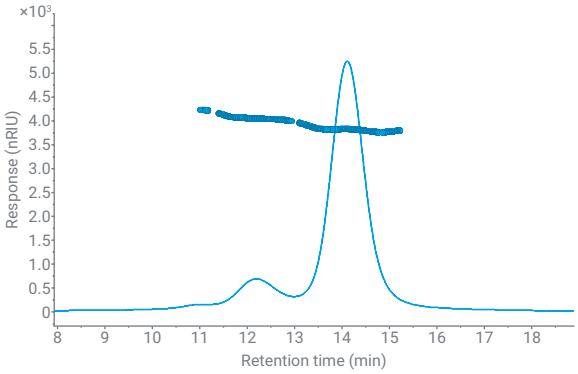
Application Note

Biopharma

Size Exclusion Chromatography-Multi-Angle Light Scattering Characterization of mRNA

Using Agilent Advanced GPC/SEC Software for OpenLab CDS



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Abstract

The characterization of messenger ribonucleic acid (mRNA) with size exclusion-multi-angle light scattering (SEC-MALS) enables the analysis of both biophysical and structural attributes of the molecules. Aggregation, molecular weight (MW), and molecular size can be determined to study the impact on these attributes from different conditions during production, formulation, and storage. The Agilent Advanced GPC/SEC Software for OpenLab CDS, in combination with the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector including the Agilent 1290 Infinity III Bio LC System, enables light scattering analysis in a compliance-supporting chromatography data system (CDS) environment and provides a single platform for advanced size exclusion chromatography (SEC) analysis of biopharmaceuticals.

Introduction

The coupling of multi-angle light scattering detection (MALS) with traditional SEC adds essential value for the analysis of in vitro transcription (IVT) mRNA. SEC can separate mRNA monomers from potential aggregated molecules, which are considered product-related impurities and therefore must be accurately quantified and identified as important quality attributes. In addition to quantitative information from the concentration detector—ultraviolet (UV) or refractive index (RI)—to determine aggregation percentage, the addition of MALS detection enables the calculation of MW and molecular size to identify the mRNA monomer molecules and aggregates. These details cannot be revealed by conventional SEC due to the difference in conformation of the used globular protein standards versus the mRNA structure in solution.

mRNAs are large RNA molecules exhibiting high MW, so SEC columns with a large pore size are required. The wide-pore Agilent AdvanceBio SEC columns meet the needs for robust, high-resolution separations with the ideal combination of small particles and large pore volumes. For molecules the size of mRNA, the wide-pore column with 1,000 Å pore size is optimally suited for a good separation of mRNA monomer and aggregates.[1]

The Advanced GPC/SEC Software for OpenLab CDS seamlessly integrates GPC/SEC light scattering capabilities in a compliance-supporting CDS environment and provides a single platform for advanced SEC analysis of biopharmaceuticals like monoclonal antibodies, mRNA, and other biopharmaceuticals. The Agilent OpenLab CDS environment supports static and dynamic light scattering data acquisition, analysis, and reporting options from Agilent light scattering solutions like:

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

In this application note, we show advanced SEC with online MALS detection for the analysis of mRNA samples, determining aggregation percentage, MW in Dalton (Da), and molecular size as radius of gyration (Rg).

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 Multi-Angle Light Scattering Detector (G7885A)
- 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 1000 Å column, 7.8 × 300 mm, 2.7 µm (part number PL1180-5302).

The column was extensively flushed with phophate buffer mobile phase 24 hours before it was attached to the 1260 Infinity II Multi-Angle Light Scattering Detector.

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.). Sodium dihydrogen phosphate, disodium hydrogen phosphate heptahydrate, and sodium hydroxide were obtained from Sigma-Aldrich (Steinheim, Germany). MabThera (rituximab) was purchased from Medizone Germany GmbH (Munich, Germany). The IVT mRNA samples were kindly provided from the Research Institute of Chromatography (Kortrijk, Belgium).

Solvent and sample preparation

A 2 L volume of 150 mM phosphate buffer was prepared using 15.22 g of sodium dihydrogen phosphate and 46.41 g of disodium hydrogen phosphate heptahydrate. The pH was adjusted to 7 using sodium hydroxide solution. The prepared phosphate buffer was triple filtered using a 0.2 µm membrane filter. 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). MabThera is formulized at a concentration of 10 mg/mL.

