Application Note

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Advanced SEC-MALS Analysis of Sodium Hyaluronates

Using the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector

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Abstract

Size exclusion chromatography on Agilent SUPREMA columns enables accurate and reliable separation of ultrahigh molar mass sodium hyaluronates (sodium salts of hyaluronic acid), exceeding the molar mass of typical standards applied to calibrate SEC columns. The Agilent 1260 Infinity II Multi-Angle Light Scattering Detector coupled to the Agilent 1260 Infinity III LC System allows molar mass determination without the need for column calibration. This provides enhanced repeatability of molar mass results. In addition to extending the calibration range, Multi-Angle Light Scattering (MALS) can provide absolute molar masses, unlike the relative molar masses obtained by column calibration using polymer standards. This reduces the uncertainty resulting from relative molar mass determination.

Introduction

Hyaluronic acid is used in a variety of cosmetic and pharmaceutical applications due to its viscoelastic properties. The ability to retain water is the reason for its application in eye drops and gels. In cosmetics, hyaluronic acid is applied to reduce wrinkles.

As with every macromolecular material, the molar mass and molar mass distribution impact the properties of hyaluronic acids. Thus, it is of high importance to reliably and precisely characterize these molecular features to gain both insight into structure-property relations, as well as in quality control (QC) to assure constant material quality.

Size-exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), is an essential tool for determination of molar masses and molar mass distributions of polymers and macromolecules. SEC separates the molecules by their hydrodynamic size in solution. By running a series of narrowly distributed polymer standards of known molar mass and assigning the peak elution volumes to the molar masses, a calibration curve is constructed. The calibration curve is then used to assign a molar mass to the eluting fraction of the polymer to be analyzed, allowing determination of the molar mass distribution and molar mass averages of unknown samples.[1,2]

As the hydrodynamic size of macromolecules in a given solvent depends on macromolecule chemical structure, only molar masses relative to the applied standards are obtained. In addition, for very large molecules like hyaluronic acid, the situation may arise that no standards of sufficient size are available to allow assigning a molar mass to the elution volume of the analyte. In these cases, parts of the analyte's chromatogram elute outside the calibrated region, resulting in unreliable molar mass determination.

The lack of sufficiently large polymer standards can be overcome by using a Multi-Angle Light Scattering (MALS) detector, which enables molar mass determination without the need for column calibration. In addition, information about the size of the eluting molecule (radius of gyration) can be obtained by MALS if the dimension of the molecule is large enough, compared to the wavelength of the laser.[2,3]

Experimental

Equipment

Agilent 1260 Infinity II//Infinity III GPC/SEC System:

- Agilent 1260 Infinity III Isocratic Pump (G7110B)
- Agilent 1260 Infinity III Vialsampler (G7129A)
- Agilent 1260 Infinity II GPC/SEC Column Thermostat (G7886A)
- Agilent 1260 Infinity III Variable Wavelength Detector (G7114A) with Standard Flow Cell (G1314-60186)
- Agilent 1260 Infinity III Refractive Index Detector (G7162A)
- Agilent 1260 Infinity II Multi-Angle Light Scattering Detector (G7885A)

Columns

3x Agilent SUPREMA Linear Ultrahigh, 8 × 300 mm, 10 µ (SUA083010LUH) with Agilent SUPREMA guard column, 8 × 50 mm, 10 µ (SUA080510)

Software

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

Chemicals, solvents, and samples

Sodium hyaluronates were sourced from Lifecore Biomedical. Desalted water was obtained using a Millipore Elix Essential water purification system. Phosphate-buffered saline (pH of 7.4) was obtained from Sigma-Aldrich.

Solvent and sample preparation

10 mM Phosphate-buffered saline (pH of 7.4, PBS) was prepared by dissolving the contents of two pouches of phosphate-buffered saline in two liters of water. Sodium hyaluronate samples were prepared in PBS at a concentration of 0.5 g/L. Samples were filtered through 1 µm PTFE Syringe Filters.

