Gel Electrophoresis as a Tool to Measure Hyaluronan Polydispersity

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Introduction

The polydispersity of hyaluronan was recognized very early after Karl Meyer's historical work on the purification of hyaluronan from vertebrate tissues and bacterial capsules in the 1930s and 40s. During the recent decades the issue of polydispersity was of less and less interest. We have reached the stage today that researchers often disregard this important characteristic of this polyacid and use the molecular weight or mass as if it represented a distinct value for a monodisperse molecular population. In reality all hyaluronan molecular populations obtained from biological sources, purified or not, are highly polydisperse. The results of molecular weight determinations by intrinsic viscosity or light scattering are often regarded as distinct molecular weights or numbers. All hyaluronan preparations above the molecular weight of 30,000 are polydisperse; therefore the molecular mass or number must be expressed as an average.

Gel Electrophoresis Technique and Limitations

The weight average (M_w) and the number average molecular weight (M_n) can be determined by gel electrophoresis. This method was introduced by Mary Cowman and her student Hong Gee Lee in their publication in 1994 for characterizing hyaluronan from tissue extracts or cell culture extracts, as well as for purified hyaluronan preparations used for therapeutic purposes[1]. What has greatly facilitated the use of this method today is the availability of standards for a broad range of molecular weights as most of these standards represents monodisperse molecular populations[2]. The use of these standards is imperative to obtain reliable M_w, M_n and polydispersity data on pure hyaluronan solutions, as well as for tissue or cell culture extracts that contain hyaluronan.

One of the limitations of this gel electrophoresis method is the presence of proteins in the sample. This is because the presence of proteins may interfere with the staining of the hyaluronan in the gel. Below a certain protein concentration (as a % of the total hyaluronan content) the staining of the hyaluronan in the gel is not affected. This is the case in the liquid vitreus and healthy human synovial fluid. But above this concentration removal of the protein, using protease treatment is necessary. The removal of the proteins does not change the Mw or Mn of hyaluronan or its polydispersity. The purification of hyaluronan solutions from protein must be done in such a way that the Mw, Mn and polydispersity of the hyaluronan population are not compromised. This is especially important when homogenized tissue or cell culture extracts are used. The technique of gel electrophoresis used for the data presented here is described in detail in an SOP, which is available on

The Matrix Biology Institute website[3]. It is essentially the same method described in the paper by Lee and Cowman in 1994[1]. After using this technique in more than 500 gel experiments in our laboratory, we concluded that it produces reproducible results, provided the concentration of hyaluronan in the samples applied to the gel, as well as the volume used, are limited to a certain range. Therefore, the concentration of hyaluronan must be determined before its use. We recommend hexuronic acid determination because it is a simple determination with good reproducibility and without interference from protein molecules. When tissue extracts or culture media or extracts are used, one must be certain that the protein in the sample does not interfere with the staining of hyaluronan. Protease digestion as described in the SOP will remove the interference, but one must be certain that it does not change Mw , Mn or the polydispersity of the hyaluronan population. The simplest way to check this possibility is to run a digested and an undigested sample in parallel on the same gel.

Polydispersity and Statistical Calculations [4, 5]

The **location** of the distribution is characterized on both the molecular weight and number scales. The visualization can be made either on logarithmic or linear scales. The **location** includes three values: *mode, mean*, and *median*. The *mode (peak)* of the distribution expresses the most common molecular weight or number. The *mean* expresses the arithmetic average of the weight or number and the *median* expresses the molecular weight or number values for which to half of the data are smaller and half of the data are larger.

The **spread** of distribution is characterized by the *standard deviation* for both the weight and number of the molecule.