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 Advanced GPC/SEC software for OpenLab CDS enables the users to acquire light scattering data and perform data processing and analysis within the compliance-supporting CDS environment of OpenLab CDS. Here, we combined the 1290 Infinity III Bio LC System with the 1260 Infinity II Multi-Angle Light Scattering Detector for SEC-MALS analysis of mRNA.

Using the wide-pore column with 1000 Å pore size, required for mRNA analysis, a typically used bovine serum albumin sample is not ideal for calibrations, as the monomeric peak cannot be resolved. As light scattering requires a single measurement with a monodispersed, baseline-resolved peak for detector setup and calibrations, a monoclonal antibody (rituximab) with a MW of ~ 145,000 Da was used for calibration, showing a clear monomeric peak with negligible amounts of aggregates.[1]

The workflow to build the correct fundament for light scattering calculations with the Advanced GPC/SEC software and OpenLab CDS is very similar to the one for proteins, which can be found in another application note.[2]

Figure 1 shows the separation of IVT mRNA on the AdvanceBio SEC 1000 Å column, revealing aggregation with elevated percentage of high molecular weight (HMW) species. The chromatogram shows the signals from MALS at 90° in blue versus the UV signal at 260 nm in green. The signal intensity in light scattering is proportional to concentration as well as MW, so signals from HMW species are usually visible in higher intensities compared to UV or RI detection; vice versa is true for smaller molecules.

With the used LC configuration in combination with MALS detection, 20 angles can be measured at the same time to calculate MW and Rg simultaneously. MW was determined by SEC-MALS with the RI used as the concentration detector, using a dn/dc of 0.172.

Table 1. Method parameters.

Parameter	Value
Flow Rate	0.6 mL/min
Mobile Phase	150 mM phosphate buffer, pH 7, triple filtered
Injection	15–25 µL
Stop Time	30 min
Needle Wash	Flush port, 3 s, water/isopropanol 80/20 (v/v)
Autosampler Temperature	8 °C
Column Temperature	30 °C
Detection UV	260 nm, peak width > 0.05 min (10 Hz)
Detection RI	Temperature: 30 °C Sampling rate: 2.31 Hz
Detection MALS	Cell temperature: 30 °C, 20 angles recorded

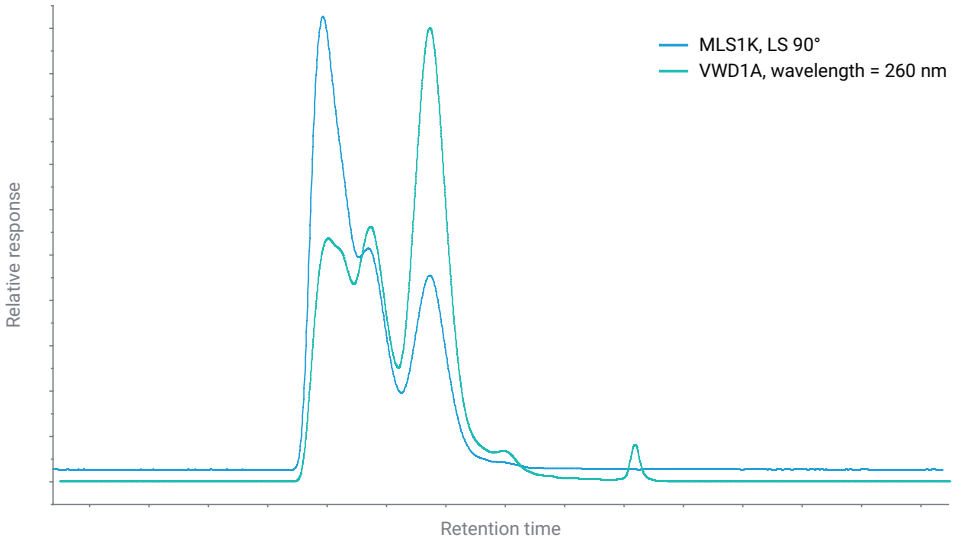

Figure 1. Separation of HMW species from the mRNA monomer with MALS at 90° in blue and UV signal at 260 nm in green.

Figure 2 shows the characterization of an IVT mRNA sample in the GPC/SEC Compound Details window. Here, the MW distribution can be displayed over the different peaks of interest. The calculated MW and Rg can be annotated directly over the peaks in the window, and all parameters are displayed in the GPC/SEC LS Results table.