Note: Phosphate-buffered solvents at physiological pH are highly prone to bacteria and algae growth and should be replaced at least every few days. 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	0.5 mL/min
Mobile Phase	10 mM phosphate-buffered saline, pH 7.4
Injection	100 µL
Run Time	120 min
Column Temperature	35 °C
Detection RID Temperature	35 °C
Detection MALS	20 angles collected
Detector Setup	Pullulan standard PSS-PUL110K (PSS-PUL110K | Agilent)

Results and discussion

Hyaluronic acids and the corresponding salts are frequently of very high molar mass. Often the pore sizes of SEC columns are not large enough to allow separating such high molar mass materials. In such cases, the very large molecules elute without separation at the column's exclusion limit, resulting in a sharp rising peak onset. In addition, not much variation in peak onset is observed for different samples of high molar mass. Figure 1 shows the RI-traces of three different high molar mass sodium hyaluronate samples. Despite their large size, differences in elution time are clearly observed. Peak onset clearly shifts, indicating that the pore size of the applied SUPREMA column combination is appropriate to separate these high molar mass sodium hyaluronates. Additionally, the chromatogram of a series of pullulan standards having molar masses of M_p = 1.45 × 10^6, 216,000, 20,700, and 991 g/mol is overlaid. The molar mass of the pullulan with M_p = 1.45 × 10^6 is among the highest molar mass standards commercially available to calibrate SEC columns in aqueous solvent. Despite the high molar mass of the pullulan, it elutes significantly later than the hyaluronates. Thus, even if using one of the highest molar mass standards available for aqueous SEC, large fractions of the hyaluronates will elute outside the calibrated region, questioning the reliability of even relative molar masses provided by SEC.

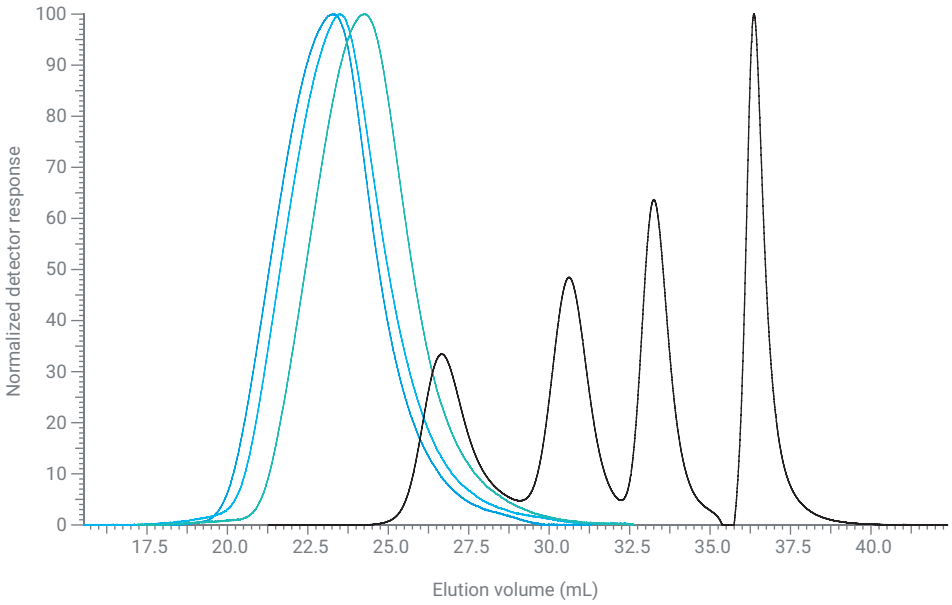

Figure 1. Normalized RI-traces of hyaluronates A, B, and C (blue) in comparison to a set of pullulan standards having molar masses of M_p = 1.45 × 10^6, 216,000, 20,700, and 991 g/mol (black).

