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The Strengths and Weaknesses of Methods for Determination of Hyaluronan Molecular Weight

Ebrahimi T, Keramati M* and Ahangari Cohan R

Nano-Biotechnology Department, New Technologies Research Group, Pasteur Institute of Iran, Iran

***Corresponding author:** Malihe Keramati, Nano-Biotechnology Department, New Technologies Research Group, Pasteur Institute of Iran, No. 69, Pasteur Ave, Tehran, 1316943551, Iran, Tel: 982164112126; Email: keramatim@pasteur.ac.ir

Review Article

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Abstract

Hyaluronan (HA) as a naturally polysaccharide has several physiological functions in health and disease. Depending on molecular weight (Mw), HA regulates different biological processes which have led to increased interest in determination of Mw and size distribution of HA. The Mw varies when HA is being extracted from different tissues or biological fluids and it is always polydisperse in molecular mass even when extracted from a single source. The molecular mass of the HA is an important aspect of its biological activity and physicochemical properties in sample characterization. In this review, we survey methods related to technologies for the detection of HA and determining its Mw and concentration and the advantages and disadvantages of each of them will be compared.

Keywords: Hyaluronan; Molecular Weight; Size Distribution; Method

Abbreviations: HA: Hyaluronan; Mw: Molecular Weight; LS: Light Scattering; SEC: Size Exclusion Chromatography; MwD: Molecular Weight Distributions; MALS: Multi-angle Light Scattering; DLS: Dynamic Light Scattering; FFF: Field Flow Fractionation; MS: Mass Spectrometry; CE: Capillary Electrophoresis; FACE: Fluorophore-assisted Carbohydrate Electrophoresis; PAGE: Polyacrylamide Gel Electrophoresis; SE-PAGE: Sensitivity-Enhanced Polyacrylamide Gel Electrophoresis; CE: Capillary Electrophoresis; EMD: Electrophoretic Mobility Diameter; ESI: Electrospray Ionization; HPLC: High-performance Liquid Chromatography; SS: Solid-state.

Introduction

Hyaluronan (HA) is a simple repeating disaccharide polymer with a broader range of sizes in tissues or fluids.

HA possesses numerous functions within the body including cell differentiation, wound repair, cell migration, and cell signaling. Due to its versatility, HA has been a significant component of biomedical research and has seen application in several fields of medicine such asvascular, cartilage, bone, tissue engineering, drug delivery and cancer treatments in various forms and sizes [1]. The physiological and biochemical functions of HA are closely connected with its chain length. HA polymer chains often exist as a wide range of different molecular weights (Mw), a quality known as polydispersity that its particular Mw are associated with specific biological activities [2,3]. High molecular weight HA (HMw HA), bigger than 5×10^5 Da mediates tissue integrity and has anti-angiogenic, immunosuppressive and anti-inflammatory properties. Under inflammatory conditions due to damage and/or degradation of HA an abundance of low molecular weight HA (LMw HA) with opposite effects

is found [2,4]. Consequently, due to the difference in size-dependent HA functions, determination of Mw is critical aspect of HA as a translational bioindicator of diseases. Hence there is a need for the development of highly sensitive methods to accurately determine the average Mw and distribution range of HA under different physiological and pathological states. Many current methods for determination of distribution of MWs (polydispersity) of HA from tissues and biological fluids have been reported Cowman MK, et al. [5].

The Methods for Mw Determination

The Mw is one of the most principal parameters in characterization of a polymer. The Mw of a molecule is closely associated with the physical characteristics such as viscosity and shear behavior in solution and optical properties. Methods that do not fractionate the sample can only provide an average of the Mw for all molecules. There are several forms of average values which can be directly obtained by different methods. The most prominent is likely the weight-average molecular weights (Mw), which can be directly obtained by light scattering measurements. The number average of molecular weights (Mn) is important for calculating the dispersity. Mn can be determined by using the colligative properties of a polymer solution, e.g., by using osmometry or by light scattering after separation or purification methods such as chromatography. The viscosity average molecular weights (Mv) measurements by viscometry that deliver only relative Mv values Oberlerchner, et al. [6].

