Application Note

Biopharma

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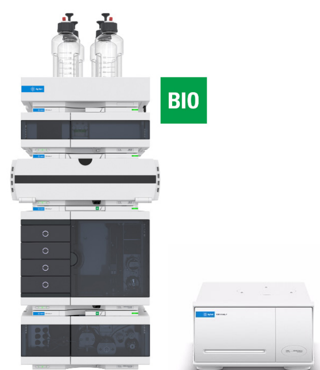
Advanced SEC-MALS Analysis of Monoclonal Antibodies with the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector



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Abstract

Size exclusion chromatography (SEC) in combination with multiangle light scattering (MALS) detection enables accurate and reliable separation and molecular weight (MW) determination of monomer and aggregate protein species. The Agilent 1260 Infinity II Multi-Angle Light Scattering detector coupled to the Agilent 1290 Infinity II Bio LC System offers the possibility to measure the intensity of the scattered light at multiple angles to gain more precise absolute MW for the analysis of biopharmaceuticals. The 1290 Infinity II Bio LC is specially designed for conditions used in biochromatography with a completely iron-free flow path and a high tolerance to salt-based buffers such as those used in SEC.

Introduction

SEC is an essential tool for characterizing biotherapeutic proteins such as monoclonal antibodies (mAbs), especially for MW, size, and aggregate ratio. The determination of these parameters is crucial in product characterization and quality control throughout all lifecycle stages. High ratios of aggregates or fragments are critical indicators of an unstable product. Detailed characterization of main product, as well as potential degradation products is therefore invaluable for a safe biopharmaceutical, and is required by regulatory agencies.[1]

In conventional protein SEC, a standard mix containing different proteins of defined MW is injected to generate a column calibration detected by a concentration detector such as an ultraviolet (UV) or refractive index (RI) detector. A critical factor for successful SEC separation is having no protein interactions with the column through "SEC unspecific" reactions such as electrostatic or hydrophobic interactions. As the column calibration is related to the elution volume, unspecific electrostatic or hydrophobic interactions may result in inaccurate MW determination.

A powerful addition to classical SEC is MALS, which enables absolute determination of MW independent of "SEC unspecific" column interactions or the amount of sample loaded onto the column. Light scattering detectors determine the MW without the need for column calibration. In addition, dimensional information about molecular size by the radius of gyration (Rg) can be obtained with MALS if the dimension of the molecule is large enough compared to the wavelength of the laser. Typically, this technique is used for molecules with an Rg greater than 10 nm (considered anisotropic scatterers). Therefore, it is not applied for isotropic scatterers such as globular proteins.[2,3]

Experimental

Equipment

Agilent 1290 Infinity II Bio LC System:

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Quick Connect Bio Heat Exchanger Standard Flow (G7116-60071)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with Bio Micro Flow Cell VWD, 3 mm, 2 µl, RFID (G1314-60189)
- Agilent 1260 Infinity II Multi-Angle Light Scattering Detector (G7885A)

Note: All measurements shown in this application note can also be performed on other Agilent Bio LC Solutions such as the Agilent 1260 Infinity II Bio-Inert LC System.

Column

Agilent AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm (PL1180-5301)

Software

Agilent WinGPC software, version 1.0, was used in this study. Later versions also apply.

Chemicals, solvents, and samples

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium dihydrogen phosphate, disodium hydrogen phosphate heptahydrate, and sodium hydroxide were obtained from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). MabThera was purchased by Medizone Germany GmbH (Munich, Germany) and was stored in a refrigerator for approximately 7 years.

Solvent and sample preparation

Two liters of 150 mM phosphate buffer were prepared using 15.22 g of sodium dihydrogen phosphate and 46.41 g of disodium hydrogen phosphate heptahydrate. The pH was adjusted to pH 7 using sodium hydroxide solution. The prepared phosphate buffer was triple-filtered using a 0.2 µm membrane filter. In addition, the samples were filtered using an Agilent Captiva premium syringe filter with a regenerated 4 mm cellulose membrane, 0.2 µm pore size (part number 5190-5106). BSA was dissolved in the prepared phosphate buffer to a concentration of 20 mg/mL. MabThera is formulized at a concentration of 10 mg/mL.

Note: Phosphate buffered solvents at physiological pH are highly prone to bacteria and algae growth and should be replaced at least every few days. In between buffer changes, the LC needs to be flushed with water/organic mixtures to prevent contamination. To avoid buffer salt crystallization, the flow should be set to a low flow rate instead of stopping the flow after analysis.

Table 1. Method parameters.