The lack of very high molar mass standards for aqueous SEC can be overcome by using a MALS detector in combination with a concentration detector, typically an RI-detector.

Figure 2 shows the RI-signal in comparison to the light scattering trace at 90° for a hyaluronate sample. The shift in the two signals is a consequence of the molar mass sensitivity of the MALS detector. At the same concentration (RI-signal height) the molar mass at lower elution volumes is higher than at higher elution volumes. Since the light scattering signal increases with molar mass, the light scattering signal at low elution volume is enhanced, compared to the light scattering signal at the same concentration with a higher elution volume.

For molecules with sizes much lower than the wavelength of the incident light, the intensity of the scattered light is independent of the angle of observation (isotropic scattering). Therefore, the molar mass derived by light scattering does not vary with the angle of observation. In contrast, for large molecules, the scattering intensity changes with the angle of observation. This needs to be taken into account to derive the correct molar mass. Figure 3 presents a plot of reduced scattering intensity as a function of the angle of observation ($\sin^2(\text{theta}/2)$) for a single slice close to the apex of the hyaluronic acid peak. To calculate the correct molar mass, extrapolation of the scattered light intensity to zero angle ($\sin^2(\text{theta}/2) = 0$) is necessary. In Figure 3, this extrapolation results in a molar mass of approximately 550,000 g/mol. If light scattering is calculated using only a single angle, incorrect molar masses may result. For example, in Figure 3, evaluating the scattering intensity only at e.g. 90° ($\sin^2(\text{theta}/2) = 0.5$) results in a calculated molar mass of $M \cong 350{,}000$ g/mol and thus heavily underestimates the true value.

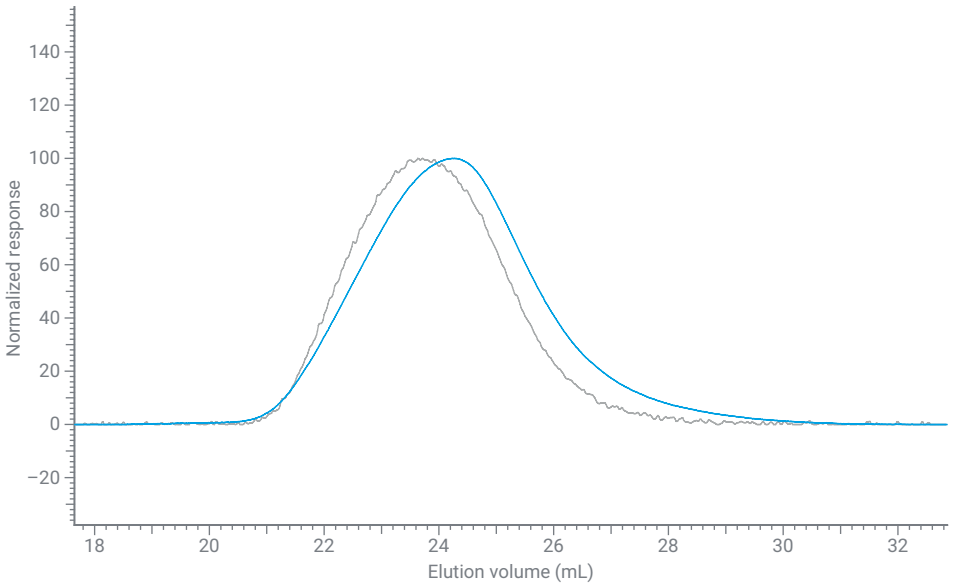

Figure 2. Comparison of normalized 90° MALS (grey) and RI-signal (light blue) for a sodium hyaluronate.