Today, Viscometry, Electrophoretic techniques, Light Scattering (LS) and Size Exclusion Chromatography (SEC) are the most widely used methods for determining the Mw of HA. The choice of method for Mw determination is influenced by factors such as information required, measurable range, cost effectiveness, and experimental conditions and requirements [7]. This review provides an overview and summary of technologies for determination of HA Mw and molecular weight distributions (MwD).

Osmometry

Osmometry is the first analytical methods used in polymer chemistry and continues to serve as sources of fundamental information. At the early of 1930s, Schulz established membrane osmometry as the standard method for determination of Mw of the polymers. In this method, the molecular mass depends on colligative properties, meaning that the number of dissolved molecules is the only factor that alters the properties of a solution. There are two common osmometry methods that are suitable for determining average Mw of polymers: membrane osmometry and vapor pressure osmometry .While the first one is suitable for Mws between 50 and 2000 kDa, the second one is applicable for 'short' polymeric chains below 40 KDa [8]. A serious disadvantage of vapor pressure osmometry is excessive sensitivity to the presence of low molar mass compounds, such as residual monomers, solvents or moisture, which can result in serious underestimation of Mn. In membrane osmometry, if the sample contains oligomeric species that can permeate through the membrane the osmotic pressure is too low and the obtained Mn is overestimated. One advantage of both methods is the independency of chemical nonuniformity and it can be used to determine an absolute Mw [8,9] (Table 1).

Table 1: Current methods for determination of HA molecular weight.

Method	Range (Da)	Advantage	Disadvantage
Osmometry	3×10^4–1.7×10^6	·Low cost	·Requires known concentration
		·Measures Number averaged Mw	· Limited effectiveness for HMW
Viscometry	10^2-3.1×10^8	·Low cost	·Large volumes of samples of known concentrations
		·Ease of use	· Low accuracy for LMW
		·Rapid	
		·High throughput	
		·Measures Viscosity-averaged Mw	
Multi-angle light scattering (MALLS)	10–100×10^6	·Measures polydispersity	·Requires known concentration
		·Sensitive	·Time consuming
		No need for calibration standards	·Extensive sample preparation and analysis.

Size exclusion chromatography (SEC)	$10^2 \times 10^7$	· Sensitive	·Requires known concentrations
		· High resolution	·Sample preparation
		· Measures polydispersity	·Required · High-cost equipment
			·Capability decreases with increasing HA MW
Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF)	up to n17	·Measures polydispersity	·Requires known concentration
		· Wide molecular weight range	· Sample preparation
			·Time consuming
			·High pure sample
			·Limited to the analysis of individual smaller oligosaccharides but not for a mixture of oligosaccharides
			·Do not have satisfactory sensitivity
horizontal Agarose Gels Electrophoresis (AGE)	$0.79–6 \times 10^6$	·Simple and inexpensive	·Limited effectiveness for oligosaccharides
		·Used for the separation of HMw HA	
Polyacrylamide Gel Electrophoresis (PAGE)	$10–100 \times 10^3$	·Simple and inexpensive	·Failure to retain species with less than about 11 disaccharides in the gel during staining processes
		·Suitable for the analysis of oligosaccharides and fragments of HA	·Insensitive and require relatively large amounts of sample
		·Calibration of gel permeation chromatography	
		columns by direct analysis of the MWD in each fraction	
Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)	2-25 disaccharide repeats	·High-resolution separation of HA oligosaccharides	·Time consuming
		Rapid	·sample preparation

Viscometry

In 1930, Hermann Staudinger for the first time recognized an empirical relationship between the relative magnitude of the increase in viscosity and the Mw of the polymers [10]. Viscometry or viscosity method is actually one of the of the simplest and rapid methods used to determine average Mn. Viscometry as a standalone method allows the determination of the HA polymer weight-average over a wide range of Mw (10^2-10^8 kDa) [11]. For unpurified HA and soluble extracts, the viscometric method can give a reasonable estimation of HA Mw, because the solution viscosity is mainly determined by the HMw polymer and not by the much smaller soluble proteins [12]. Viscometric assays can be performed on crude fermentation broths, the equipment required is inexpensive and the procedure is simple and not excessively time-consuming [13]. For pure HA sample, viscosity is an attractive identification method and already is a leading method in industrial applications for determination of Mw. However, viscometry has several practical challenges, including the requirement for large amount of sample (up to 1 g of HA, depending on chain size) and variability induced by ionic strength and temperature because of the dependence of viscosity on experimental factors [9]. Upon an increase of ionic strength, influenced by temperature, the hydrogen bonds present at the intermolecular structure promote conformational change due to the self-aggregation and increase the viscosity accordingly Vega ED, et al. [14].

