Analysis of Hyaluronic Acid using the EcoSEC® GPC System coupled to Differential Viscometry

EcoSEC GPC System
APPLICATION NOTE

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Introduction

Hyaluronic acid or hyaluronan is a naturally occurring linear polysaccharide composed of alternating repeating D-glucuronic acid and D-*N*-acetylglucosamine units. Hyaluronic acid contains between 500 and 50,000 monosaccharide units per molecule, thus has a molar mass that can range from 10^4 to 10^7 g/mol with a polydispersity index (*PDI*) >1.3.[1-3] This polysaccharide is widely distributed in mammalian cells and tissue but is primarily found in synovial fluid and loose connective tissues such as the umbilical cord, dermis and arterial walls. The roles which hyaluronic acid plays within the body and its unique physicochemical properties, e.g. viscoelastic properties and water retention capacity, have led to interest in the characterization of the polysaccharide, as hyaluronic acid has potential applications in drug delivery, as a surgical aid for ophthalmology and potential for treatment of arthritis.[1]

There are two forms of hyaluronic acid, linear and cross-linked, which are common components of cosmetics, personal care products, dietary supplements, medical products, and medical devices. Different applications of hyaluronic acid require different configurations, e.g. hyaluronic acid must adopt different degrees of cross-linking depending on the application of interest. Linear non-crosslinked hyaluronic acid is typically employed in ophthalmic device materials while crosslinked hyaluronic acid is employed in structure implants.[2] Along with the degree of cross-linking, the molar mass of hyaluronic acid plays a role in its elastic characteristics, as the higher the molar mass the higher the viscosity of the solution.

The unique physicochemical properties of hyaluronic acid are governed greatly by molar mass and molar mass distribution, thus a reliable method needed for determining the molar mass is important. Traditionally the molar mass of hyaluronic acid has been determined using peak-position calibration size exclusion chromatography (SEC) with a refractive index detector (RI) or UV detector based on the standards such as pullulan or dextran. Another way of determining molar mass, which is more absolute (independent of the chemistry or architecture of the samples and standards), is universal calibration. Here we have used a dual detector SEC set-up encompassing the dual flow RI in the EcoSEC GPC System and a ViscoStar® differential viscometer to determine the molar mass averages and polydispersity of a crosslinked and non-crosslinked hyaluronic acid sample. In addition to the molar mass averages, the dual detector SEC set-up allows for the determination of other physicochemical properties such as polymeric size, confirmation, and intrinsic viscosity.

Experimental Conditions

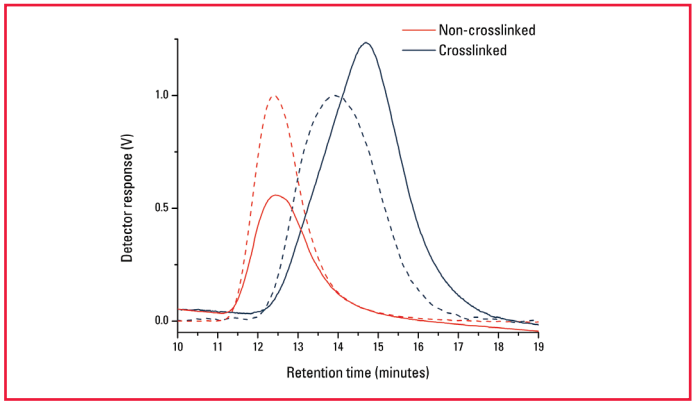
Dual detector SEC analysis was performed using the EcoSEC GPC System (HLC-8320) equipped with a refractive index detector (RI) (Tosoh Bioscience LLC) coupled in series to a ViscoStar Viscometer (Wyatt Technology Corporation). Separation of unfiltered 250 µL injections occurred over a column bank consisting of two 7.8 mm ID × 30 cm, 13 µm particle size TSKgel® GMPW$_{XL}$ columns (separation range ~1,000 to 5 × 10^6 g/mol (Tosoh Bioscience LLC). The mobile phase and solvent were water with 0.1 mol/L $NaNO_3$ and 0.02% NaN_3 at a flow rate of 1.0 mL/min. Detector, pump oven, and column oven were maintained at 35 °C. Data was processed using Wyatt's ASTRA 6.1 software. Two hyaluronic acid samples, crosslinked and non-crosslinked, were prepared with a final concentration of 0.20 g/L. All chromatographic determinations are averages of three injections from one sample dissolution.

Molar mass averages obtained from dual detector SEC experiments were determined based on an universal calibration curve created using eight pullulan standards (Polymer Standard Service) ranging in molar mass from 5.0 × 10^3 to 7.1 × 10^5 g/mol under the same experimental conditions as sample analysis. The universal calibration curve data was fitted with a linear function and had a R^2 value of 0.999. For the dual detector SEC experiments the interdetector delays were calculated using a virtually monodisperse pullulan standard with a molar mass of 4.6 × 10^4 g/mol (Polymer Standard Service).

Results and Discussion

As described above, an EcoSEC GPC System equipped with an internal dual flow refractive index (RI) detector was coupled to a differential viscometer (VISC) to characterize crosslinked and non-crosslinked hyaluronic acid samples. The SEC elution profile, as monitored by the RI and VISC, is shown in *Figure 1*. The SEC elution profile as measured by both detection methods displays a shorter retention time for the crosslinked hyaluronic acid sample than the non-crosslinked hyaluronic acid sample. The difference in retention times in the GPC elution between the two samples is an indication that the crosslinked hyaluronic acid sample is smaller in polymeric size than the non-crosslinked hyaluronic acid sample.