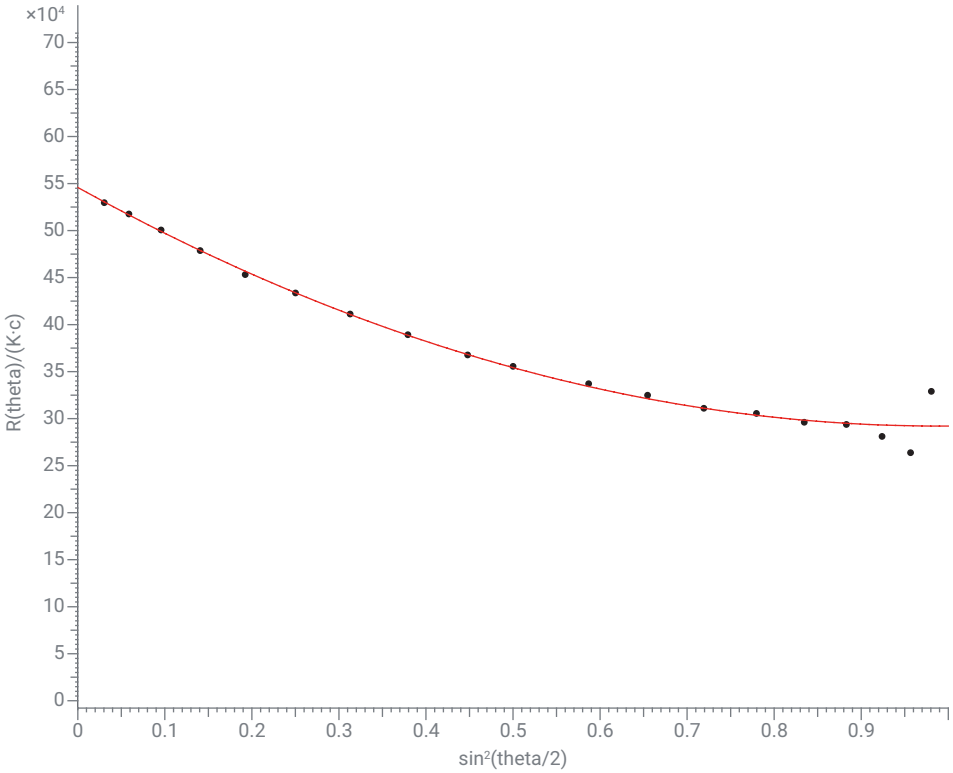

Figure 3. Reduced angular dependence of scattered light as function of scattering angle for a GPC/SEC slice close to the apex of hyaluronic acid sample B having a molar mass of approximately 540,000 g/mol.

In addition, determining the slope of the angular dependence allows calculating the mean squared radius of gyration, which is a measure of the size of the molecule. Both the correct molar mass of large molecules, as well as a determination of the radius of gyration can only be achieved when the scattering intensity can be determined with high accuracy at a sufficiently high number of scattering angles (MALS).

The combination of a light scattering and a concentration detector allows for determination of the molar mass at each elution volume, independent of polymer standards eluting at similar elution range. Figure 4 shows the molar masses derived by MALS for a hyaluronate and a broadly distributed pullulan sample. In addition, the corresponding concentration profiles are provided. For both samples, molar masses are derived across the elution range of the sample chromatograms. As expected for a SEC separation, the molar masses decrease with increasing elution volume, providing evidence that indeed a molar mass separation is achieved using the applied column combination. Furthermore, the molar mass obtained for pullulan at a specific elution volume is significantly higher than the molar mass obtained for the hyaluronate. This is attributed to the different coil expansions of pullulan and hyaluronate. Although both compounds are composed of linked saccharide subunits (Figure 5) the difference in the type and linkage of the subunits results in significantly different coil sizes in solution. The larger size of the hyaluronate at a given molar mass results in stronger exclusion from the pores, and thus in an earlier elution as compared to the pullulan of the same molar mass. The molar mass evaluation of a sodium hyaluronate using a pullulan calibration would result in a 2- to 3-fold overestimation of the true molar mass of the hyaluronate.