Light Scattering and Size Exclusion Chromato graphy

Light scattering (LS) is a primary and fundamental method for determining the Mw distribution of a macromolecule like

a polymer or a protein in solution. The first report of using LS for Mw HA referred to sixty-eight years ago. The LS method reduces not only errors due to shearing force by very high Mw that leading to a lower apparent MW value, but also sample size rather than other physicochemical methods [15]. LS as a relatively sensitive process which determines absolute Mw, MwD and conformations of polysaccharides, has the advantage of not requiring comparison to a standard [16]. Two modes of LS, multi-angle light scattering (MALS) and dynamic light scattering (DLS) is used for the characterization of macromolecules Mw analysis (from 7 kDa to several MDa HA) and for particle sizing, respectively Hokputsa S, et al. [17]. One challenge with MALS approaches is their requirement for known concentration of purified HA sample, dependent on Mw and size distribution. For example, 5–20 μg of monodisperse HA synthesized chemoenzymatically (>500 kDa) would be required, whereas polymers <50 kDa may require 75–100 μg but in physiological HA, ~2–5 folds more sample may be needed. In MALS, larger molecules scatter light more strongly and contaminants in sample can change the results by changes in the intensity and angular distribution of scattered light [16]. MALLS can report on HA MW, but is not intrinsically quantitative andhas limited precision, and is relatively insensitive to low-MW fragments [17].

LS gives a weight-average Mw and when used in conjunction with size exclusion chromatography (SEC) the complete MwD for HA determined [16]. SEC appeared in the late 1950s, is the most powerful and, commonly-applied method, for determination of Mw and Mw distribution of HA and its fragments [18,19]. This method has some advantages such as less expensive nature, tolerance to partially purified samples, and ease of use [18,19] (Table 1). Although SEC method is extremely reproducible, it is a relative method and needs Mw standards for calibration to obtain the relation between elution volume and Mw, as a consequence, the difference between experimental MwD and true MwD for HA may be dramatic [20,21]. This method enables discrete size ranges of HA to be quantified, but long run times places practical constraints on the number of fractions and samples that can be examined. Typically, most modern MALS instruments used a separation method such as HPLC-SEC or Field flow fractionation (FFF) approaches as a separation method, pre-filter and assess size distribution. MALS is intrinsically rapid but is limited by this separation steps. The absolute Mw obtained through MALS is widely recognized as the gold standard, but the accuracy of the Mw distributions is somewhat limited by the separation method resolution. SEC-MALLS technique provides accurate and informative data regarding the size distribution of purified HA samples without the need for calibration standards [22]. In addition SEC-MALLS capable of measuring the samples with Mw < 1 $\times 10^6$ Da and showed improved separation for Mw > 1 × 10^6 Da [23].

Other macromolecule size characterization methods include LS, osmometry, and viscometry require significant sample quantities and relatively high concentrations and are limited to low resolution or, in some cases, to the measurement of a single moment of the size distribution (Table 1). When multiple components are present or suspected, these methods generally deliver ambiguous results. To date, the most widely used methods, for size distribution analysis of imperfectly pure HA are SEC [24], and agarose or polyacrylamide gel electrophoresis with staining or with blotting and specific detection [12,25]. The mass spectrometry (MS), electrophoresis, and SEC, have been used to determine the molar mass of HA fragments [22].