Parameter	Value
Flow Rate	0.6 mL/min
Mobile Phase	150 mM Phosphate buffer, pH 7, triple filtered
Injection	5 to 20 µL
Stop Time	22 min
Needle Wash	Flush port, 3 s, water:isopropanol 80:20 (v:v)
Autosampler Temperature	8 °C
Column Temperature	30 °C
DAD Detection	280 nm, peak width > 0.05 min (10 Hz)
MALS Detection	Cell temperature 30 °C, 20 angles collected

Results and discussion

Static light scattering requires a single measurement for detector calibration procedures as well as for interdetector delay setup. For proteins, the molecule of choice is often BSA. Figure 1 shows the separation of the BSA monomer, dimer, trimer, tetramer, and higher aggregates on the AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm. Excellent resolution was found between the monomer and the aggregates. The BSA monomer was used for calibration and detector setup. The values calculated for the different aggregate species were found to match at approximately a multiple of the value of the monomer. Figure 1 shows the UV signal of the VWD, as well as three exemplary MALS signals with 90°, typically used for protein analysis together with the smallest (12°) and the greatest (164°) available angle of the 1260 Infinity II MALS detector.

The bigger the molecules, the more sensitive the reaction of the MALS detector.[4] MALS is a molar-mass sensitive detector, where the signal intensity depends on the concentration of the samples but also on molar mass. The higher the molar mass, the higher the signal intensity. In contrast, the signal intensity of concentration detectors, such as UV detectors, only depends on sample concentration, together with a sample constant such as the extinction coefficient at the measured wavelength. The higher aggregates of BSA are clearly visible in the MALS signals, compared to very low UV signals (Figure 2). Moreover, the signals of the small angles show the highest sensitivity for large aggregates of BSA.

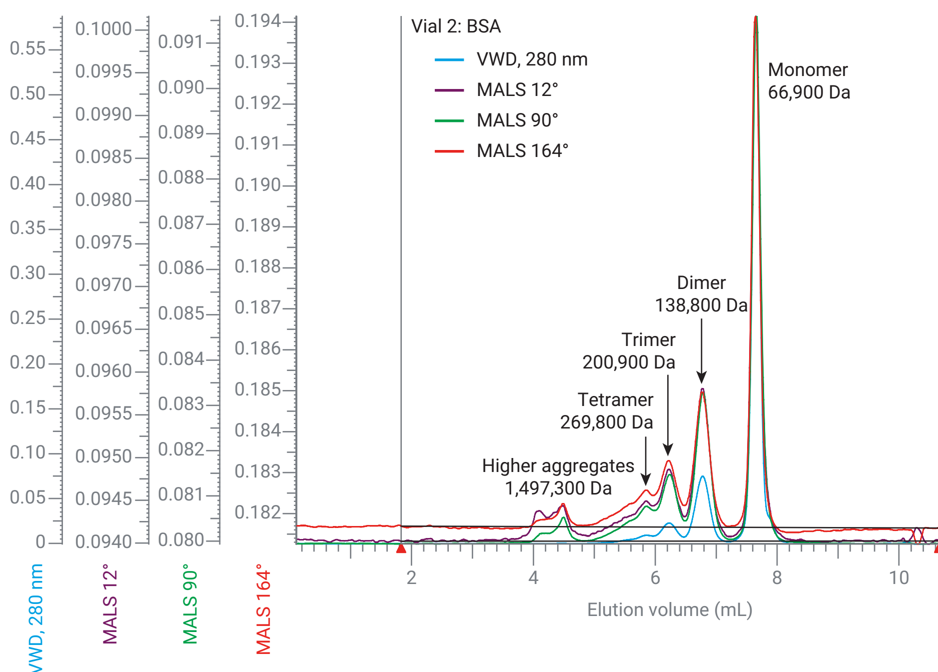

Figure 1. SEC MALS analysis of BSA. VWD at 280 nm (blue) and examples of three MALS angles at 12° (purple), 90° (green), and 164° (red) are displayed together with the corresponding MW results for monomer, dimer, trimer, tetramer, and higher aggregates.