The **shape** of the distribution is characterized by two parameters: *skewness* and *kurtosis. Skewness* **i**s a non-dimensional number that measures the unbalance of the distribution to the right (positive *skewness*) or to the left (negative *skewness*). For normal distribution *skewness* is zero. *Kurtosis* is also a non-dimensional number that expresses the relative peakfulness or flatness of the distribution relative to a normal distribution. The normal distribution has a *kurtosis* value of three. To determine monodispersity only the values for location are needed (*mode, mean, median*). When the values for location are the same one can assume the molecular distribution is monodisperse. We use an ImageMaster® software program to determine the positions of Mode (peak) for each standard and samples obtained from the gel densitometric profiles. The peak position data obtained from Hyalose, LLC Standard Ladders are used to produce a slope that converts migration distance to molecular weight for each densitometric profile. Two Excel programs are employed to collect, sort and analyze this data where the

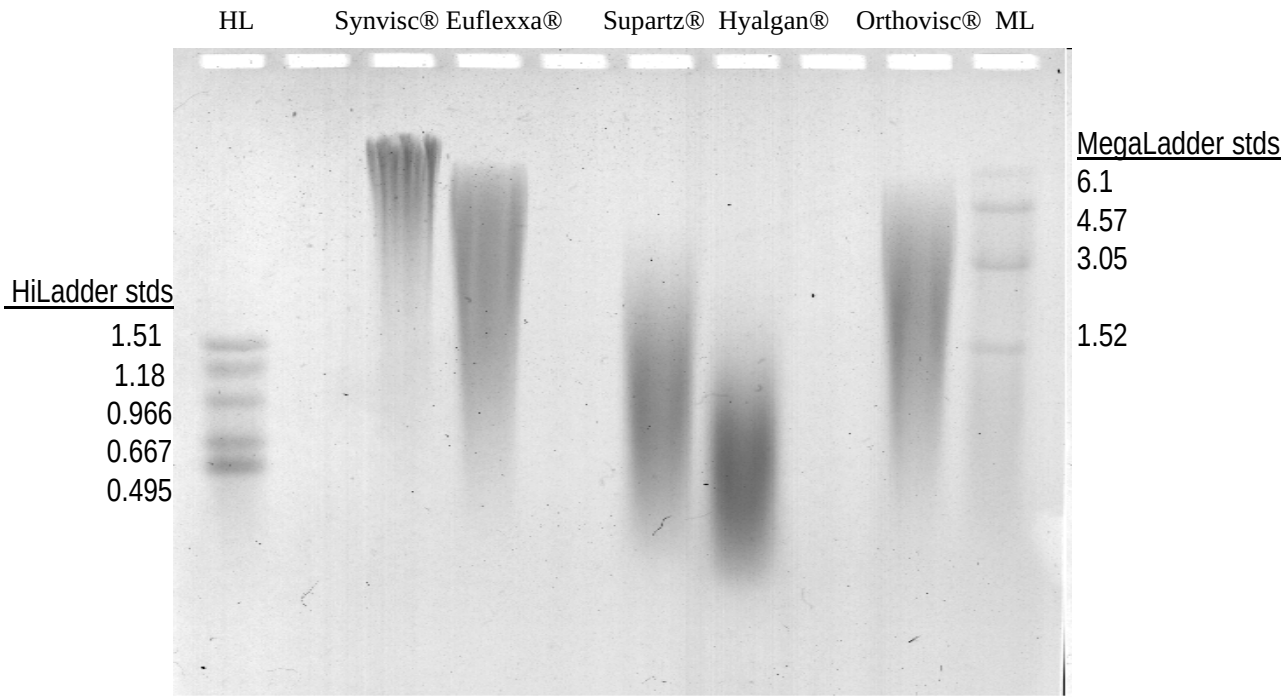
distributions can be plotted in both linear and logarithmic scale. The full range of statistical parameters described here can be calculated from each sample profile (See Table).

The electrophoresis data allow calculation of both the weight fraction distribution and the number fraction distribution. Both are necessary and useful. The weight values reflect the size of the molecules. The number values reflect the number of molecules or moles present at each molecular weight. When exploring questions concerning synthesis and degradation of hyaluronan, the number of molecules present relates to the kinetics underlying the generation of the distribution. In this case, the number distribution parameters are pertinent. However, when the coiling and entanglement coupling of the largest molecules dominate the viscoelastic properties of hyaluronan, the weight distribution parameters are pertinent.