Gel Electrophoresis

Currently electrophoretic techniques as a high sensitivity, simple and inexpensive method used for characterization of the mean Mw and distribution of Mws (polydispersity) HA ranging from oligosaccharides to polymers with Mw up to about 6 × 10^6 Da [12]. In this method, a solid gel made of polyacrylamide or agarose is molded and suspended in buffer [13]. The charge-to-mass ratio is constant for HA molecules, thus, like DNA or denatured proteins, migration of HA through a gel matrix allow sieving on the basis of size [16]. Gel electrophoresis methods by staining with a nonspecific cationic dye like Stains-All (3,30-dimethyl-9- methyl-4,5,40,50-dibenzothiacarbocyanine) allows sufficiently sensitive detection on the microgram scale without labeling (2.5 μg for agarose and 0.5 μg on polyacrylamide gels) [26].

The method is characterized by sufficient sensitivity for the detection of HA from biological samples, at microgram level, with-out need of any modification [27]. In this method HA standards must be co-electrophoresed in the same gel used for unknown samples for size analysis [9]. These methods are resistance against sample impurities, so HA does not need to be highly pure to be analyzed by gel electrophoresis. However, some impurities such as strongly bound proteins and non-HA anionic contaminants cause an electrophoretic mobility shift and non-specific staining (like Stains-All or Alcian Blue with or without silver staining) respectively [28,29].

The most widely used HA assessment approach is agarose or poly-acrylamide gel electrophoresis, through which band intensity and position can be analyzed to denote a size distribution. Although simple and inexpensive to implement, this method is slow and labor intensive and must be calibrated. However, its effectiveness depends on relatively high concentrations and innate molecular charge in order to detect the polymer within the gel [9]. Although

simple and inexpensive to implement, this method is slow, requires large sample size (fluid volume and HA mass), requires calibrated standards, usually by the use of markers and provides only semi-quantitative data.

Agarose Gel Electrophoresis

In 1982 agarose gel medium has been shown to be useful in analyzing HMw HA. Lee and Cowman used adapted methods in the electrophoretic separation of HMW nucleic acids for the separation of HMw HA [29]. Agarose electrophoresis is a facile method for the determination of the MwD of HA. Gels with a large average pore size such as agarose gels are used for the separation of HMw HA [30]. These methods have employed particular electrophoretic conditions, different agarose concentrations and buffer systems in which HA of different sizes can be accurately determined with a linear relationship between electrophoretic mobility and the logarithm of the weight-average Mw [25]. Generally, agarose gel system is appropriate size-separated and analyzed method for HA greater than ~100 kDa in the range of 0.1–7 μg, depending on polydispersity and staining method [12]. The applicable Mw range depends on agarose concentration and buffer. Sample loads of approximately 4–7 μg were required for polydisperse samples and the separated pattern was visualized by staining with the Stains-All dye [25]. The gel electrophoresis using staining with toluidine blue followed by Stains-All procedure can detect as little as 10 ng of a single species, and can be used to stain a few micrograms of a complex HA polysaccharide mixture [31]. Higher agarose concentrations were used by Pummill and DeAngelisto optimize the separation of HA in the 0.2×10^6–1×10^6 Da range [25].

The methods of viscometry, SEC-MALLS and gel electrophoresis all provided accurate measurements when Mw of HA was below approximately 2×10^6 Da. When Mw > 2×10^6 Da, viscometry, SEC-MALLS underestimate the values of Mw in comparison with agarose gel electrophoresis technique but electrophoresis provide superiority for measuring polydispersity in comparison with other techniques [23]. There have been numerous recent reports that fragments of HA have different properties compared to the intact molecule [32]. In order to obtain an accurate view of the HA molecule, it is necessary to analyze both the oligomeric and the polymeric levels. So HA Mw can be also determined by the separation and quantitation of oligosaccharide products with identical repeating disaccharide structures and different Mw [5]. The capillary electrophoresis (CE) [33], fluorophore-assisted carbohydrate electrophoresis (FACE), and polyacrylamide gel electrophoresis (PAGE) [34] are alternative techniques appropriate for the separation of HA oligosaccharides with wider size ranges.

