rEX UHPLC detectors determine absolute solution molecular weight and molecular size of each eluting species, yet UHP-SEC-MALS experiments are completed in five minutes, making this an ideal technique for process monitoring.

The two antibodies in this study mimic different conditions for which high-speed monitoring with UHP-SEC-MALS may be appropriate. mAb1 represents multiple fractions collected from a purification process. The molar mass of each peak, measured by MALS, helps to identify each species. The results indicate that UHP-SEC-MALS may be implemented in routine in-process monitoring.

MAb2 represents a molecule undergoing stability testing. The combination of multiple formulations and stability conditions can easily result in hundreds of samples to be characterized. The improved throughput of UHP-SEC-

MALS analysis relative to traditional HPLC-SEC-MALS facilitates characterization of degradants such as aggregates and fragments. We found that different fragments may be generated by condition 3 compared to conditions 1 and 2 based on the measured molar masses of these species. This sample underscores the need for MALS detection in UHP-SEC, as the two stability conditions result in monomers with different elution times but identical molar masses as determined by MALS.

Materials and Methods

UHP-SEC was performed with an Acquity UPLC pump and autosampler (Waters Corporation) and BEH SEC column (200 Å, 1.7 µm, 4.6 mm x 150 mm; Waters). The effluent of the SEC column flowed through an inline UV/Vis detector (Waters), Wyatt µDAWN multi-angle light scattering detector with internal WyattQELS™ dynamic light scattering detector, and Optilab UT-rEX dRI detector. Phosphate buffered saline (PBS) was used as the mobile phase at a flow rate of 0.5 mL/min. For each chromato-gram, 2 µL to 5 µL sample was injected with an overall run time of 5 minutes per sample. Data collection and analysis was performed with ASTRA software (Wyatt). The samples are summarized in Table 1.

Monoclonal antibody 1 (mAb1) represents various phases of the purification process, ending with purified sample. Monoclonal antibody 2 (mAb2) has undergone various stability testing processes. In addition, bovine serum albumin (BSA) was used as a control to demonstrate no loss of resolution or data quality as a function of flow rate.

Sample	Description
mAb1	Purified antibody 1
mAb1-C1	mAb 1, process condition 1
mAb1-C2	mAb 1, process condition 2
mAb2-C1	mAb 2, stability condition 1
mAb2-C2	mAb 2, stability condition 2
mAb2-C3	mAb 2, stability condition 3

Table 1. Antibody samples

Results and Discussion

No loss of resolution with flow rate

Peak resolution, molar mass distribution, and calculated mass were compared for BSA at multiple flow rates. As shown in Figure 1, there is no appreciable change in peak shape or resolution when the separation is performed at 0.5 mL/min compared to 0.3 mL/min. Furthermore, the measured molar masses for the monomer, dimer, and trimer are in good agreement, and the eluted mass for each species and overall eluted mass is the same at both flow rates (Table 2).

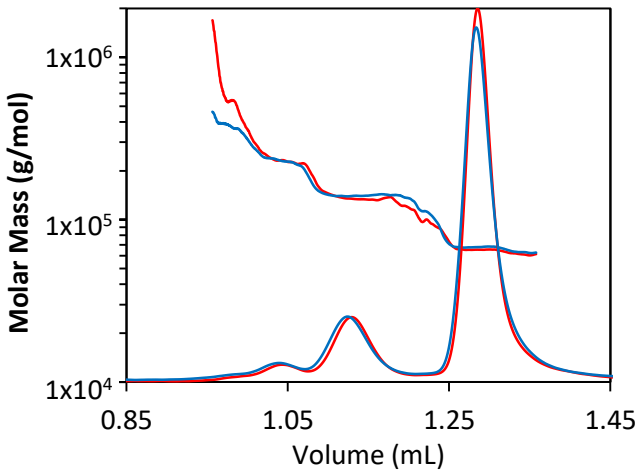


Figure 1. Light scattering chromatogram for BSA separation at 0.3 mL/min (blue) and 0.5 mL/min (red). The molar mass measured by MALS is overlaid for each chromatogram.

	0.3 mL/min	0.5 mL/min
Monomer	86.4%	85.5%
Dimer	8.2%	8.1%
Trimer	1.4%	1.6%

Table 2. Mass fraction of each species at the two flow rates shown in Figure 1.

In-process monitoring of mAb1 purification

Once we established that the resolution and molar mass accuracy did not change as a function of flow rate, we used high-speed UHP-SEC-MALS to quantify each phase of the purification of mAb1. The starting material (mAb1-C1) contains approximately 70% monomer, with the rest of the mass represented by aggregate and fragment species. Sample mAb1-C2 represents an intermediate purification process, resulting in a solution of 85% monomer.

The final purified antibody (mAb1) is >95% monomer by mass.