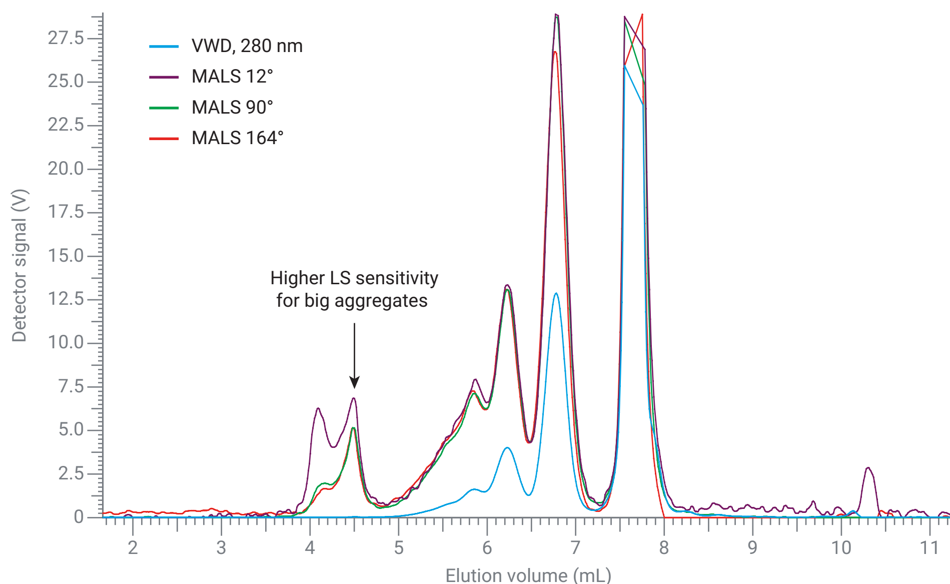

Figure 2. SEC-MALS analysis of BSA – zoomed in. VWD at 280 nm (blue) and examples of three MALS angles at 12° (purple), 90° (green), and 164° (red) show that MALS versus UV sensitivity increases with increasing molecule size.

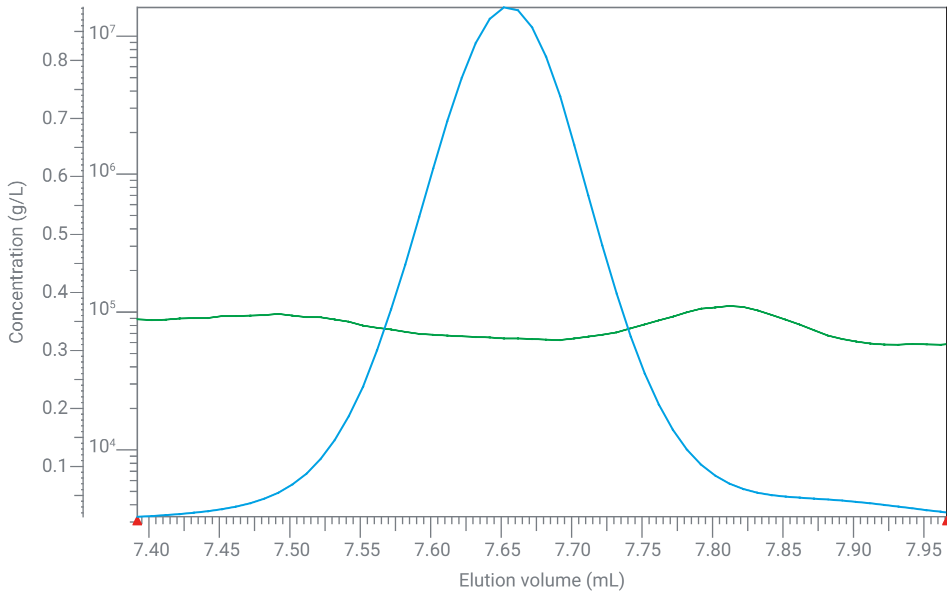

Figure 3. BSA monomer (blue UV signal) that shows a monodisperse peak (flat green signal).

Pure monomeric proteins such as the BSA monomer generate monodisperse peaks in the SEC chromatogram, containing one type of molecule with a defined MW (Figure 3, flat green line within the peak limits).

Figures 4A and 4B show a SEC-MALS characterization of the mAb MabThera (rituximab). The MALS analysis revealed a MW of 144,000 Da for the monomeric peak, which is in agreement with the value found in literature.[5] The precision of the MW determination of 10 consecutive runs was excellent, with 0.139% relative standard deviation (RSD). Figure 4B shows a closer look into the aggregates and fragment section, which enables the determination of dimer and higher aggregates. In addition, a peak with 103,400 Da was detected, implying a possible heavy chain fragment. A shoulder at the main peak was detected as the mass of the monomeric peak.