Figure 1. SEC elution profile of non-crosslinked (red) and crosslinked (blue) hyaluronic acid as monitored by the RI (solid) and VISC (dash).



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Molar Mass

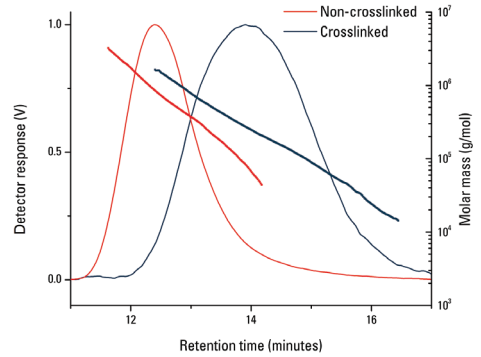
The molar mass averages and polydispersity index of the hyaluronic acid samples were determined via universal calibration via dual detector SEC and are given in *Table 1*. The molar mass averages for the non-crosslinked hyaluronic acid sample were about an order of magnitude larger than that of the crosslinked hyaluronic acid sample. The molar mass distribution of the non-crosslinked hyaluronic acid sample extends further in the high molar mass direction, M_z, while the molar mass distribution of the crosslinked hyaluronic acid sample extends further in the low molar mass direction, M_n, *Figure 2*. For a majority of the molar mass distribution, the two hyaluronic acid samples have the same molar mass. The molar mass polydispersity was > 1, an indication that hyaluronic acid is polydisperse in molar mass no matter the degree of crosslinking.

Table 1. Molar mass averages and polydispersity index of hyaluronic acid samples via SEC/RI/VISC.

Sample	M_n (g/mol)	M_w (g/mol)	M_z (g/mol)	PDI[a]
Non-crosslinked	3.18×10^5	8.58×10^5	1.50×10^6	2.727
Crosslinked	7.22×10^4	2.14×10^5	4.96×10^5	2.977

[a] $PDI = M_w/M_n$

Figure 2. GPC elution profile of non-crosslinked (red) and crosslinked (blue) hyaluronic acid as monitored by VISC and molar mass distribution across the elution profiles as determined by universal calibration.

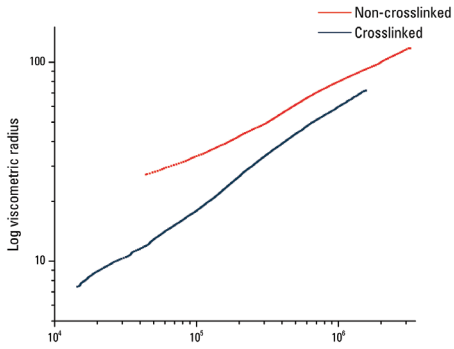


Structure

The dual detector SEC experimental set-up employed here also allows for the determination of several parameters that can provide structural comparisons between the two hyaluronic acid samples, e.g. intrinsic viscosity, viscometric radius, conformation plots, and Mark-Houwink plots. The intrinsic viscosity of a sample is defined as the amount a dissolved molecule contributes to the overall viscosity of the solute and can be thought of as an inverse density. The intrinsic viscosity is an indication of how extended a polymer is in solution, the larger the value of the intrinsic viscosity the more extended or the less dense a polymer is in solution. As expected, the intrinsic viscosity of the hyaluronic acid identified as being non-crosslinked is significantly (almost an order of magnitude) larger, 3,000 g/mL, than the intrinsic viscosity of the hyaluronic acid sample identified as being crosslinked, 500 mL/g .

The viscometric radius of a sample scales proportional to the intrinsic viscosity, as the viscometric radius, R_η, is the radius of a solid sphere that increase the viscosity of the fluid by the same amount as does a macromolecule. The viscometric radius of the non-crosslinked hyaluronic acid sample is almost three times that of the crosslinked hyaluronic acid, 68 and 25 nm, respectively. The distinct differences between the intrinsic viscosity and viscometric radius for the two hyaluronic acid samples confirm that the two samples vary in the degree of crosslinking. The structural differences between the two hyaluronic acid samples can also be seen through the conformation plot, *Figure 3*, which plots the viscometric radius vs the molar mass. At any given molar mass the viscometric radius for the crosslinked hyaluronic acid sample is smaller than that of the non-crosslinked hyaluronic acid sample.

Figure 3. Conformation plot of non-crosslinked (red) and crosslinked (blue) hyaluronic acid.



Conclusions

Two hyaluronic acid samples were analyzed using a dual detector SEC set-up encompassing the dual flow refractive index detector in the EcoSEC GPC System and a ViscoStar differential viscometer. The molar mass of the two hyaluronic acid samples were determined using universal calibration. The two hyaluronic acid samples were determined to be similar in molar mass but different in structure. The addition of the differential viscometer to the EcoSEC GPC System allowed for the structural differences of the two hyaluronic acid samples to be determined as a function of molar mass. The non-crosslinked hyaluronic acid sample was determined to have a larger viscometric radius and higher intrinsic viscosity than the crosslinked hyaluronic acid sample, two indications that the non-crosslinked hyaluronic acid is more extended than the crosslinked hyaluronic acid. The use of an SEC/RI/VISC experimental set-up permits for the determination of molar mass averages based on universal calibration, a method shown to be independent of the chemistry and architecture of a polymer, and physicochemical properties such as polymeric size, confirmation, and intrinsic viscosity.

References

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