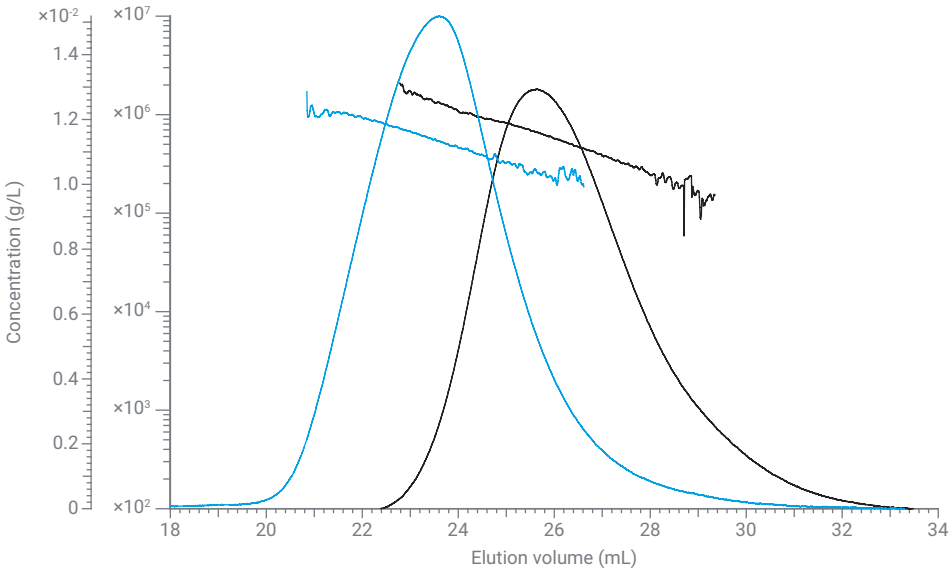

Figure 4. Concentration profiles and molar masses derived by SEC-MALS for hyaluronic acid B (blue) and a broadly distributed pullulan sample (black).

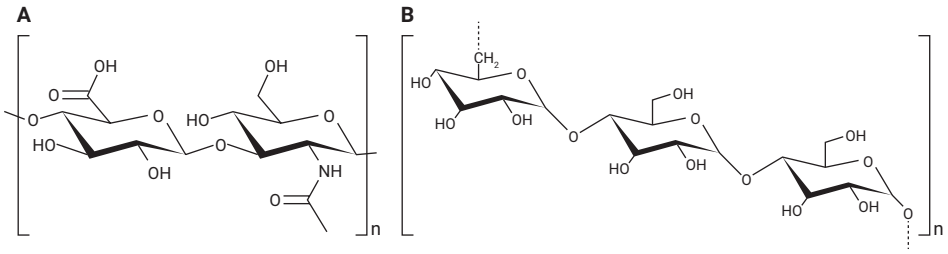

Figure 5. Comparison of chemical structures of hyaluronic acid (A) and pullulan (B).

Conclusion

Agilent SUPREMA ultrahigh columns provide very large pores which allow for the separation of the sodium salt of high molar mass hyaluronic acids. The reduced risk of exclusion peaks lowers molar mass uncertainties resulting from insufficient separation of the high molar mass fraction of the molar mass distribution.

The large number of scattering angles in the Agilent 1260 Infinity II Multi-Angle Light Scattering Detector allows reliable extrapolation to zero angle required for correct molar mass and size determination.

Using a 1260 Infinity II Multi-Angle Light Scattering Detector in combination with an RI-detector allows molar mass determination without establishing a GPC/SEC calibration curve based on polymer standards. Therefore, reliable molar mass determination of hyaluronic acids can be achieved even if no calibration standards of suitable molar mass or suitable chemical structure are available. This reduces the uncertainties in molar masses resulting from different experimental conditions and calibrants used in different laboratories. The determination of absolute molar masses derived by light scattering between laboratories enables comparability and consistency when providing molar masses e.g. for registration purposes.

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