Application Note

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Size Exclusion Chromatography Light Scattering Analysis of Monoclonal Antibodies

Using Agilent Advanced GPC/SEC Software for OpenLab CDS

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Abstract

Size exclusion chromatography (SEC) in combination with static light scattering (SLS) and dynamic light scattering (DLS) detection enables accurate and reliable separation and molecular weight (MW) and molecular size determination of monomer and aggregate protein species. The Agilent Advanced GPC/SEC Software for OpenLab CDS in combination with the Agilent 1260 Infinity II Bio-SEC Multidetector System featuring dual-angle and DLS detection enables light scattering (LS) capabilities in a compliance-supporting chromatography data system (CDS) environment and provides a single platform for advanced SEC analysis of biopharmaceuticals.

Introduction

The determination of molecular weight and size are some of the critical quality attributes (CQAs) to evaluate stability and integrity of biopharmaceutical relevant proteins, like monoclonal antibodies (mAbs). In addition, high ratios of aggregation and fragmentation products indicate an unstable product. Detailed characterization of the product and potential degradation products is therefore invaluable for a safe biopharmaceutical, and is required by regulatory agencies.[1]

Traditionally, these parameters can be evaluated using SEC by injecting standard mixtures of proteins with defined molecular weights to generate a column calibration detected by a concentration detector such as an ultraviolet (UV) or refractive index (RI) detector. A powerful addition to classical SEC is SLS and DLS detection, enabling absolute determination of MW and molecular size, independent of "SEC unspecific" column interactions or amount of sample loaded onto the column.

The Advanced GPC/SEC Software for OpenLab CDS seamlessly integrates gel permeation chromatography/size exclusion chromatography (GPC/SEC) LS capabilities in a compliance-supporting CDS environment, and provides a single platform for advanced SEC analysis of biopharmaceuticals like mAbs and other proteins. The OpenLab CDS environment supports SLS and DLS data acquisition, analysis, and reporting options from Agilent LS solutions like:

- Agilent 1260 Infinity II Bio-SEC Multidetector System featuring dual-angle (SLS) and DLS detection
- Agilent 1260 Infinity II Multi-Angle Light Scattering Detector

The precise determination of CQAs like absolute MW in Dalton (Da) and size as hydrodynamic radius (Rh) in nm ensures the quality targets of the product are met.

In this application note, we show advanced SEC analysis with online LS/DLS detection for the characterization of a rituximab biosimilar, determining MW in Da and size as Rh.

Experimental

Instrumentation

In this application note, the Agilent 1290 Infinity III Bio LC System was used, consisting of:

- Agilent 1290 Infinity III Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity III Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (option number 101)
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent Quick Connect Bio Heat Exchanger Standard Flow (G7116-60071)
- Agilent 1290 Infinity III Variable Wavelength Detector (G7114B) with Agilent InfinityLab Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID (G1314-60189)
- Agilent 1260 Infinity II Bio-SEC Multidetector System featuring dual-angle (SLS) and DLS detection (G7805AA and G7809A)
- Agilent 1260 Infinity III Refractive Index Detector (G7162A)

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

Column

This application note utilized the Agilent AdvanceBio SEC 300 Å column, 7.8 × 300 mm, 2.7 µm (part number PL1180-5301).

The column was extensively flushed with phosphate buffered saline (PBS) 24 hours before it was attached to the 1260 Infinity II Bio-SEC Multidetector System featuring dual-angle and DLS detection.

Software

The software used was Agilent OpenLab CDS, version 2.8, plus Agilent Advanced GPC/SEC Software for OpenLab CDS (or later versions).

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, U.S.). The PBS tablets and the bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). The prepared PBS buffer was filtered threefold using a 0.2 µm membrane filter. BSA was dissolved in the filtered PBS buffer to a concentration of 20 mg/mL. A rituximab biosimilar (Reditux by Dr. Reddy's, formulized in a concentration of 10 mg/mL) was kindly provided by Agilent India.

Note: Phosphate buffered solvents at physiological pH are highly prone to bacterial and algal growth and should be replaced 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.

Method parameters are outlined in Table 1.

Results and discussion

The combination of Advanced GPC/SEC software and OpenLab CDS enables the user to acquire LS data and perform data processing and analysis within the compliance-supporting CDS environment of OpenLab CDS.