Area percentage, MW, Rg, and corresponding reproducibility were analyzed in a sequence with 10 consecutive runs of the IVT mRNA sample, and the results are summarized in Table 2. The results were found to be highly reproducible with RSD values below 5.4% for the MW and below 7.3% for the Rg values. The mean MW value of the monomer peak with 1,464 kDa over 10 consecutive runs was in near-perfect agreement with the theoretical value of the mRNA nucleotide sequence with a length of 4,546 nucleotides. In addition, two HMW species were found eluting before the mRNA monomer.

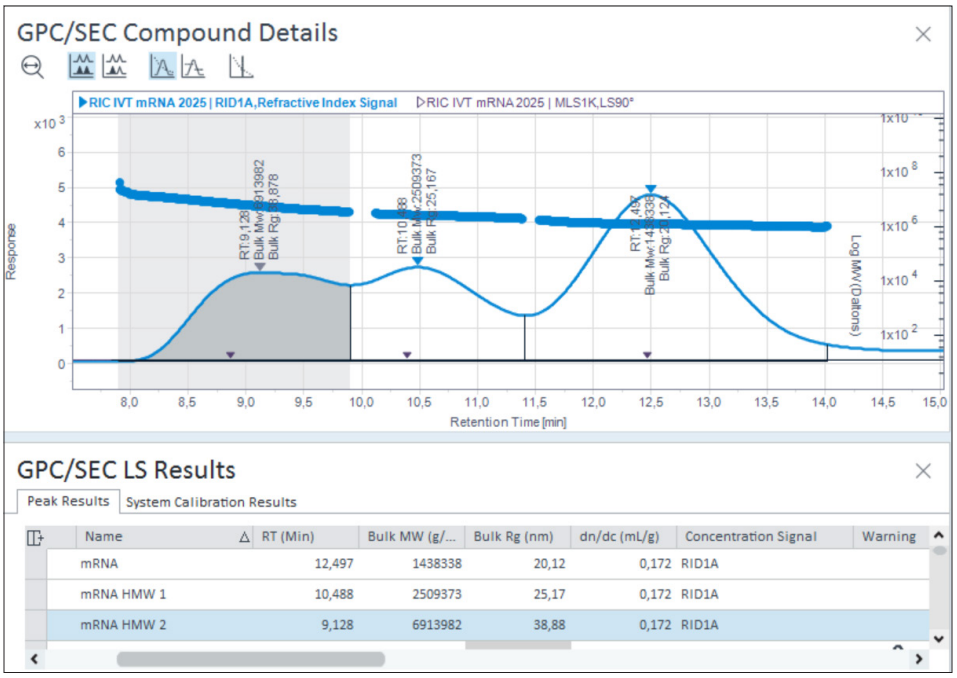

Figure 2. Screenshot of the GPC/SEC Compound Details window as well as the GPC/SEC LS Results table.

Table 2. Area percentage, mean MW, mean Rg, and corresponding reproducibility analyzed in a sequence with 10 consecutive runs for IVT mRNA sample.

	Area (%)	MW (kDa)	RSD MW (%)	Rg (nm)	RSD Rg (%)
IVT mRNA Monomer	49.09	1,464	5.38	19.2	7.28
HMW 1	24.62	2,532	3.69	23.6	4.79
HMW 2	26.29	7,033	1.26	38.8	0.65

Figures 3A and 3B show the analysis of two different mRNA examples: A) with the IVT mRNA generously provided by the Research Institute of Chromatography in Belgium, and B) showing the analysis of firefly luciferase (fLuc) mRNA—a commonly used test sample for mRNA analysis. The mRNA samples differ in their mass, with a MW of about 1,464 kDa for the IVT mRNA and around 611 kDa for the fLuc mRNA. A similar size was found for both with an Rg of ~ 18 nm for the monomer. Both plots showed a typical MW distribution over the different peaks of the mRNA samples, starting with HMW that decreased over time from HMW to the monomer.

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 plus Agilent 1260 Infinity II Multi-Angle Light Scattering Detector—provides a powerful solution for the characterization of biopharmaceuticals. Critical quality attributes like aggregation percentage, MW, and molecular size can be simultaneously and precisely characterized to ensure the highest quality standards of the produced biopharmaceuticals. The 1260 Infinity II Multi-Angle Light Scattering Detector with 20 angles enables highly precise calculations of the absolute molar mass. Especially for higher protein aggregates, the MALS detector shows higher sensitivity compared to UV detection, adding valuable information to a conventional SEC setup.

