Application Brief

Materials Testing & Research

Agilent

Trusted Answers

GPC/SEC-MALLS Analysis of Hyaluronic Acid

Authors

Jasmin Preis*,
Friedhelm Gores, and
Günter Reinhold
*Agilent Technologies, Inc.

Abstract

This application brief describes the robust GPC/SEC-MALLS analysis of hyaluronic acid. The advantage of this setup is that the absolute molar mass is obtained, while conventional GPC/SEC with concentration detectors only provides apparent molar masses.

Introduction

Hyaluronic acids (HA) are macromolecules composed of disaccharides of linked glucuronic acid and N-acetyl-D-glucosamine units. The molecular weights of HAs range from 5,000 to 20,000,000 Da, where up to 25,000 disaccharide units can be linked together. Hyaluronic acid is heavily used in medical and cosmetic applications.

GPC/SEC is the method of choice for measuring the molar mass distribution of hyaluronic acid. True molar masses can be obtained when online multi-angle light scattering is used in combination with a refractive index detector. The refractive index increment (dn/dc) for light scattering data evaluation can be determined online, offline (using dedicated instrumentation), or taken from the literature.[1]

Experimental

See Table 1.

Results and discussion

GPC/SEC analysis of high molar mass samples requires lower flow rates and concentrations in GPC/SEC to avoid shear-induced chain elongation and overloading effects. Thus, GPC/SEC conditions and columns need to be optimized in terms of loading, flow rate, and particle and pore size when analyzing high molar mass HAs.

Larger particle (10 µm) and large porosity Agilent SUPREMA columns are used to avoid these problems and provide interaction-free chromatography, advantageous for accurate GPC/SEC measurements when using PBS pH 7.4 as the mobile phase.

Table 1. Instrument and sample conditions.

	Conditions
Pump	Isocratic pump Flow rate: 0.5 mL/min Mobile phase: phosphate buffered saline (PBS), pH 7.4
Injection System	Autosampler Injection volume: 100 µL
Columns	Agilent SUPREMA 10 µm precolumn, 8 × 50 mm (p/n SUA080510) Agilent SUPREMA 10 µm 30 Å, 8 × 300 mm (p/n SUA0830103e1) Agilent SUPREMA 10 µm 10,000 Å, 8 × 300 mm (p/n SUA0830101e4) Agilent SUPREMA 10 µm 10,000 Å, 8 × 300 mm (p/n SUA0830101e4)
Sample Concentration	0.5 mg/mL
Detectors	Refractive index (RI) detector Multi-angle light scattering detector (MALLS) at λ = 638 nm
Detector Setup	Agilent ReadyVLS-Kit, Dextran/Pullulan (p/n PSS-VLSKITR1PD20)
Software	Agilent WinGPC

Due to the lack of high molar mass calibration standards, the use of light scattering detection is recommended. This detection technique also enables measurement of true molar masses. When determining molar masses by GPC/SEC-MALLS, the water content of HA, which could be as high as around 12%, was taken into account.

A dn/dc value for hyaluronic acid of 0.165 was used to evaluate the light scattering data.[2]

Figure 1 shows the slice concentration measured by the RI detector as well as the online-determined molar mass. Sample recovery was nearly 100%, showing that all material eluted from the column. The clear decrease in molar mass with elution volume provides evidence that separation with respect to molar mass was achieved.

Figure 2 shows the mass distribution including the cumulative distribution of a hyaluronic acid sample.