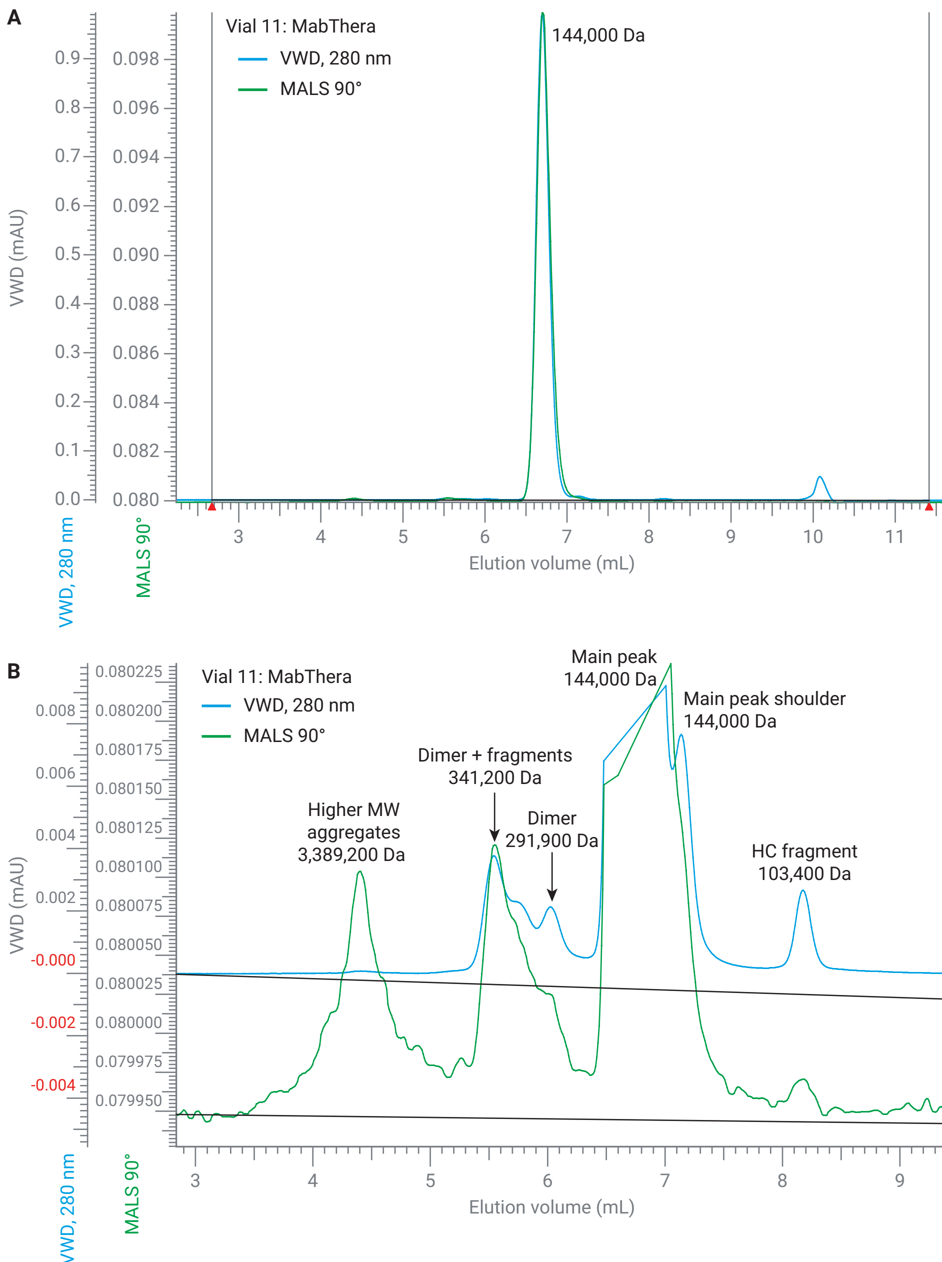

Figure 4. (A) SEC MALS analysis of MabThera (rituximab) with UV (blue) and MALS signal at 90° (green). The MW was determined at 144,000 Da for the monomer peak. (B) A zoomed in version, with more details of aggregates and fragments.

Conclusion

The possibility of using a MALS detector with 20 angles enables highly precise calculations of absolute molar mass. The MALS detector shows higher sensitivity than UV detection especially for higher protein aggregates, adding valuable information to a conventional SEC setup. The smaller angles allow the determination of higher molecular mass aggregates with high sensitivity and precision. The excellent resolution of the Agilent AdvanceBio SEC 300 Å column enables clear separation of monomer, dimer, higher aggregates, and mAb fragments, leading to unique MW determinations for the separated peaks.

The combination of the Agilent 1290 Infinity II Bio LC System with the Agilent 1260 Infinity II Multi-Angle Light Scattering detector provides a complete biocompatible flow path to ensure trusted results for challenging solvent conditions or iron-reactive samples.

References

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