UHP-SEC-MALS provided rapid quantitation of each mAb sample, making this technique perfect for ALPM. All the species elute in less than three minutes (Figure 2), and the solvent peak is visible in the RI data at ~4 minutes elution time, allowing a complete UHP-SEC-MALS experiment to be performed in 5 minutes.

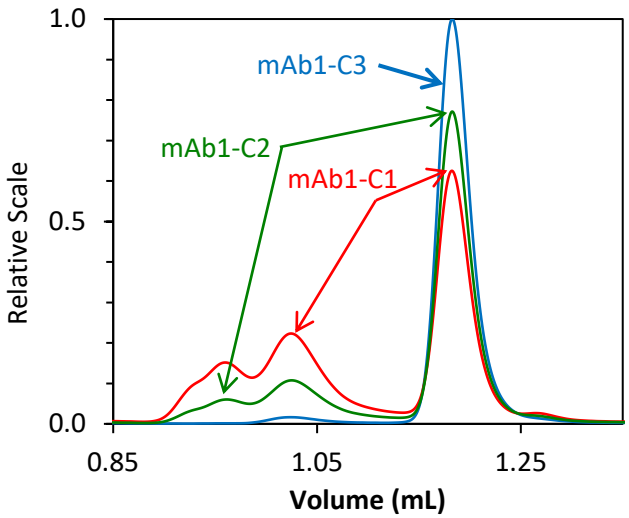


Figure 2. Overlay of light scattering chromatograms for mAb1 undergoing different stages of purification.

Not only is the UHP-SEC-MALS method fast, it is also reliable. Figure 2 and Figure 3 show the chromatograms for replicate injections of each sample. The light scattering data (Figure 2) and concentration measured by refractive index (Figure 3) overlay exactly for each replicate. Molar mass is critical to establishing the identity of the eluting species. The molar mass measured by the µDAWN and UT-rEX is also extremely robust and reliable, despite the low concentration of certain peaks. For example, in the case of the purified mAb1 (blue lines, Figure 2 and Figure 3), even though the maximum concentration of aggregate is only 0.8 µg/mL as it elutes from the column, the measured molar mass is within 2% of the molar mass measured for conditions 1 and 2, where the eluting concentration is significantly higher (Peak 2 M_w, Figure 4).

Five different species were defined based on the mAb1-C1 chromatogram. The peak definitions for these species are shown in Figure 4. The mass fraction of each species was calculated by integrating the RI signal for each peak. The table in Figure 4 shows how effectively the different aggregate fractions are removed, while the monomer is enriched, as the purification process progresses. The molar mass of each species also appears consistent for collected fraction (Figure 3 and Figure 4). These molar mass data provide important information about the purification process or the nature of each aggregate, such as whether the aggregates are sheared during the process or whether new aggregate species are formed.

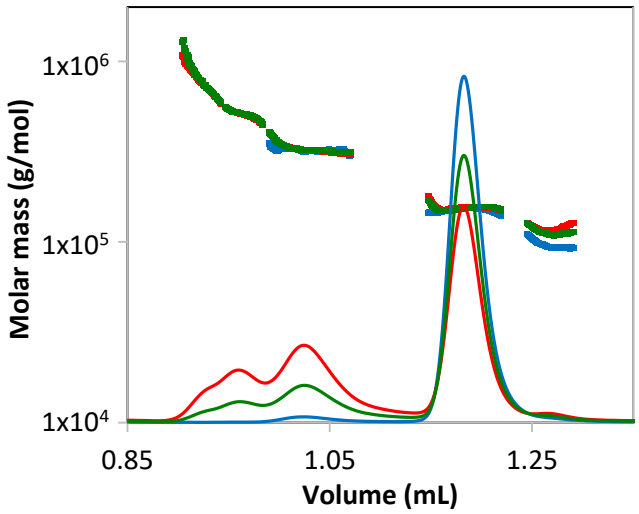
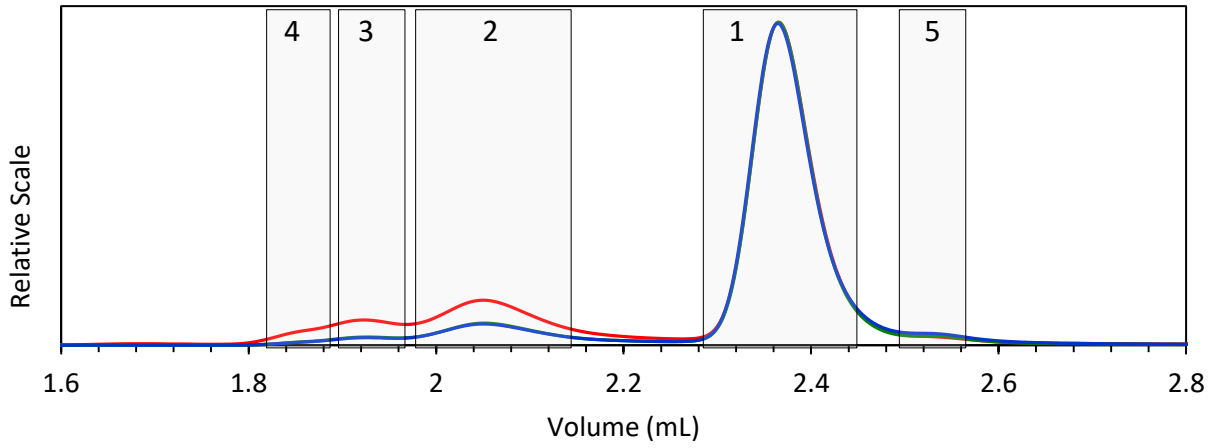