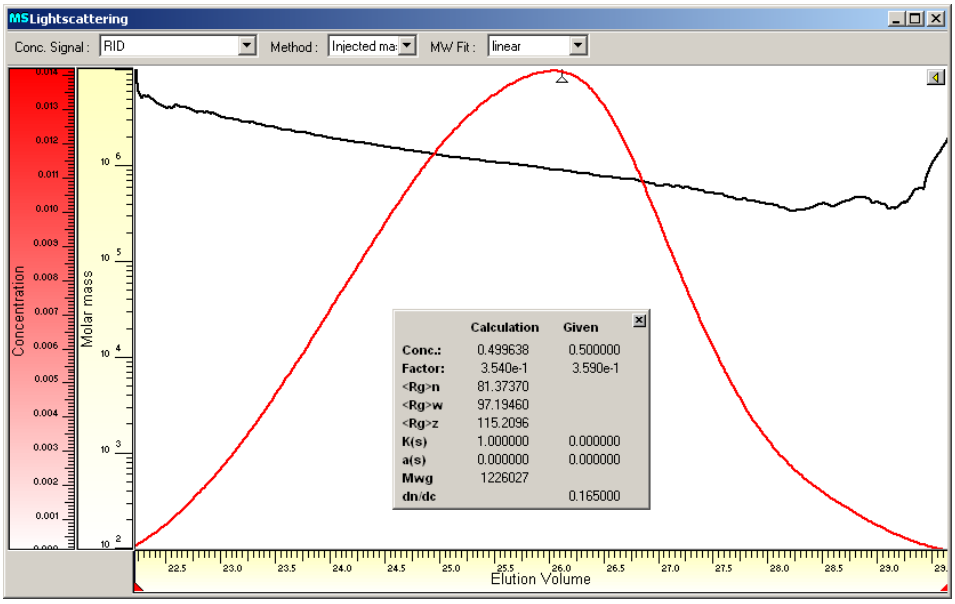

Figure 1. Elugram of a hyaluronic acid sample (red: RI-detector trace (concentration), black: measured molar mass (MALLS)).

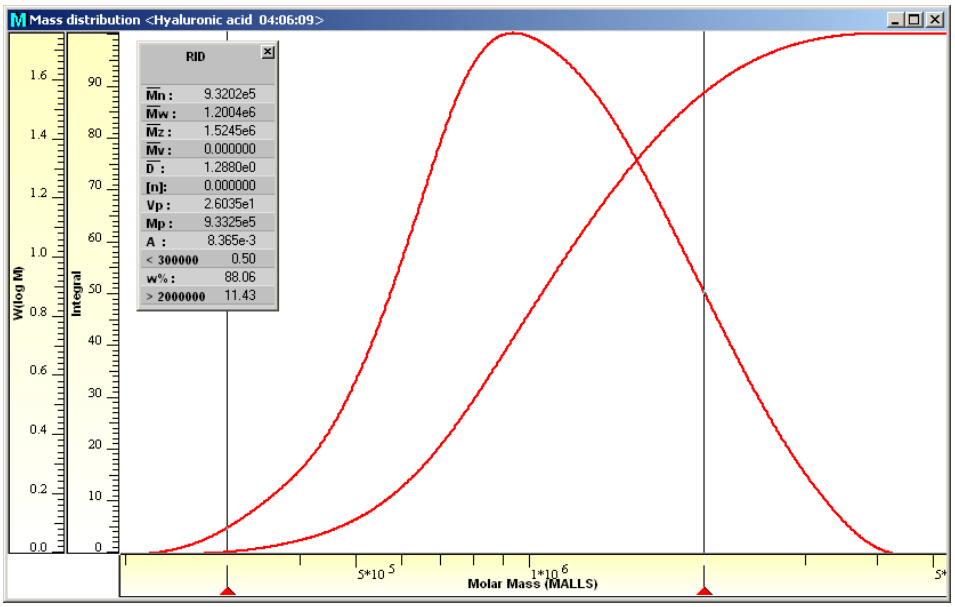

Figure 2. Molar mass distribution and cumulative distribution of a hyaluronic acid sample.

Table 2 shows the molar mass averages of the hyaluronic acid sample obtained from conventional calibration (RI only, apparent molar masses) using pullulan standards and from GPC/SEC-MALLS analysis. The weight average molecular weight (Mw) measured with light scattering is three times lower compared to the value obtained with a conventional pullulan calibration curve. As GPC/SEC separates based on hydrodynamic size in solution, the difference in the molar masses obtained by conventional calibration and GPC/SEC-MALLS analysis indicates that the hydrodynamic size of a pullulan chain at a given molar mass is significantly smaller than that of HA of identical molar mass.

Table 2. Comparison of the molecular weights measured by RI detection and MALLS.

	Pullulan Calibration*	MALLS
Mn [Da]	1,224,000	932,000
Mw [Da]	3,818,000	1,200,000
Mp [Da]	1,398,000	933,000

* Highest molecular weight standard: Mp = 2,500,000 Da

Conclusion

Hyaluronic acid can be analyzed with GPC/SEC-MALLS using Agilent SUPREMA columns, with optimized particle and pore sizes to deliver robust separations and reliable results. Conventional calibration based on pullulan reference material with RI detection overestimated the Mw by a factor of three. True average molecular weight values are measured when using absolute detection such as MALLS.

References

1. Gupta, R. C. *et al.* Veterinary Pharmacology and Toxicology, *Volume 6*, **2019**.
2. Lavrenko, P. N.; Linow, K-J; Gornitz, E. *In* Analytical Ultracentrifugation in Biochemistry and Polymer Science, Royal Society of Chemistry, Cambridge, **1992**, pp 517–531.

www.agilent.com

RA44973.5730439815

Printed in the USA, March 2, 2023
5994-5701EN

Agilent
Trusted Answers