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Agarose and Polyacrylamide Gel Electrophoresis Methods for Molecular Mass Analysis of 5–500 kDa Hyaluronan

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Abstract

Agarose and polyacrylamide gel electrophoresis systems for the molecular mass-dependent separation of hyaluronan (HA) in the size range of approximately 5–500 kDa have been investigated. For agarose-based systems, the suitability of different agarose types, agarose concentrations, and buffers systems were determined. Using chemoenzymatically synthesized HA standards of low polydispersity, the molecular mass range was determined for each gel composition, over which the relationship between HA mobility and logarithm of the molecular mass was linear. Excellent linear calibration was obtained for HA molecular mass as low as approximately 9 kDa in agarose gels. For higher resolution separation, and for extension to molecular masses as low as approximately 5 kDa, gradient polyacrylamide gels were superior. Densitometric scanning of stained gels allowed analysis of the range of molecular masses present in a sample, and calculation of weight-average and number-average values. The methods were validated for polydisperse HA samples with viscosity-average molecular masses of 112, 59, 37, and 22 kDa, at sample loads of 0.5 µg (for polyacrylamide) to 2.5 µg (for agarose). Use of the methods for electrophoretic mobility shift assays was demonstrated for binding of the HA-binding region of aggrecan (recombinant human aggrecan G1-IGD-G2 domains) to a 150 kDa HA standard.

Keywords

hyaluronan; glycosaminoglycan; electrophoresis; molecular mass; molecular weight; electrophoretic mobility shift assay

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Introduction

The biological functions of the glycosaminoglycan hyaluronan (HA1; poly[(1→3)-β-D-GlcNAc-(1→4)-β-D-GlcA-]) are closely connected with molecular mass. In tissues and biological fluids, HA molecules can occur with molecular mass ranging from greater than 6000 kDa polymers to 0.8 kDa tetrasaccharides [1–13]. Under normal physiological conditions, HA is synthesized predominantly as the high molecular mass polymer. Degradation to lower molecular mass can be enzymatically mediated by the hyaluronidases, but can also be caused by reactive oxygen and nitrogen species, especially in inflammatory processes [13–16]. HA can also be synthesized as lower molecular mass polymers, depending on the differential expression of three hyaluronan synthase enzymes [17–18].

Cell surface receptor proteins such as CD44, TLR 2/4, and RHAMM respond differentially to HA, depending on its molecular mass [19–28]. Low mass HA can result in changes in gene expression for mediators of host defense against microbes, mediators of inflammation response, and proteins connected with cell migration. Most of the studies documenting the signaling properties of low mass HA have relied on the use of exogenous HA that has been degraded to lower molecular mass. There is a need for the development of improved and highly sensitive methods to accurately analyze the molecular mass of HA as it exists in tissues under different physiological and pathological states. Since HA samples always occur as polydisperse mixtures of molecules with a range of molecular masses present, and molecules of different mass can have different biological properties, both the average molecular mass and the molecular mass distribution are equally important to document. Gel electrophoresis methods are capable of providing the necessary separation of HA on the basis of size, and allow sufficiently sensitive detection that HA isolated on the microgram scale from biological samples can be analyzed without labeling [29].

A number of agarose gel electrophoresis methods for the separation of HA have been developed. For high molecular mass HA (ca. 200–6000 kDa), we developed a 0.5% agarose gel system that has been shown to provide excellent size-based separation and accurate calculation of the weight-average and number-average molecular mass [11] Cowman et al., accompanying paper). Many other agarose gel compositions and electrophoretic conditions have been reported [17, 30–50]. These methods have employed different agarose sources, concentrations, and gel dimensions. The voltage and time for the separation have been varied. Changes in the staining procedure, and more importantly the destaining procedure, both of which must be properly controlled to yield quantitative densitometric scans, have been made. Many of the newer methods give apparently excellent separation of high to moderate molecular mass HA, but validation of accuracy with respect to the molecular mass distribution detected has not been provided.