Polyacrylamide Gel Electrophoresis (PAGE) of HA Oligosaccharides and Fragments

The first reports of the use of PAGE for analysis of HA oligosaccharides back to1985 [28]. PAGE techniques have a tighter network structure than agarose gels which have been developed for the separation of LMw HA samples (~ 5–100 kDa) using total HA mass of ~ 0.5–1.0 μg [35,36]. A ladder-like series of bands is observed, in which each corresponding to a unique Mw species. The gel composition varied from 10 to 25% acrylamide and the separation patterns are visualized either by fluorography (for radiolabeled samples) or by staining with a cationic dye such as Stains all, Alcian blue and Acridin orange. Simple gels containing 5%, 10%, and 15% polyacrylamide separate HA well but did not resolve a sufficiently broad molecular mass range in the short gels. For higher resolution separation covering a broader range of molecular masses, long gradient PAGE gels (e.g., 4–20% acrylamide) were superior in separation with a sample load of 0.5 μg for each polydisperse HA sample [25]. The previous PAGE systems [35,37,38] required high sample load (5 μg per band, or up to 200-500 μg total for highly polydisperse samples) that reduced resolution, so that species >30-40 disaccharides could not be separated into discrete bands [36]. One additional property of HA which appears to be Mw dependent is the ability to bind and aggregate cationic dyes. Unlike LMw HA (approximately 12-13 disaccharides in length), HMw HA preparation binds acridine orange and induced optical activity in the visible region absorption band of the dye. Turner and Cowman investigated the interaction between HA oligosaccharides and Alcian blue. They showed that short oligosaccharides (less than about eight disaccharides in length) were not visualized using Alcian blue as a cationic dye in staining [28].

Several studies describe different modifications of the electrophoretic technique which result in improved resolution and sensitivity. Min and Cowman developed an improved procedure using long thin gels and a two-step staining process, combined Alcian blue/silver stain staining to increase sensitivity to approximately 100-fold, and results in resolution of individual HA fragments (8 up to at least 250 disaccharides in length) [36]. Sensitivity-Enhanced Polyacrylamide Gel Electrophoresis (SE-PAGE) method is excellent systems for separation of HA and sulfated glycosaminoglycan oligosaccharides. The smaller sample load (50 ng or 2-5 μg for polydisperse samples) employed in the SE-PAGE method minimizes band diffusion and overlap and fragmented HA samples show ladder-like patterns of bands in a short analysis time [36].

Due to the use of large-slab gels, the PAGE method still takes a long time for electrophoresis and it is particularly troublesome to handle such large gels in the staining process.

The trend toward mini-gel systems and staining using Alcian blue/silver were exploited in the development of a rapid method for HA fragment analysis. Ikegami-Kawai and Takahashi used mini-gel PAGE method to increase sensitivity and results in the resolution of individual HA oligosaccharides of 5 to more than 50 repeating disaccharide units in length in a short electrophoresis time [39]. The detection limit in this mini-gel PAGE method is less than 1 ng per band, for 11 repeating disaccharide units, indicating 50 folds higher sensitivity than SE-PAGE [39]. The PAGE techniques are not suitable for the quantitative analysis of polydisperse HA samples containing species with fewer than about 11 disaccharides by dyes but can be analyzed by the fluorophore-assisted carbohydrate electrophoresis (FACE) method [13]. HA oligosaccharides smaller than 11 disaccharides (4.4 kDa) cannot be detected quantitatively (142) (possibly due to poor immobilization in the gel) (78, 147, 164–170), which uses reducing end labeling of the oligosaccharides with a fluorescent dye before electrophoresis so that no additional staining and de-staining procedure is needed [16].

Fluorophore-Assisted Carbohydrate Electro phoresis (FACE) of Short HA Oligosaccharides

FACE is an alternative sensitive gel electrophoretic procedure that would allow for detection of HA oligosaccharides (1pmol or less range) smaller than ~ 4.4 kDa that cannot be detected quantitatively by Cationic dyes [40,41]. Fluorescent labeled HA fragments (2 to 25 disaccharides) were separated into discrete bands in the mini PAGE gels for short run times with no additional staining and de-staining procedure gel [42]. In this system, the fluorescent labels allow immediate visualization of the separation, so that even small mono- or oligosaccharides remain trapped in the gel [43,44]. The FACE method is approximately two times more sensitive for n6-mer and 20 times less sensitive for n11-mer than the present PAGE method, but this method needs a lengthy sample pre labeling process [40]. One of the disadvantages of FACE is that it requires a lengthy sample prelabeling process of 16 h with specific fluorophores reporters, while PAGE method is finished in a shorter time. So PAGE method seems to be more suitable for the analysis of HA oligosaccharides from enzymatic digestion than the FACE method [39] (Table 1).