LS instruments, like the 1260 Infinity II Bio-SEC Multidetector System, featuring dual-angle and DLS detection, and the 1260 Infinity II Multi-Angle Light Scattering Detector, are set up and displayed in the same way as the other modules in the OpenLab data acquisition. The method parameters for the LS instruments can be easily set up in the analysis method without the need for additional software packages. Additional LS parameters to calculate MW (in Da) as well as the hydrodynamic radius (Rh) can be already entered in the injection list; for example, parameters like the RI increment dn/dc, extinction coefficient, MW of the calibrants, and so on.

In the data analysis GPC/SEC layout, all GPC/SEC-related tabs like GPC/SEC LS Results and LS Results Plot, GPC/SEC Calibrations, GPC/SEC Compound Details, GPC/SEC Distributions, and LS-related functions are included to provide a clearly arranged user interface, as shown in Figure 1.

To build the correct fundament for LS calculations, the analysis type and the detector can be set up in the GPC/SEC General tab of the processing method, as shown in Figure 2. To enable the calculation of MW with LS detection, select **Advanced LS Bulk**.

Table 1. Method parameters.

Parameter	Value
Flow Rate	0.6 mL/min
Mobile Phase	PBS pH 7.4, triple filtered
Injection	20–30 µL
Stop Time	20 min
Needle Wash	Flush port, 3 s, water/isopropanol 80/20 (v/v)
Autosampler Temperature	8 °C
Column Temperature	30 °C
Detection UV	280 nm, peak width > 0.05 min (10 Hz)
Detection RI	Temperature: 30 °C Sampling rate: 2.31 Hz
Detection LS/DLS	Temperature: 30 °C Sampling rate: 5 Hz Correlator run time: 5 s Correlator function clip time: 10 µs (recommended for protein sized molecules)

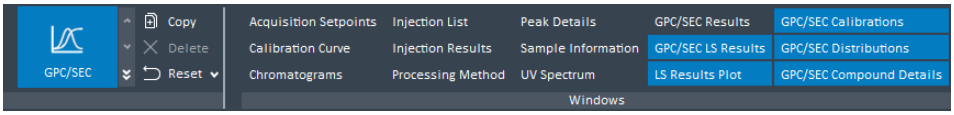


Figure 1. GPC/SEC layout options.