For low molecular mass HA, polyacrylamide gel electrophoresis (PAGE) systems have been developed. The PAGE methods of Cowman et al. [51], Hampson and Gallagher [52], and Knudson et al. [53], especially as modified by Min and Cowman [54] and Ikegami and Takahashi [55], are excellent systems for separation of HA and sulfated glycosaminoglycan oligosaccharides. The validity of 10% PAGE with Alcian Blue and silver staining for the accurate densitometric analysis and weight-average molecular mass calculation of fractionated low mass HA samples with average sizes of 7–20 kDa was confirmed by low angle laser light scattering [56]. Kooy et al. [57] recently reported the use of 20% PAGE

¹Abbreviations: HA, hyaluronan; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GlcA, D-glucuronic acid; M_i, molecular mass; M_w, weight-average molecular mass; M_n, number-average molecular mass; M_v, viscosity-average molecular mass; TAE, Tris-acetate-EDTA buffer; TBE, Tris-borate-EDTA buffer

stained with Stains-All for chemoenzymatically synthesized low molecular mass HA, and reported the use of the densitometric profile to calculate molecular mass averages.

A further improvement in resolution has been reported for structurally heterogeneous sulfated glycosaminoglycans by using gradient PAGE gels [58–63]. Bourguignon et al. [64] have reported the use of 5–15% PAGE for HA, but did not report calculation of molecular mass averages from densitometric data.

Our goal in the present study was to conduct a systematic study of the applicable molecular mass range for HA analysis by gel electrophoresis on agarose and polyacrylamide, as a function of gel and buffer compositions and electrophoretic conditions. Optimized methods for HA in the molecular mass range associated with receptor-mediated signaling were chosen and validated by analysis of polydisperse HA samples of independently determined molecular mass.

As an illustration of the use of gel electrophoresis for purposes other than molecular mass analysis, the detection of an electrophoretic mobility shift of moderate mass (ca. 150 kDa) HA by noncovalent binding to a recombinant human HA-binding domain of aggrecan is reported. This finding complements previous reports of changes in the electrophoretic mobility of short HA oligosaccharides upon complexation with HA-binding domains of aggrecan or versican [65–68], and the slow electrophoretic migration of high molecular mass HA that is covalently modified by the heavy chains of IaI [69, 70]. The detected shift for a noncovalent HA-protein complex also serves as a reminder that HA must be purified free of binding proteins prior to electrophoretic analysis of molecular mass.

Materials and Methods

Materials

Polydisperse bacterial HA samples of high purity and known average molecular mass (based on measurement of intrinsic viscosity) were purchased from Lifecore Biomedical. Chemoenzymatically synthesized HA standards of low polydispersity and known molecular mass based on size exclusion chromatography with multi-angle light scattering (Select-HA™: specific HA sizes and mixtures of sizes as HiLadder and LoLadder) were obtained from Hyalose LLC. Low molecular mass HA oligosaccharide standards were also prepared by testicular hyaluronidase digestion of HA and subsequent fractionation by size exclusion chromatography as previously described [56]. Recombinant HA binding protein, containing the G1-IGD-G2 domains of human aggrecan, was obtained from R&D Systems. Unless otherwise stated, agarose was obtained from GE Healthcare (agarose NA, $-m_r = 0.10$). Additional agarose samples were GE Healthcare agarose IEF, ($-m_r = 0.00$), Bio-Rad Laboratories Low Range Ultra agarose ($-m_r \leq 0.12$), and Lonza NuSieve 3:1 agarose ($-m_r \leq 0.13$). Polyacrylamide gels in TBE containing a 4–20% gradient in acrylamide concentration and a constant 2.6% bisacrylamide concentration were obtained from Invitrogen. Stains-All dye (3, 3'-dimethyl-9-methyl-4, 5, 4', 5'-dibenzothiacarbocyanine) and bromophenol blue tracking dye were obtained from Bio-Rad laboratories. Equivalent material is available from other suppliers. All other chemicals were reagent grade quality.

Agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer

Electrophoresis of HA on agarose gels cast and run in TAE buffer (40 mM Tris, 5 mM CH_3COONa , 0.9 mM EDTA, pH 7.9) was performed using the method of Lee and Cowman [11], as adapted to a minigel format (Cowman et al., accompanying paper). The agarose gel concentration was varied from 0.5–2.0%.

Agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer

Agarose gels with final concentrations of 0.5–2.0% (w/v) were prepared by dissolving 0.2–0.8 g of agarose in 40 ml TBE buffer (100 mM Tris-borate, 1 mM EDTA, pH 8.3), using brief heating in a microwave oven. The agarose solution was transferred to a 48°C water bath for 15 min to equilibrate prior to pouring the gel.