Densitometric Scan and Graphic Representations of Hyaluronan Polydispersity

Densitometric Scan of Some Commercially Available Hyaluronans

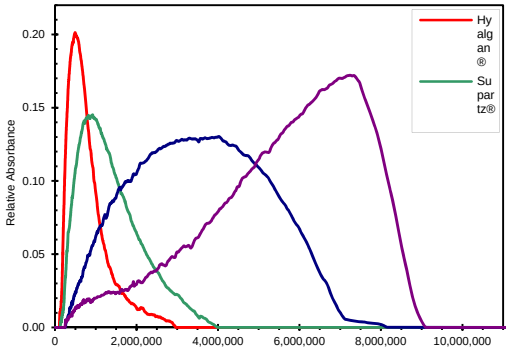
M_w in millions



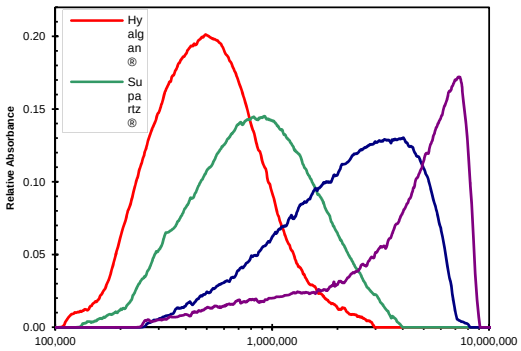
An Excel Program is used to convert densitometric scans (above) into molecular weight using the slope calculated from the peak positions of the standards. Statistical Data is based on the linear scale of the distributions except for the median value, where the logarithmic scale is representative. The logarithmic scale emphasizes the lower molecular weight portion of the distribution. We prefer the linear scale, but either is appropriate.

Both Linear and Logarithmic Representations of Some Commercially Available Hyaluronans

Linear Scale

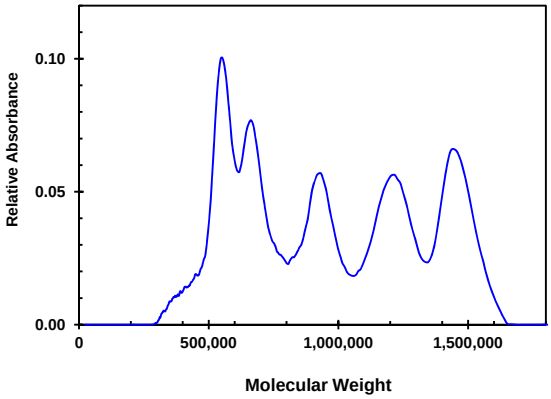


Logarithmic Scale



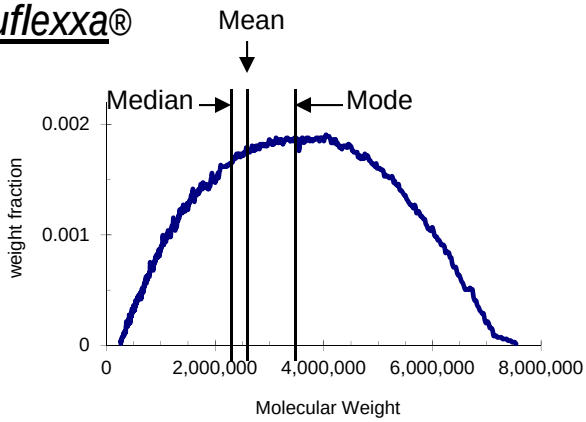
Examples of Hyalose, LLC Standard Ladder Distribution

The peaks do not resolve to the baseline because the samples are too close together and overlap. This does not affect their use because the value of the Mode (peak) is the only data used in the calculation.