High-performance Capillary Electrophoresis of HA

Since the introduction of capillary electrophoresis (CE) by Jorgenson and Lukacs in 1981, CE has evolved into a highly versatile separation technique in the high-performance separations of biological ionic polymers such as proteins, nucleic acids and polysaccharides [45]. CE is an analytical technique performed in a thin diameter glass tube that separates molecules based on their mobility under the influence of an applied voltage. The first reported application of CE in HA oligosaccharide separation was in 1991 by Stephen, et al. [46]. This is a powerful technique due to its high resolution, low sample and solvent consumption and high sensitivity. The CE technique is limited to pure HA samples and minimizes diffusion and increases separation by size, such separations have the potential to replace the PAGE method for HA oligosaccharide analysis [47]. Due to its high separation efficiency, CE has been shown to be applicable for the characterization of oligosaccharides [48]. CE method possesses high sensitivity but requires pure sample and is restricted to determine HA samples with MM<10 kDa [27]. CE has provided little information on the purity of the oligomer samples and has limited capabilities to determine the Mw of longer oligosaccharides greater than ~HA 16 mers [32]. The sensitivity at low macromolecule concentrations is limited unless molecule-specific labeling methods are implemented or unless it is combined with mass spectrometry (MS) [49]. CE has a greater peak capacity than HPLC, meaning the separations are more efficient and more peaks can be detected so compared to liquid chromatography, CE separations are often faster and more efficient. HPLC is more versatile and many stationary and mobile phases have been developed for different types of molecules.

Gas-phase Electrophoretic Mobility Molecular Analysis (GEMMA)

GEMMA was first introduced in 1996 by Kaufman et al. to determine molecular weights and demonstrate it for globular proteins. The method measures the electrophoretic mobility of molecules and constructs in gas phase to estimate Mw [3]. Malm L, et al. [20] in 2012 demonstrated that the logarithmic relation of electrophoretic mobility diameter (EMD) for HA can be used to estimate MW in the whole physiological range but not for very small oligo-HA [20]. This method requires a single calibration of analyte electrophoretic mobility rather than routine calibrations in contrast to HPLC-SEC. Thus, once the electrophoretic mobility of HA has been calibrated, no further calibration of the GEMMA instrument is needed. The resolution of the GEMMA system is comparable with that of but its ability to produce a full Mw distribution is limited. The system measures a complete distribution of HA sample over a size range corresponding to molecular masses from 30-2400 kDa in a few minutes [20,50]. The extremely high sensitivity and small sample volumes make GEMMA an excellent tool for both Mw determinations and estimation of concentration of very low concentration of HA samples , but it requires pure HA [20]. One intrinsic limitation of the GEMMA approach is the assumption of spherical molecules. Molecules having shapes that deviate from spheres is likely to have a larger apparent EMD value than their spherical counterparts with the same volume.

Therefore, variation in instantaneous polymer conformation may cause measurement inaccuracies. The technique has been shown the EMD values of HA >70 kDa underestimate the Mw of the molecule [5]. Larger HA molecules, having a larger aspect ratio, will be more aligned in the DMA than small HA molecules. GEMMA method is applicable for size determination of other glycosaminoglycans, which may or may not be sulfated, provided that their shape dependence of the EMD is calibrated [20].