For 3.0–4.0% agarose gels in TBE buffer, a modified procedure was employed. The method was based on a protocol developed by Lonza Rockland, Inc. (document # 18103-0807-8). Agarose powder (1.2–1.6 g) was slowly sprinkled into 40 ml of chilled TBE buffer, while stirring using a magnetic stir bar. The mixture was allowed to continue mixing by stirring for 15 min at room temperature, and the weight recorded. The flask was covered with plastic wrap or parafilm (with a single perforation) and heated in a microwave oven at medium power for 1.5–2 min, interrupted periodically for gentle mixing by swirling the flask. Further heating at high power for 1–2 min with intermittent swirling continued until there were no visible particles. The mixture was weighed, and the lost water (approximately 4–7 ml) was replaced with hot deionized water. A small excess of water (approximately 1 ml) was added in anticipation of further losses, and the mixture was heated again at high power for approximately 30 sec. The weight was rechecked and adjusted if necessary by further heating or water addition. The agarose solution was thoroughly mixed and transferred to a 58°C water bath for 15 min to equilibrate prior to pouring the gel.

Dissolved and equilibrated agarose solution was poured into a 10 cm × 6.2 cm Bio-Rad Mini Sub Cell GT gel casting tray (nominally 10 × 7 cm) to form a 6.5 mm thick gel. An 8-tooth well-forming comb was set at a height of 1 mm from the bottom, creating wells of 1 × 5 × 5.5 mm. Any trapped air bubbles were moved to the sides using a glass rod or pipette. The gel was allowed to set for 20 min, then covered with 40 ml TBE buffer, and stored under plastic wrap at room temperature overnight. For electrophoresis, the comb was removed from the gel, and the gel with its supporting plate was transferred to a Bio-Rad Mini Sub Cell GT electrophoresis unit. The unit was filled with ca. 245 ml TBE buffer (at room temperature), resulting in an approximately 4 mm thick layer of buffer above the gel. For the 3.0% and 4.0% gels, pre-electrophoresis was performed at 40 V for 20 min to cause mobile impurities in the agarose to migrate ahead of samples. This procedure is usually not necessary in the lower concentration gels.

Samples of polydisperse HA were usually prepared at a concentration of 0.3–0.5 µg/µL in 150 mM NaCl, but phosphate-buffered saline (PBS, 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4) or water may be used. A 5 µl aliquot of the HA sample was mixed with 10 µl water and 3 µl of loading buffer (0.02% bromophenol blue, 2 M sucrose in 1X TBE) in order to load 1.5–2.5 µg HA. Select-HA™ low polydispersity standards and mixtures of standards (HiLadder, LoLadder) were prepared in water at concentrations of 0.04–0.1 µg/µl per HA component. A 5 µl aliquot of the HA standard or standard mixture was mixed with 10 µl water and 3 µl of loading buffer, in order to load 0.2–0.5 µg HA per band.

Electrophoresis was carried out at room temperature at a constant voltage of 20 V for 0.5 h, then 40 V for 3.5 h (for 0.5–2.0% gels) or 4 h (for 3.0–4.0% gels).

Immediately after the run, the gel was placed in approximately 500 ml of solution containing 0.005% Stains-All in 50% ethanol (stored protected from light). The gel was stained overnight under light-protective cover at room temperature. For destaining, the gel was transferred to 10% ethanol solution and stored in the dark for one day, with at least one change of destaining solution. Final destaining of any residual background was accomplished by placing the gel on a light box for a few minutes. (Destaining the gel by

photobleaching alone can be used for qualitative work.) The final gel was scanned in transmission mode using a GE Healthcare ImageScanner III, controlled using LabScan v.6 software. A red source was used to enhance the detection of the blue-stained HA. Quantitative analysis of the calibrated image was accomplished using ImageQuant TL software. Background subtraction was accomplished by comparing the sample lane profile to that of an empty lane. The corrected lane profiles were exported to Excel for data analysis and plotting. A calibration plot of the logarithm of the HA standard molecular mass versus migration distance (pixel number in the scan data) on the gel was prepared for each gel. In order to determine which HA standards to include in the calibration plot, the standard deviation of each data point from the line was determined, and points deviating by more than two standard deviations were excluded. From the linear portion of the calibration plot, an equation was generated to allow conversion of migration distance to molecular mass for all HA sample densitometric profiles in that gel. For ease in comparing densitometric profiles between samples in the same plot, data were scaled vertically, usually by normalizing all sample profiles to the same total area. Thus the vertical scale in relative absorbance is arbitrary unless otherwise noted. This scaling procedure has no effect on the calculated molecular weight for a given sample, since every point in the profile is scaled by the same factor. Instructions for creation of Excel spreadsheets for plotting densitometric profiles and calculating molecular weight averages have been previously provided (supplementary material in Cowman et al., accompanying paper).