Figure 3: Concentration data from refractive index for mAb1 undergoing different stages of purification. The measured molar mass is overlaid for each peak. Chromatograms are colored as in **Error! Reference source not found.**.

Stability testing of mAb2

In addition to purification, ALPM is desirable in accelerated stability tests used to optimize formulations. Stability testing may generate hundreds of samples, and the ability to quantify them accurately and rapidly is of paramount importance. Antibody sample mAb2 underwent three stability-testing conditions. For each condition, we quantified the molar mass and mass fraction of monomer, dimer, and fragment using the same chromatography conditions as for mAb1.

Each of the three stability conditions resulted in different monomer, dimer, and fragment profiles. Conditions 1 and 2 produced similar chromatograms; however, mAb2-C2 contained a slightly higher fraction of dimer compared to mAb2-C1 (Figure 5 and Table 3). In contrast, condition 3 resulted in relatively little dimer but a larger fraction of fragment (Figure 6 and Table 3).



		Peak 1 (monomer)		Peak 2		Peak 3		Peak 4		Peak 5 (fragment)	
		MW (kDa)	Mass (%)	MW (kDa)	Mass (%)	MW (kDa)	Mass (%)	MW (kDa)	Mass (%)	MW (kDa)	Mass (%)
mAb1-C1	1	155.4	70.7%	328.1	18.5%	507.3	4.7%	720.5	2.0%	120.0	4.2%
	2	155.3	70.6%	329.7	18.6%	510.9	4.8%	735.9	2.0%	119.9	4.0%
mAb1-C2	1	153.6	85.2%	329.7	8.8%	510.5	1.9%	729.2	0.7%	114.3	3.5%
	2	153.3	85.1%	329.9	8.9%	510.8	1.9%	730.0	0.7%	115.4	3.4%
mAb1-C3	1	152.4	96.0%	323.4	1.1%	--	<0.1%	--	<0.1%	98.2	2.8%
	2	152.4	96.1%	335.9	1.2%	--	<0.1%	--	<0.1%	102.9	2.7%

Figure 4: Peak definitions (top) and resulting molar mass and mass fraction (bottom) of each species. Two injections were performed for each mAb1 sample. Peak areas were defined at identical elution times for all six injections, as shown in the top figure (mAb1-C1). The weight-average molar mass and mass fraction for each peak are listed in the table. Plots correspond to light scattering (red), RI (blue) and UV (green) signals.

Stability condition 3 underscores the need for MALS in conjunction with SEC with two striking differences compared to conditions 1 and 2 (Figure 6). First, the monomer does not elute at the same time for condition 3 as it does for conditions 1 and 2; however, the measured molar mass of this peak has not changed (Table 3). Relying only on the elution time, one might conclude that the antibody has been degraded. However, by measuring an identical molar mass for all three conditions, we hypothesize that the late elution time may be due to a change in conformation (e.g., partial unfolding) or column interactions (e.g., resulting from a hydrophobic patch or other residue being exposed during the stability test). Second, the molar mass of the fragment is significantly higher than it is for conditions 1 and 2. This may be due to coelution with the monomer or may suggest that this stability condition produces a different fragmentation pattern compared to conditions 1 and 2.

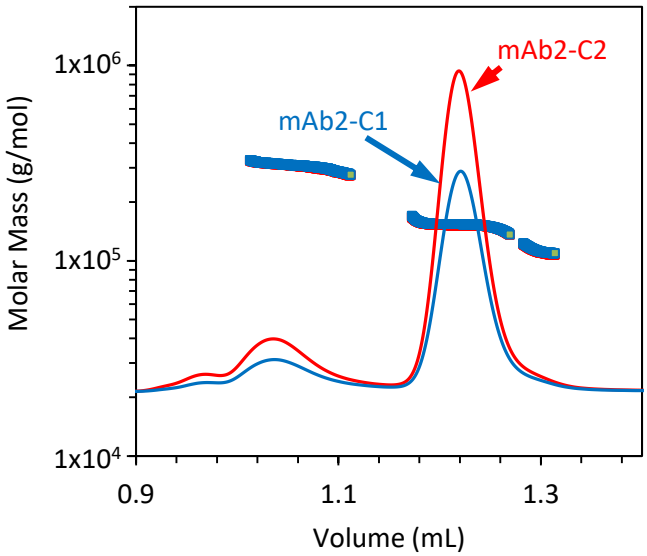


Figure 5: Refractive index chromatograms for mAb2 under stability conditions 1 (blue) and 2 (red). The molar mass of the monomer, dimer, and fragment peaks has been overlaid.