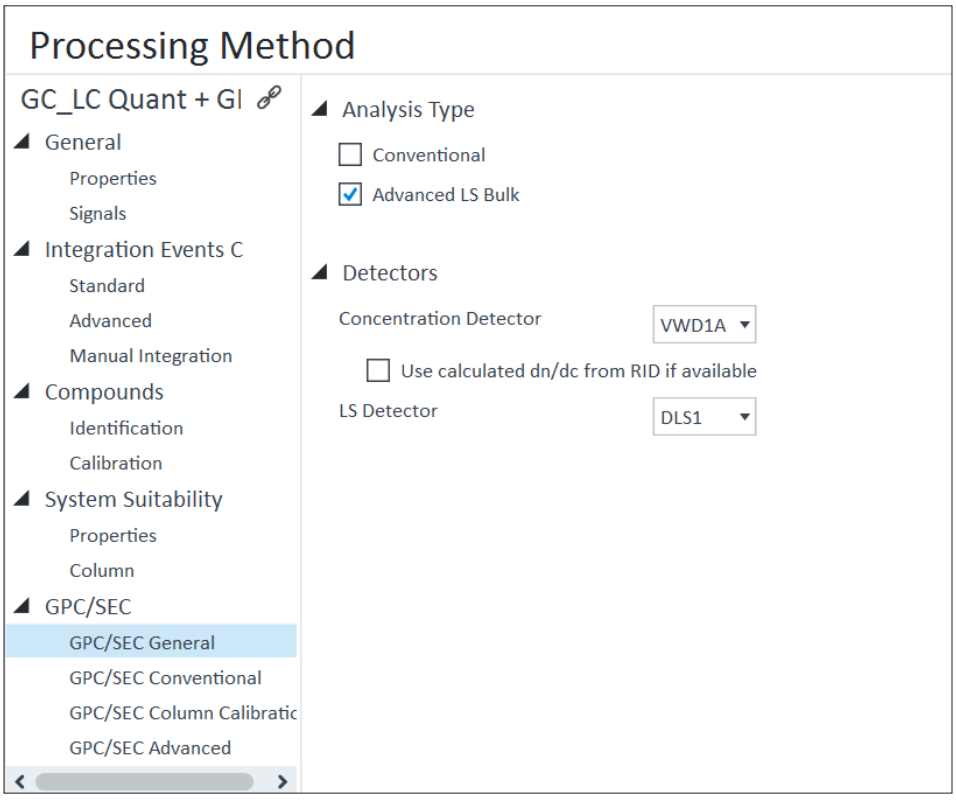
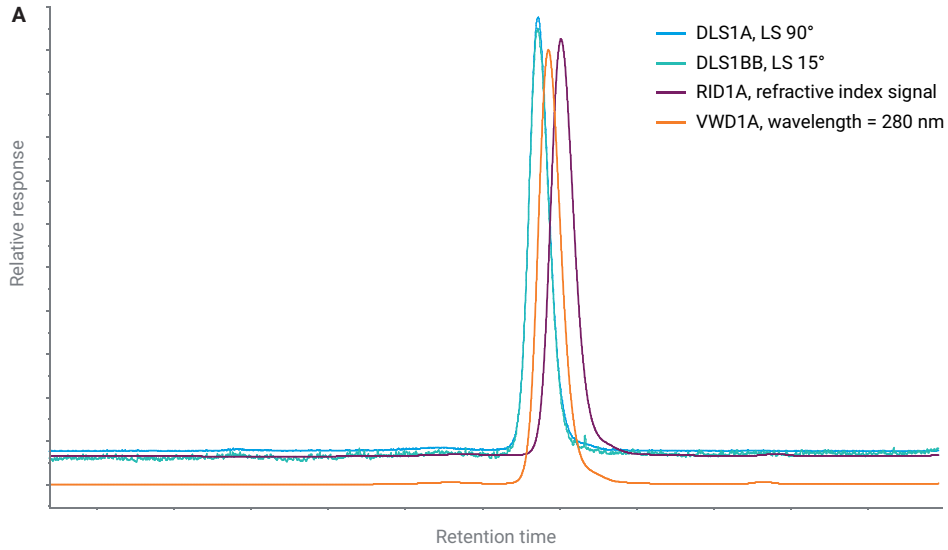
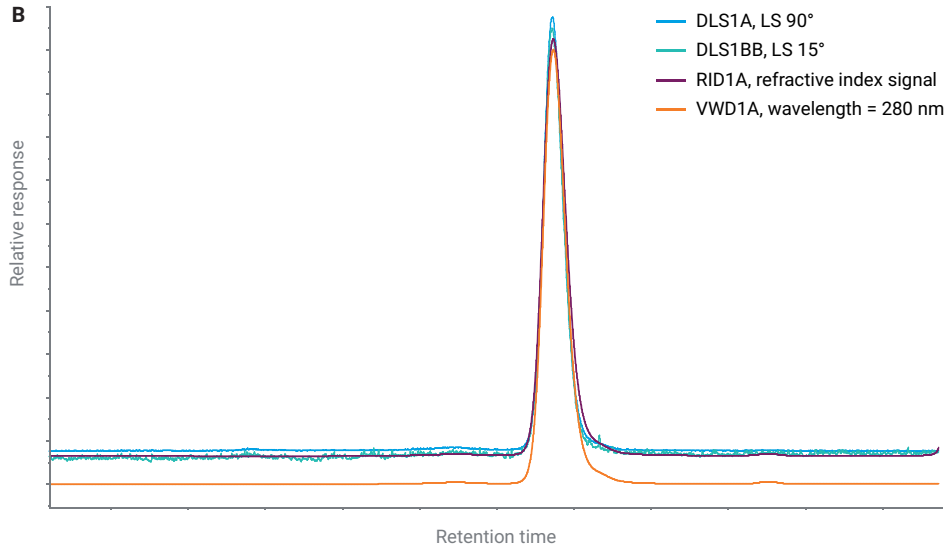


Figure 2. GPC/SEC General tab of the Processing Method.

One of the first steps in a GPC/SEC workflow is the calibration of the system, as well as ensuring signal alignment for successful MW calculations. The detectors are connected over capillary connection with specific volumes, which leads to different retention times (RT) for each signal. Signal alignment is easily done in the processing method by selecting Use delay for successful alignment after reprocessing, as shown in Figures 3A (before reprocessing) and 3B (after reprocessing). In this case, the LS signals (90° and 15° angles), as well as two concentration detectors (RI and UV), are aligned.





