Application Note

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SEC-MALS for mRNA Characterization with the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector

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Abstract

The characterization of messenger ribonucleic acid (mRNA) with mass spectrometry can be particularly challenging due to the complex and large structure of RNA molecules. The combination of multiangle light scattering (MALS) detection with size exclusion chromatography (SEC) can successfully be applied to the analysis of mRNA, delivering biophysical and structural information.

The Agilent 1260 Infinity II Multi-Angle Light Scattering Detector coupled to the Agilent 1290 Infinity II Bio LC System enables the determination of molecular weight (MW) in addition to aggregate percentage information from the concentration detector. The 1290 Infinity II Bio LC is specifically designed for conditions used in biochromatography, with a completely iron-free flow path and high tolerance to the salt-based buffers typically used in SEC.

Introduction

mRNA-based biopharmaceuticals have been in development as a new class of drugs for over three decades.[1] They came into focus during the COVID pandemic with the development of mRNA-based vaccines. This evolution has led to the need for analytical methods to evaluate the critical quality attributes of mRNA.[2,3]

mRNA is produced by in vitro transcription from a linear DNA template and is chemically modified at the regulatory elements present at the mRNA ends (5' cap and poly(A) tail) to improve efficiency and stability during translation.

High-performance liquid chromatography (HPLC) is especially advised for mRNA purity analysis by the USP guidelines.[2] Beyond the analysis of purity, capping efficiency, and poly(A) tail mostly with ion pairing reversed-phase (IP-RP) HPLC, an important characteristic of mRNA is the analysis of aggregates. Aggregates are considered product-related impurities, and their quantification and identification are important quality attributes.

In addition to quantitative information from the concentration detector (ultraviolet, or refractive index (RI)) as aggregation percentage, the addition of MALS detection to SEC enables the determination of MW for the identification of the large-sized 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.

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 InfinityLab Quick Connect Heat Exchanger 1290 Bio Standard Flow (part number G7116-60071)
- Agilent 1290 Infinity II Refractive Index Detector (G7162B)
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID (part number 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 systems such as the Agilent 1260 Infinity II Bio-Inert LC System or the Agilent 1260 Infinity II Prime Bio LC System.

Column

Agilent AdvanceBio SEC 1000Å, 7.8 × 300 mm, 2.7 µm (part number PL1180-5302)

Software

The software used in this study was Agilent WinGPC software, version 1.0. 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, U.S.). Sodium dihydrogen phosphate, di-sodium hydrogen phosphate heptahydrate, sodium hydroxide, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Steinheim, Germany). MabThera (rituximab) was purchased from Medizone Germany GmbH, Munich, Germany. An mRNA sample was kindly provided from the Research Institute of Chromatography, Kortrijk, Belgium.

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 di-sodium 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). 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 Volume	5 to 20 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 RID	30 °C, Peak width > 0.05 min (9.25 Hz)
Detection UV	260 nm, Peak width > 0.05 min (10 Hz)
Detection MALS	Cell temperature 30 °C, 20 angles recorded

For the MW calculation, a dn/dc value of 0.186 mL/g was used for proteins, and 0.172 mL/g was used for mRNA.

Unlike in conventional SEC, the MW analysis using an SEC light scattering detector requires knowledge of the sample concentration. There are different options to determine the concentration. If the extinction coefficient of the sample is known, using a calibrated UV detector and the extinction coefficient (ε or dA/dc) is a reliable way to determine the concentration of the analyzed molecule. This calculation method is often applied in protein analysis. If the extinction coefficient is not known, the sample concentration can also be determined using the RI increment (dn/dc) and a calibrated RI detector. RI detection is an alternative and more general detection method, and can also be applied to molecules without UV chromophores. If the dn/dc is also not known, it can be determined with RI detection using a dilution series of the molecules with known concentration, as described in the literature.[4,5] In the case of the used mRNA sample, the extinction coefficient was not known, so RI detection was used for concentration calculation with a dn/dc of 0.172 mL/g. This value was also used for MW calculation.