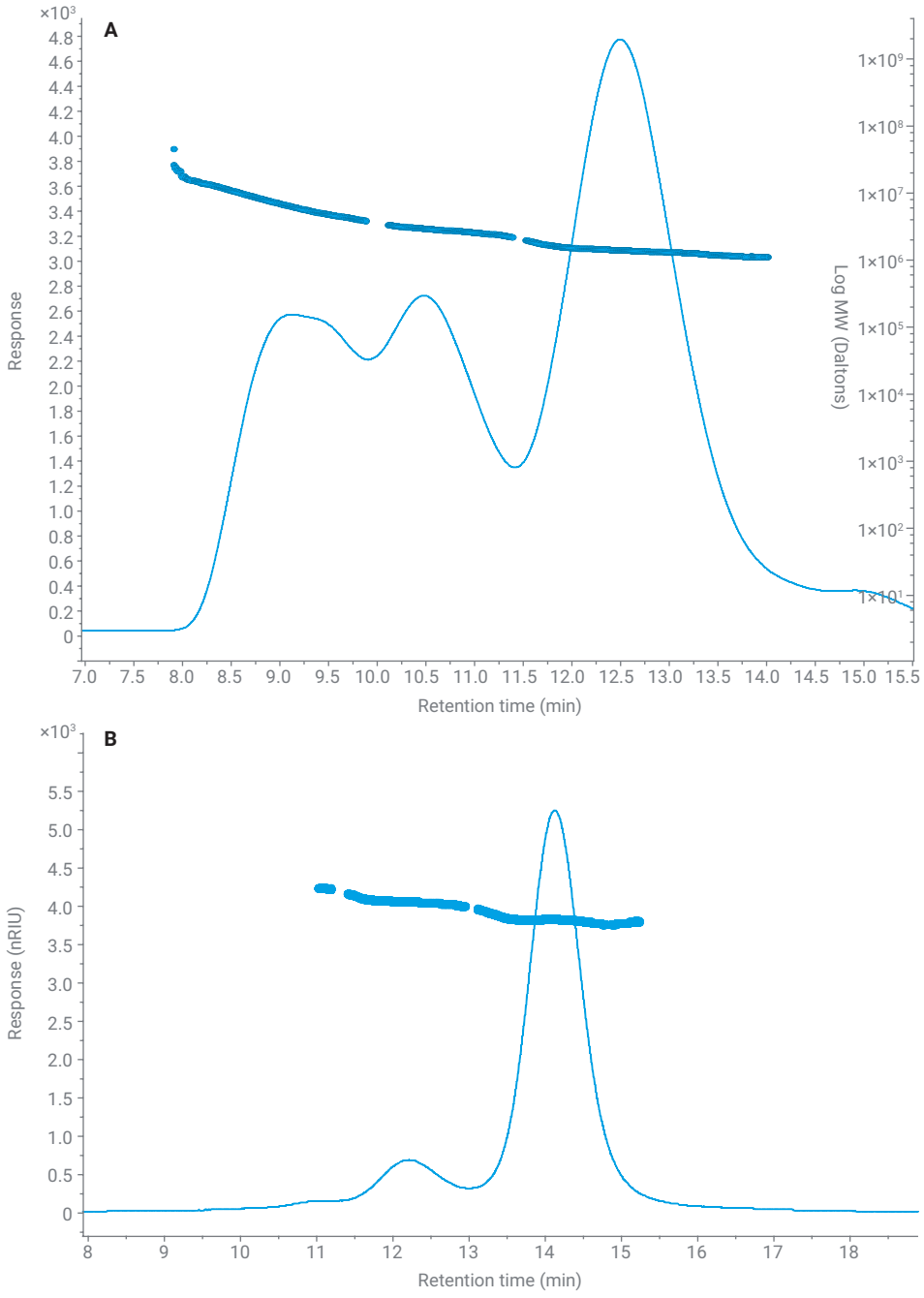

Figure 3. GPC/SEC Compound Details window for (A) IVT mRNA sample and (B) fLuc mRNA sample.

References

1. Schneider, S.; Rieck, F. SEC-MALS for mRNA Characterization with the Agilent 1260 Infinity II MultiAngle Light Scattering Detector, *Agilent Technologies application note*, publication number 5994-7745EN, **2024**.
2. Schneider, S. SEC Light Scattering Analysis of Monoclonal Antibodies with Advanced GPC/SEC Software for OpenLab CDS, *Agilent Technologies application note*, publication number 5994-9014EN, **2026**.

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