C

Processing Method

GC_LC Quant + GPC_SEC

- General
  - Properties
  - Signals
- Integration Events ChemStatio
  - Standard
  - Advanced
  - Manual Integration
- Compounds
  - Identification
  - Calibration

Scaling | Alignment | Smoothing | Blank Subtraction | Reference Chromatogram

Calculate delay from RT

Use delay	Detector	RT (min)	Delay (min)
☑	DLS1	12,048	0,000
☑	RID1	12,344	0,296
☑	VWD1	12,181	0,133

Figure 3. (A) Before signal alignment. (B) After signal alignment. (C) Signal Alignment in the Processing Method.

In the GPC/SEC Advanced tab of the processing method, different parameters for LS data processing and calculations are found, like System Calibration Settings and Analysis Settings (see Figure 4). For example, in Analysis Settings, the decision can be made if the calculation of the MW is based on the sample concentration or sample properties (dn/dc for RI or extinction coefficient for UV detection). Here, a calculation of MW was chosen based on Sample Property, as shown in the bottom of Figure 4.

SLS requires a single measurement for detector calibration. When analyzing proteins, the molecule of choice is often BSA. Figure 5 shows the separation of the BSA monomer, dimer, trimer, tetramer, and higher aggregates on the AdvanceBio SEC 300 Å column with UV, RI, and LS signals for 90° and 15°. Excellent resolution was found between the monomer and the aggregates. The BSA monomer was used for calibration and detector delay alignment. As the commonly available BSA mostly consists of a mixture of monomer and several aggregates, and only the total concentration was known of this mixture, it was important to take this into consideration for successful MW calculation. To overcome this hurdle, the software enables the user to select **Use all compounds concentration area** to use the corrected concentration for the BSA monomer (see Figure 4).

OpenLab CDS provides the streamlining of user workflows by automatically processing entire sample sets with a single predefined method, delivering up to a 10-fold reduction in data analysis time for a typical 10-sample run.

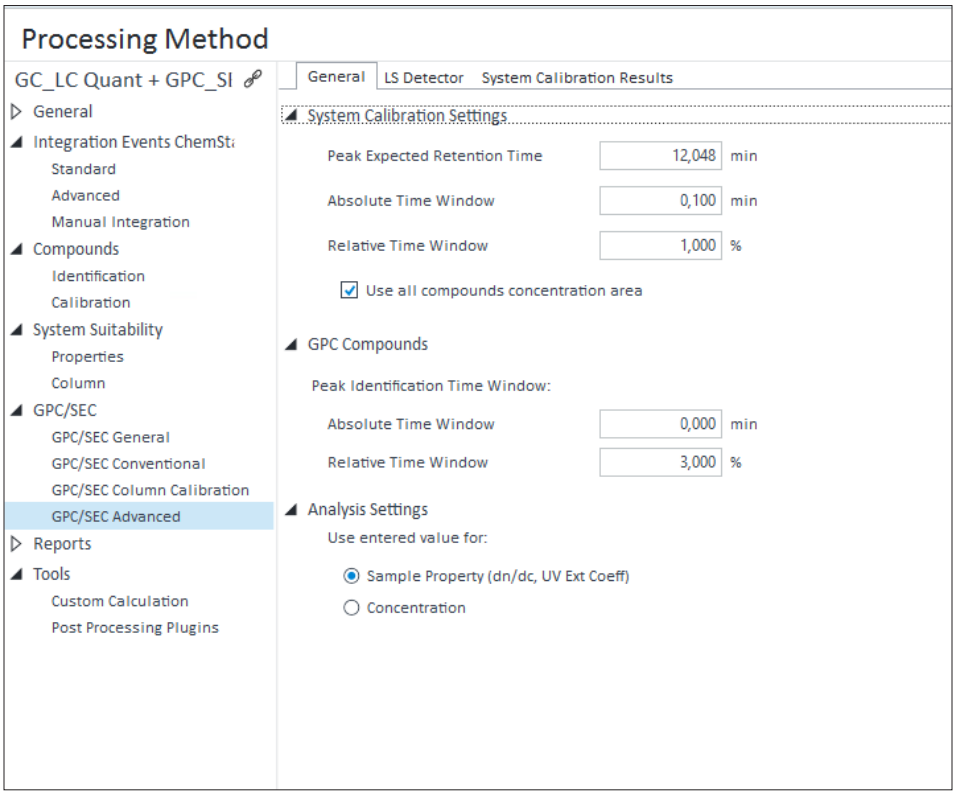


Figure 4. GPC/SEC Advanced tab of the Processing Method.