The LC configuration consisted of two concentration detectors, UV and RI detection, in combination with MALS detection. Due to pressure restrictions and to minimize peak dispersion, the detectors were installed in the following order: UV-MALS-RI. The UV signal at 260 nm detects mRNA with high sensitivity, enabling the exact determination of aggregation percentage.

Results and discussion

mRNA are large RNA molecules exhibiting high MWs, so SEC columns with 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. Static light scattering requires a single measurement with a monodisperse, baseline-resolved peak for interdetector delay calibration and light scattering detector constant determination. For SEC-MALS analysis of proteins, the BSA monomer is often used as a calibrant. With the Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column, excellent resolution is achieved between the BSA monomer and dimer as shown in previous publications.[6] With the wide-pore column of 1,000 Å pore size required for mRNA analysis, equal resolution for BSA cannot be achieved. Instead, the monoclonal antibody rituximab with a MW of 145,000 Da was used for calibration, showing a clear monomeric peak with negligible amounts of aggregates (Figure 1).

Figure 2 shows the separation of the analyzed mRNA monomer and its aggregates. Good separation of monomer and dimer was achieved on the AdvanceBio SEC 1000Å, 7.8 × 300 mm column. Based on the UV signal at 260 nm, the aggregation percentage was calculated to be 74% for the monomer, 20% for the dimer, and 6% for the higher aggregates. The MALS MW determination was calculated as bulk MW based on a dn/dc of 0.172 mL/g. For a length of 4,546 nucleotides for the monomer, an MW of 1,453,000 Da was calculated. This value is in near-perfect agreement with the theoretical value of the mRNA nucleotide sequence. The precision was also very high, with an RSD of ~2% for the MW values. With the determination of the MW by MALS, the dimer peak could be identified with a MW of 3,205,000 Da. In addition, higher MW species eluting in front of the dimer peak were detected.

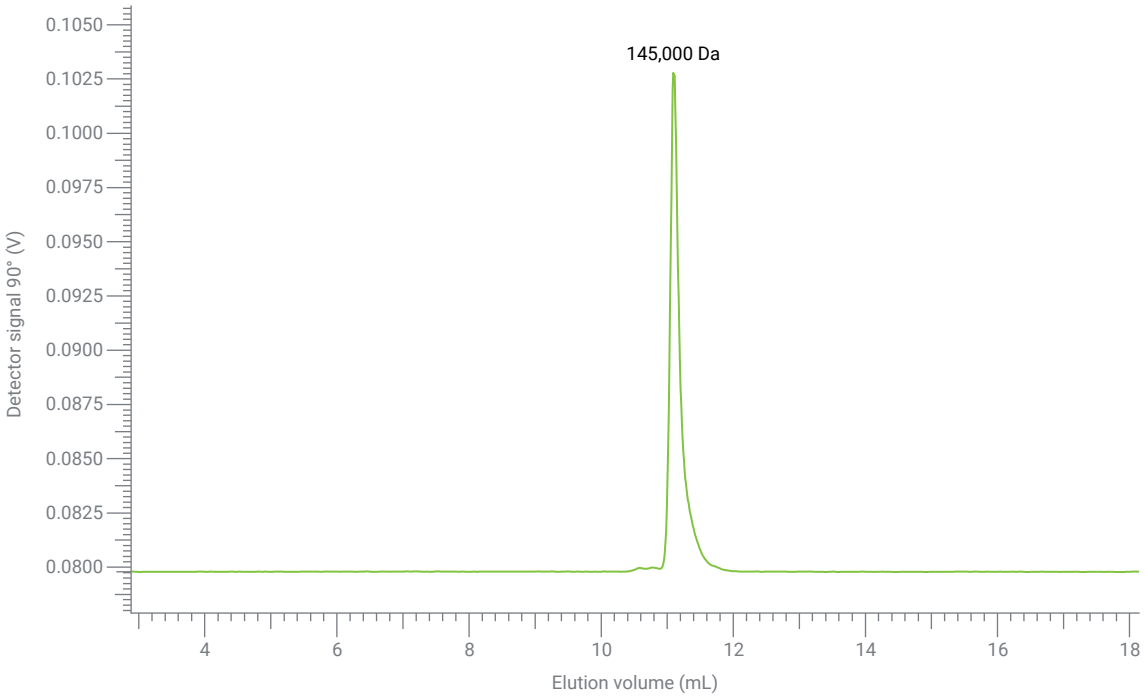

Figure 1. SEC-MALS analysis of rituximab at 90°. A monomeric peak is observed with only minimal amounts of aggregates, making it an excellent option for calibration purposes.