Polyacrylamide gel electrophoresis in Tris-borate-EDTA (TBE) buffer

The method of Min and Cowman [54] was modified for use with commercial precast minigels. Invitrogen TBE gels (nominally 10 cm × 10 cm) containing a linear 4–20% concentration gradient of polyacrylamide were 8 cm × 8 cm, 1.0 mm thick, with 10 wells. They were stored at 4°C. Prior to electrophoresis, the wells of the gel were rinsed 2–3 times with pre-cooled (ca. 4–8°C) TBE buffer. The gel was placed in an Invitrogen X-cell SureLock™ Mini Cell apparatus. The buffer core chamber was filled with approximately 200 ml of cold TBE buffer to a height of approximately 1 cm above the tops of the well teeth. The outer buffer chamber was filled with approximately 600 ml of cold TBE buffer.

HA samples were usually prepared at a concentration of 0.1–0.2 µg/µL in PBS or water. The HA sample was mixed with loading buffer (2 M sucrose in TBE) in a 5:1 volume ratio. Sample loads usually contained approximately 0.25–1.0 µg HA per lane (0.5 µg for a highly polydisperse sample). One or two empty lanes of the gel were loaded with 1–2 µL of the tracking dye bromophenol blue, dissolved at a concentration of 0.02% in 2 M sucrose in TBE.

Electrophoresis was carried out with the apparatus at room temperature, but using pre-cooled TBE buffer at a constant voltage of 400 V for 28–40 min. The tracking dye moved to the bottom of the gel in approximately 28 min. After the run, the gel was removed from the cassette and transferred to approximately 200 ml of staining solution containing 0.005% Stains-All in 50% ethanol (stored protected from light). The gel was stained for 1 h under light-protective cover at room temperature. For destaining, the gel was transferred to 10% ethanol solution and stored in the dark for 10 minutes with constant shaking (to avoid rolling of the gel). The gel was scanned in transmission mode using a GE Healthcare ImageScanner III running LabScan v.6, and the data were analyzed as described above for the agarose gels, with the following alteration. During the staining procedure the high ethanol concentration caused shrinkage of the thin polyacrylamide gel, which was most severe in the top of the gel where the polyacrylamide concentration was lowest. This shrinkage was only partially reversed during destaining, and all gel scans were analyzed using lane profiles fit to the shape of the gel.

We found our own sample buffer and running buffer preparations were superior to those available from the gel manufacturer which, in our hands, caused the sample to concentrate into a thin dark vertical line in each sample lane. We also tested gradient and single concentration polyacrylamide gels from Bio-Rad, but obtained superior separation over a broader range of HA molecular weight with the Invitrogen gradient gels.

For comparison with the commercial precast polyacrylamide gels, we also prepared and used single concentration gels according to the method of Min and Cowman [54], adapted to the minigel size.