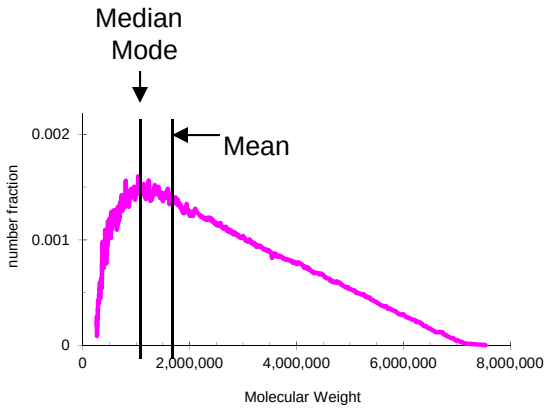


Examples of Polydispersity [4, 5]

Euflexxa®

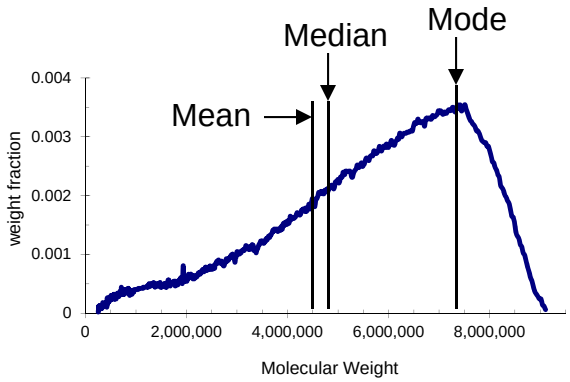


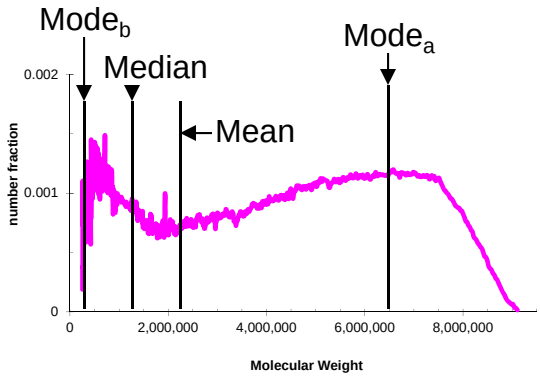
Kurtosis ~3, almost normal
Skewness ~0, almost normal



Kurtosis >3, sharper than normal

Synvisc®





Skewness < 0, tail to left

Skewness > 0, tail to right

Kurtosis < 3 for both weight and number, flatter than normal

Table of Statistical Parameters for 2 Commercially Available Hyaluronans and Standard

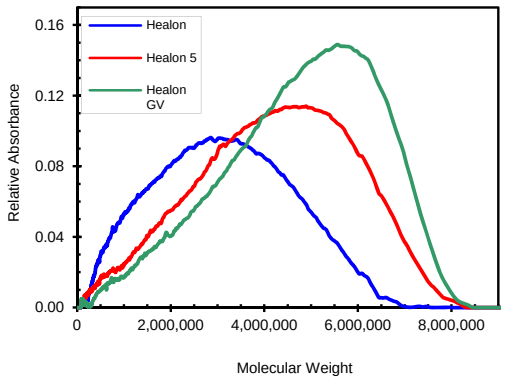
	Mean	Mode	Median	**S	Skewness	Kurtosis
	in millions					
Euflexxa®						
Weight	2.7	3.6	2.4	1.6	0.56	2.43
Number	1.7	1.0	1.2	1.3	1.40	4.57
*Synvisc®						
Weight	4.6	7.4	4.8	2.4	-0.18	1.86
Number	2.4	6.5_a, 0.5_b	1.3	2.3	1.05	2.87
HiLadder 1,500,000 standard						
Weight	1.5	1.5	1.5	0.06	0.64	3.10
Number	1.5	1.5	1.5	0.06	0.66	3.15