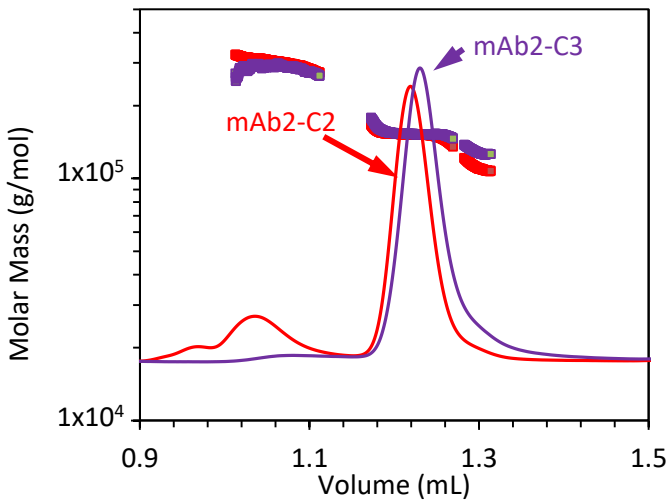


Figure 6: Refractive index chromatograms for mAb2 under stability condition 3 (purple). Molar mass values from MALS of the monomer, dimer, and fragment peaks have been overlaid. The chromatogram and measured molar mass for mAb2-C2 (red) are included for comparison.

In addition to molar mass from MALS, the µDAWN enables measurement of hydrodynamic radius by DLS. As shown in Figure 7, the hydrodynamic radius for all three monomers is R_h = 5.1 ± 0.1 nm. This suggests that mAb2-C3 is not significantly unfolded compared to mAb2-C1 and mAb2-C2, and the late elution time is likely due to column interactions.

		Monomer		Dimer		Fragment	
		MW (kDa)	Mass (%)	MW (kDa)	Mass (%)	MW (kDa)	Mass (%)
mAb4-C1	1	152.8	87.7%	308.4	8.4%	114.4	4.0%
	2	1524	87.5%	305.4	8.7%	111.1	3.8%
mAb4-C2	1	151.5	87.1%	306.6	9.3%	112.9	3.7%
	2	151.4	87.2%	303.1	9.2%	114.0	3.7%
mAb1-C3	1	153.0	90.2%	286.7	1.2%	132.1	8.6%
	2	152.7	90.2%	285.1	1.5%	130.2	8.3%

Table 3: Evolution of monomer, dimer, and fragment under different stress conditions for mAb2

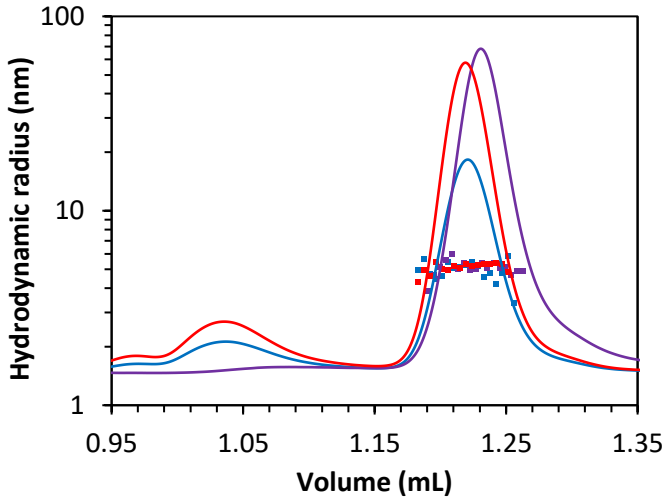


Figure 7: Measured hydrodynamic radius data for the mAb2 monomer under stability test 1 (blue), 2 (red), and 3 (purple) overlaid on light scattering chromatogram

Conclusions

In summary, we demonstrated the use of high-speed UHP-SEC combined with MALS and DLS for process monitoring of antibody samples. With this technique, measurements may be performed in as little as 5 minutes, enabling monitoring of purification and other processing steps, as well as high-throughput stability screening. The µDAWN and UT-rEX provided essential and reliable quantification of molar mass and hydrodynamic radius of aggregates and fragments that would not be possible with analytical UHPLC alone.

References

Mou X, et al. *Pharm. Bioprocess.* (2014) **2**(2), 141-156.