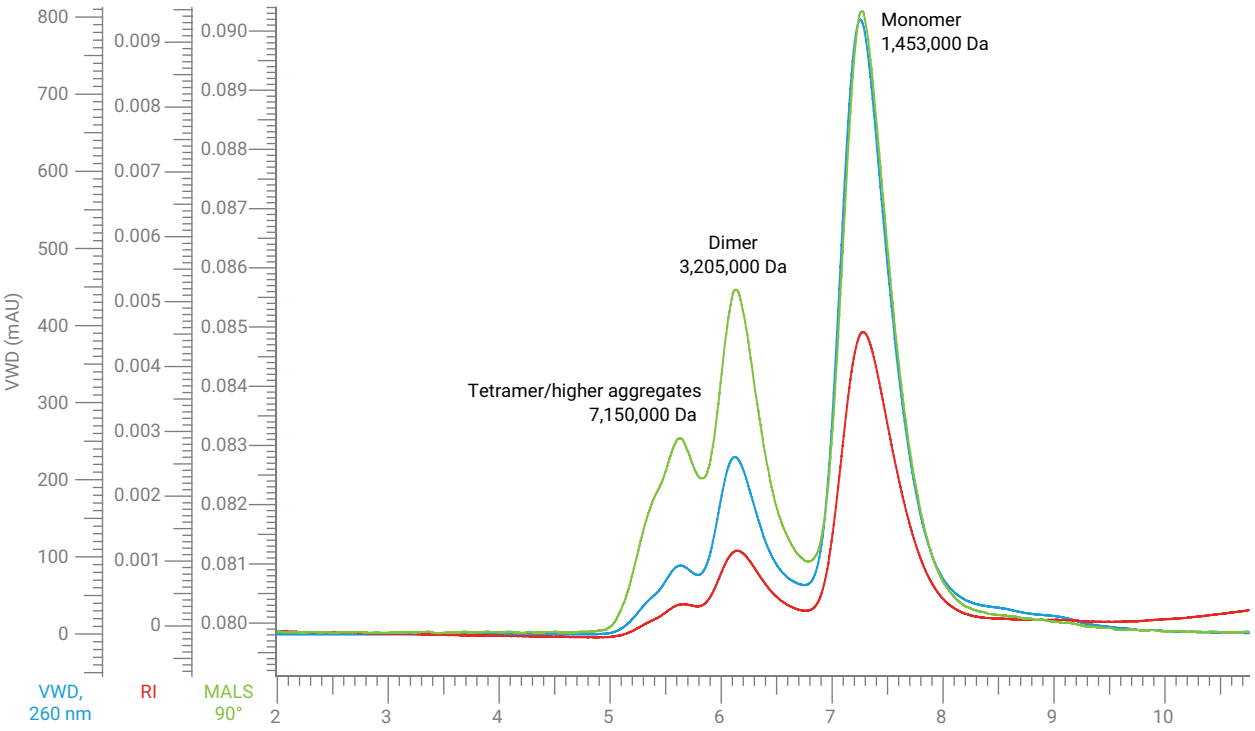

Figure 2. SEC-MALS analysis of an mRNA sample with 4,546 nucleotide length. The resulting concentration signals (UV: blue, RI: red) are displayed together with the 90° MALS signal (green).

Conclusion

An Agilent 1260 Infinity II Multi-Angle Light Scattering Detector coupled to an Agilent 1290 Infinity II Bio LC System provided biophysical information for the analysis of large mRNA molecules. The precise determination of the molecular weight was in near-perfect agreement with the theoretical value of the mRNA sequence. Good resolution was found between monomer and dimer of mRNA with an Agilent AdvanceBio SEC 1000Å column, leading to unique MW determination for the separated peaks.

The combination of the 1290 Infinity II Bio LC System and the 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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