*Fluid portion of Synvisc® only tested

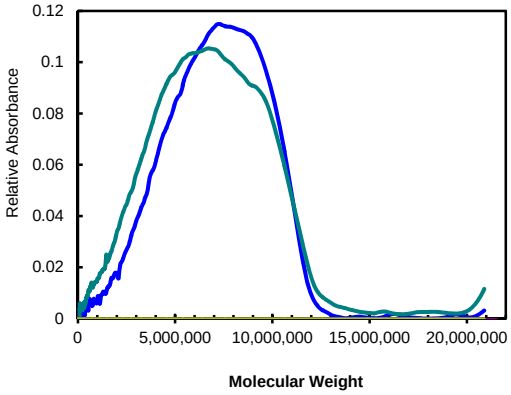
** Standard Deviation

Examples of Commercially Available and Natural Source Hyaluronans

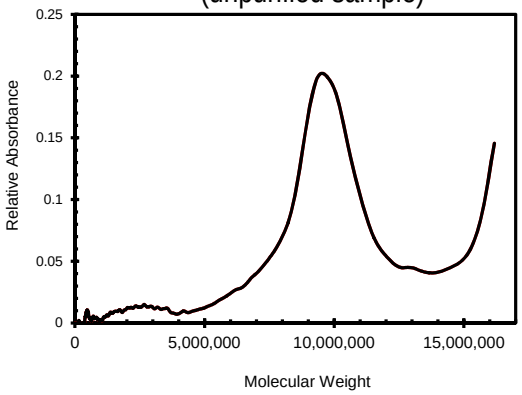
Healon®, Healon® 5, Healon® GV
used in viscosurgical procedures in the eye



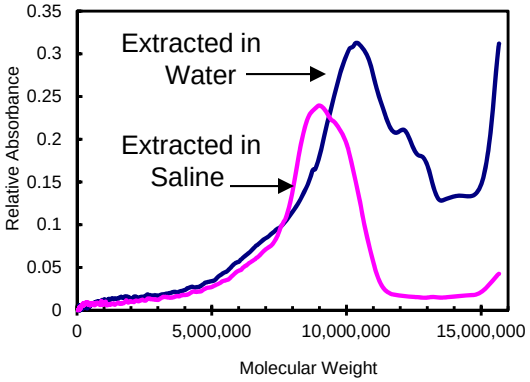
Liquid Vitreus from 2 Adult Owl Monkey Eyes
(unpurified sample)



Ichthyosan A from Tuna Aqueus
Collected from the Anterior Chamber of Tuna Eye,
(unpurified sample)

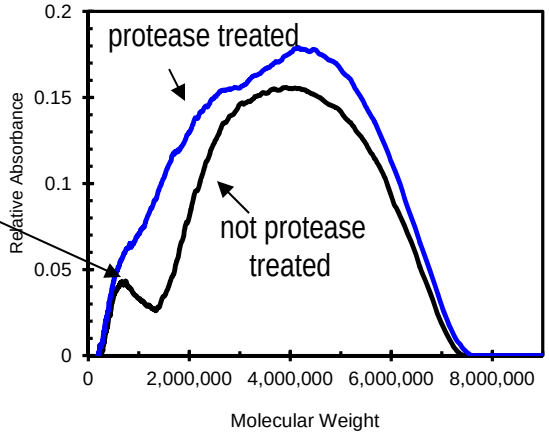


Rooster Comb Extract: in Water or Saline
(unpurified sample)



Example of a sample that required protease treatment before running on the gel; the Mode in the untreated sample is an artifact due to the presence of protein. The protease-treated sample does not show this Mode.

Pathological Human Synovial Fluid



References

(1) Lee, H.G., Cowman, M.K., Anal. Biochem., 219:278-287, 1994

(2) *www.hyalose.com*

(3) *www.matrixbiologyinstitute.org, HA References, Gel Electrophoresis, click on document.*

(4) Evans, M., Hastings, N., Peacock, B., Statistical Distributions, Wiley-Interscience, 3rd edition, 2000.

(5) Rudin, A., Elements of Polymer Science & Engineering: An Introductory Text and Reference for Engineers and Chemists, Academic Press, 2nd edition, 1998.