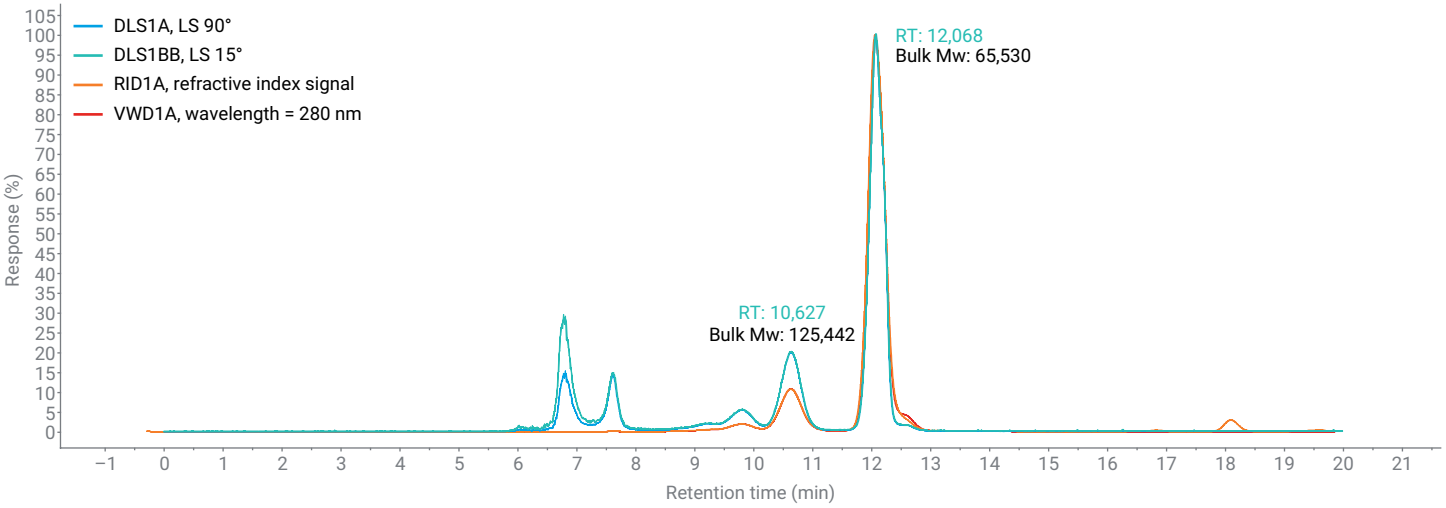


Figure 5. BSA separation on Agilent AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm.

Figures 6A and 6B show the characterization of the mAb rituximab in the GPC/SEC Compound Details window. Here, the MW distribution as well as the Rh values can be displayed over the different peaks of interest. The LS analysis revealed a MW of about 144 kDa for the monomeric peak, which was in agreement with the value found in literature.[2] The precision of MW determination of seven consecutive runs was excellent, with 0.253% RSD. Figure 6B shows a closer look into the aggregates and fragment section, which enabled the separation of the mAb dimer and MW determination with 308 kDa. In addition, the aggregation percentage was found to be low, with an aggregation percentage of less than 1%.

In addition to the SLS analysis for calculating molecular weight, the DLS detector implemented in the 1260 Infinity II Bio-SEC Multidetector System, featuring dual-angle and DLS detection, can measure the molecular size as hydrodynamic radius. DLS detects the fluctuations of the scattered light intensity due to the Brownian motion of the molecules in solution. The Rh of the mAb monomer was detected at a mean of 5.5 nm, which was in perfect agreement with literature.[3] The precision with an RSD of 1.25% over seven consecutive runs was excellent for this kind of measurement.