Electrophoretic Mobility Shift Assay

The effect of HA binding protein on the electrophoretic mobility of a 150 kDa HA was tested using agarose gel electrophoresis. Recombinant HA binding protein, consisting of the G1-IGD-G2 domains (approximate molecular weight of 74 kDa) from human aggrecan (rHuHABP) was dissolved in PBS at a concentration of 1.25 $\mu\text{g}/\mu\text{l}$. Aliquots were further diluted to concentrations of 0.625 $\mu\text{g}/\mu\text{l}$ and 0.125 $\mu\text{g}/\mu\text{l}$. Select-HATM with a molecular mass of approximately 150 kDa (actual weight-average mass of 162 kDa) was dissolved in PBS at a concentration of 0.25 $\mu\text{g}/\mu\text{l}$. Equal volume mixtures of rHuHABP and the HA resulted in samples with weight (protein/HA) ratios of 5/1, 2.5/1, and 0.5/1, corresponding to approximate molar protein/HA ratios of 10/1, 5/1, and 1/1. Each mixture was incubated at room temperature for 30 min. Samples containing 0.625 μg of HA, with or without the protein, were electrophoresed on 1.5% agarose in TAE buffer as described above. Equivalent results were obtained by mixing 1 part HABP in 4 M GuHCl, 50 mM Na acetate, 1 mM EDTA, pH 5.8, with 9 parts HA in 50 mM Na acetate, 1 mM EDTA, pH 5.8. The mobility shift of a 70 kDa HA, and the effect of boiling on the shift, were tested using polyacrylamide gel electrophoresis. A 1.25 μl aliquot of the rHuHABP at a concentration of 1 $\mu\text{g}/\mu\text{l}$ in PBS was mixed with 1 μl of HA at a concentration of 0.5 $\mu\text{g}/\mu\text{l}$ in PBS, corresponding to a molar ratio of 2.3/1. The mixture was incubated at room temperature for 1 h, then diluted with 20 μl of PBS and incubated a further 1.5 h. An identical sample was further subjected to boiling for 14 min to test the ability to disrupt the complex by heat.

Figure preparation

All gel images are unmodified with respect to contrast or brightness.

Results

Agarose gel electrophoresis: Effect of agarose concentration and buffer composition on molecular mass range of HA separated

We have previously shown ([11]; Cowman et al., accompanying paper) that 0.5% agarose gels, cast and run in a Tris-acetate-EDTA (TAE) buffer, can be used to electrophoretically separate HA over a molecular mass range of approximately 200 kDa to at least 6000 kDa. In order to find a suitable gel composition for separation of lower molecular mass HA, we compared the 0.5% agarose gel with higher agarose gel concentrations of 1.0, 1.5, and 2.0% in the TAE buffer (Figure 1, panels A–D). Chemoenzymatically-synthesized HA standards with nearly monodisperse molecular masses ranging from 30–1510 kDa were used to test the molecular mass dependence of the mobility. Gels were run under identical conditions of voltage and time, but sample loading order in the gel lanes was variable. In all cases, HA mobility decreased with increasing molecular mass. As a function of increasing agarose concentration, the mobility of all HA standards was reduced, but higher molecular mass HA standards were progressively more retarded in mobility relative to lower molecular mass HA. Higher agarose gel concentration also increased resolution of bands by more strongly sieving low molecular mass HA, and by limiting sample diffusion in the gel. Thus HA

standards with molecular masses in the 30–200 kDa range, which were poorly sieved in 0.5–1.0% gels, were better resolved in 1.5–2.0% gels.

The buffer used for preparation and electrophoresis of the agarose gels can also affect the separation achieved. Tris-borate-EDTA (TBE) buffer causes agarose gels to have a tighter gel network, with enhanced sieving power. (For that reason, the 0.5% agarose gels used for analysis of very high molecular mass HA are run in TAE buffer.) When HA of very high molecular mass is not present, it is preferable to use TBE buffer for two reasons. First, the buffer does not suffer exhaustion during the 4 h electrophoresis period, and the normal minigel apparatus with small buffer tanks may be used without significant change in pH. (In TAE buffer, the minigels should be run in a large format apparatus with large buffer tanks.) Second, there is significantly reduced background staining in gels cast and run in TBE buffer. Figure 1, panels E–H, show HA standards electrophoresed on agarose gels of 0.5 to 2.0% concentration, all in TBE. The clarity of the stained gels is greatly improved in TBE relative to the corresponding gels run in TAE. It is important to note that the background staining for agarose gels in either buffer increases with increasing gel concentration. No contrast or brightness enhancement was used to alter any of the gel images.

Agarose gels with very high concentrations of 3.0–4.0% in TBE buffer are shown in Supplemental Figure 1. These gels show excellent separation of HA fragments as small as 9 kDa, but the background staining due to impurities in the agarose can be problematic. Pre-electrophoresis of the gels is needed to cause the impurities to migrate ahead of sample.