Analysis of HA Oligomers by Mass Spectrometry

Mass spectrometry (MS) has emerged in 1997 as efficient and sensitive methods for characterization of HA and HA derivatives [51]. Matrix-assisted laser desorption ionization time of flight (MALDI–TOF) and electrospray ionization (ESI) are the most widely used ionization methods for the analysis of HA with high sensitivity [52]. Using ESI or MALDI-TOF, HA fragments up to 8 kDa or 41 kDa, respectively, can be observed [53]. Acidic nature of HA allows efficient ionization by ESI for small HA fragments with the (DP) up to 8kDa, because higher oligo HA with GlcNAc at reducing end are less stable and suffer from in-source fragmentation losing GlcNAc residue. MALDI-MS is more suitable for analysis of long oligo HAs and enables analysis in a wide range of degree of polymerization (4-34). Direct analysis of a highly polydisperse mixture of long HA fragments (>10 kDa) is complicated due to the presence of short fragments suppressing the ionization of high mass species. If LMW oHA are removed and polydispersity of HA sample is held under 1.2, HA fragments up to the 41 kDa can be identified by MALDI-MS. Direct mass determination of HMw HA fragments by MALDI-MS allows their application as calibration standards for SEC chromatography. Consequently, improved SEC methodology offers more reliable results than SEC methods calibrated with conventional dextran or pullulan standards [53]. ESI-MS can be easily combined with on-line liquid-phase separation techniques, such as high-performance liquid chromatography (HPLC) and capillary electrophoresis. With HPLC/ESI-MS complete identification and structural information for each HA oligomer species (4/6- to 18/20-mers) is obtained [52]. MS methods can deliver very accurate molecular masses from very small samples but at relatively high capital and operating cost. Its dynamic range has an upper limit of ~10–50 kDa so it typically requires complete hydrolysis of the HA, similar to HPLC. MS method is capable of detecting HA with high sensitivity, distinguish HA from various GAG structures, directly determine Mw, enhancing the capabilities of SEC for determination of Mw distribution, and even visualizing the spatial distribution of HA [53]. Although MS is capable of resolving MW differences, but in addition to requiring expensive and complex instrumentation, cannot probe HA larger than ~100 kDa [52] (Table 1). Size distribution of HA analyses by conventional methods remains challenging because of not sufficiently sensitive, have limited dynamic range, and/or are only semi-quantitative. Solid-state (SS-) nanopores are an emerging platform for sensitive molecular analysis [54].

Nanopore Analysis

Solid-state (SS-) nanopores composed of single apertures fabricated in a thin-film membrane have been applied to HA measurement [54]. In this method a thin membrane containing a nanometer-scale aperture is positioned between two reservoirs of electrolyte solution and an applied voltage is used to transport HA through the pore electrophoretically. The translocation of the molecule creates a translocation signal which can be used to determine HA Mw. A full distribution can be obtained by analyzing a representative sampling of HA in a specimen (500-1000) from HA samples as small as 10 ng total [54]. HMwHA (> 20MDa) can promote transient clogging of the pore due to entanglement, potentially resulting in an overestimated Mw. SS-nanopores yield accurate size distributions for mixtures of HA from a variety of sources and HA synthesized in vitro [55-57]. In addition, SS-nanopores could deliver both Mw distribution and quantification in a single measurement. SS-nanopore technology has advantages such as short measurement time, high sensitivity, broad dynamic range for Mw determination, and potential for integrative assessment and automation. The flexibility of this platform enables both detection and MW discrimination across a broad range of molecular sizes and its speed and quantitative output indicate a direct route to translational applications [54].

Conclusion

HA is an extremely versatile material with various unique properties. In general, the biological functions of HA are closely related to the whole distributions of the Mw and the size of the macromolecules. The numberless physiological roles of HA drive a rising interest in the molecule and underscores the need for rapid, sensitive and powerful analytical approaches to characterize HA. Many current methods for determination of the Mw of HA have been optimized. However, innovations with other existing technologies continue to be developed, suggesting that improvements in the overall capabilities and possible of HA evaluation can be expected. Therefore, it is clear that HA size analysis techniques will continue to provide new information about a variety of healthy biological processes and disease pathology. Despite having different characterization techniques for Mw characterization, the utility of many of these options is limited by high costs, low throughput, and the need for large sample volumes and long preparation times. Furthermore, they require previous knowledge on the precise concentration of sample being analyzed, which is

often difficult to determine for small volume samples.

HA and its versatile applications in multiple fields of medicine have been extensively explored over the past several years and continue to be the subject of extensive review. While the information on HA is immense, there is still much to learn about this biopolymer and its different applications, considering the importance of HA size.

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