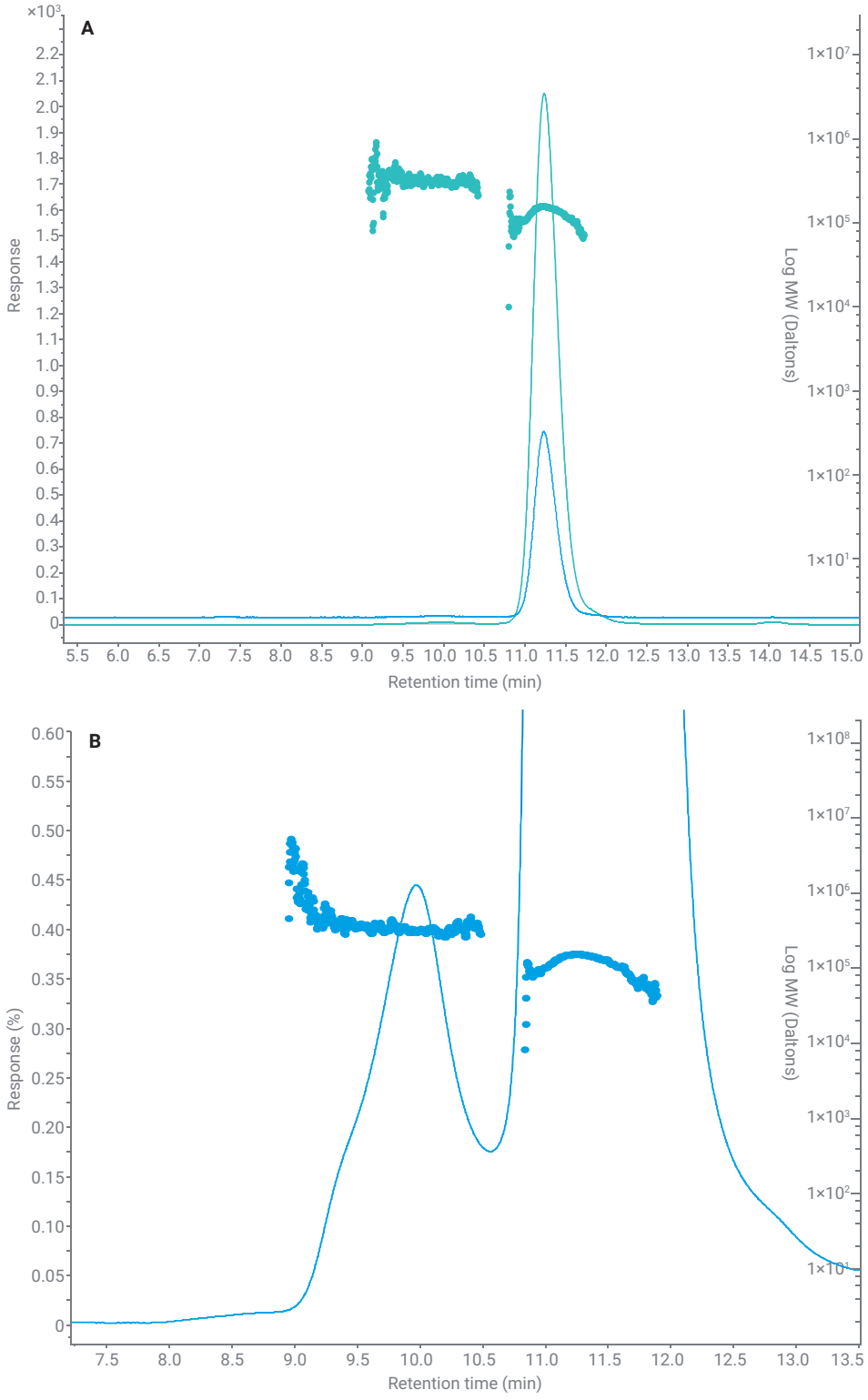


Figure 6. (A) SEC LS analysis of rituximab biosimilar with UV (green) and LS signal at 90° (blue) in the GPC/SEC Compound Details window, with displayed MW distribution over the peaks. The MW was determined at ~148 kDa for the monomer peak. (B) A zoomed in version, with more details.

Conclusion

The combination of the Agilent Advanced GPC/SEC Software for OpenLab CDS plus biocompatible hardware solutions—the Agilent 1290 Infinity III Bio LC System and the Agilent 1260 Infinity II Bio-SEC Multidetector System featuring dual-angle and DLS detection—provides a powerful and compliance-supporting solution for the characterization of biopharmaceuticals. CQAs like aggregation percentage, MW, and molecular size can be simultaneously and precisely characterized to ensure the highest quality standards of the produced biopharmaceuticals.

References

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2. Flores-Ortiz, L. F.; Campos-García, V. R.; Perdomo-Abúndez, F. C.; Pérez, N. O.; Medina-Rivero, E. Physicochemical Properties of Rituximab. *J. Liq. Chromatogr. R. T.* **2014**, *37(10)*, 1438–1452.
3. Andersen, C. B.; *et al.* Aggregation of a Multidomain Protein: A Coagulation Mechanism Governs Aggregation of a Model IgG1 Antibody Under Weak Thermal Stress. *Protein Science* **2010**, *19*, 279—290.

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