Calibration plots (Figure 2) for HA electrophoresed on agarose gels in either TAE buffer (Figure 2A) or TBE buffer (Figure 2B) show that HA in the molecular mass range below ca. 200 kDa has an improved separation with increasing gel concentration, and that the calibration is more linear for this size range for gels run in the TBE buffer. In contrast, the highest molecular mass HA standards were less well separated (noted as an upward curvature in the calibration plot) with increasing agarose concentration. Thus the choice of a preferred gel composition depends on the desired molecular mass range to be analyzed.

An example of experimental densitometric profiles for HA standards run on 3% agarose in TBE are shown in Figure 3, accompanied by the linear calibration plot used to convert mobility to molecular mass in the profiles. The linear calibration plot was fit for data of HA standards from 495 to 9 kDa. Higher molecular mass HA was poorly separated in the tight network (due to chain orientation in the direction of migration), and such samples were not included in this gel. When run on 3.0% gels, HA with a molecular mass greater than 500 kDa is very poorly separated, remains near the origin, and the molecular mass cannot be accurately determined.

In order to analyze HA greater than 500 kDa, a 1.5% agarose gel in TAE buffer may be used. A nearly linear relationship between migration position and logarithm of the molecular mass is observed for HA standards from 1510 kDa down to 68 kDa (Supplemental Figure 2A). Lower molecular mass HA was less strongly sieved by this gel system, and the corresponding data points fell below the calibration line. This effect results in an artifactually high apparent molecular mass for the smallest HA standards in the densitometric profiles (Supplemental Figure 2B).

We compared agarose from several commercial suppliers, with respect to resolving power and background staining. All of these experiments were performed in TAE buffer. Relative to the separation obtained with GE agarose NA or GE IEF agarose, Bio-Rad Ultra agarose and Lonza NuSieve agarose gave similar resolution. In some gel types there was significantly greater background staining (see Supplemental Figure 3).

Polyacrylamide gel electrophoresis of HA

Polyacrylamide gels have a tighter network structure than agarose gels. We previously showed [54] that a 32 cm long 10% polyacrylamide gel could be used to obtain high resolution separation of HA fragments containing approximately 10–100 disaccharides (4–40 kDa) over the length of the gel, with distinctly separate bands for fragments differing in length by one disaccharide. Banding patterns for longer HA fragments up to 250 disaccharides (100 kDa) in length could be obtained by extending the electrophoresis time and allowing shorter fragments to elute from the gel. A combination staining protocol, using Alcian Blue to fix the HA and a subsequent silver stain, allowed analysis of 2–5 μ g of polydisperse samples. In the present study, we adapted our previous methods to a vertical minigel format. Gels containing 5%, 10%, and 15% polyacrylamide were found to separate HA well, but did not resolve a sufficiently broad molecular mass range in the short gels. An improved separation covering a broader range of molecular mass was obtained using a commercial 4–20% gradient polyacrylamide gel. The Alcian Blue – silver stain protocol, which causes some band spreading and loss of resolution in the short gels, was replaced by use of Stains-All dye. A sample load of 0.5 μ g for each polydisperse HA sample was found to be appropriate. This is equal to the sensitivity reported by Ikegami-Kawai and Takahashi [55] for gels stained with Alcian Blue and silver stain.

Figure 4 shows the result from 4–20% gradient polyacrylamide gel electrophoresis for 28 min of chemoenzymatically-synthesized HA standards ranging in size from 495 to 30 kDa, as well as lower molecular mass HA standards prepared by enzymatic digestion of polymeric HA and fractionation by size exclusion chromatography. The smallest HA standards appear as sets of bands, which correspond to fragments differing in length by one disaccharide. Figure 5 presents the densitometric profiles of the samples, and the calibration graph used to convert migration distance (pixel position) to molecular mass. The calibration was linear over a size range of approximately 110 to 8 kDa. Higher molecular mass HA was poorly separated as the molecules become oriented in order to move through the network. This causes severe underestimation of the molecular weight of such species in the densitometric profile. At the low molecular mass end, HA fragments as small as 11 disaccharides (4.4 kDa) can be easily observed as discrete bands in the ladder-like series (data not shown), and can therefore also be analyzed by this method. HA smaller than that size was not easily detectable using our staining procedure, although HA oligosaccharides containing 9 or 10 disaccharides (3.6–4 kDa) could be faintly stained.

Electrophoresis for a longer time period allows improved resolution of HA of higher molecular mass. Supplemental Figure 4A shows the result of electrophoresis of HA standards for a time period (40 min) that allowed shorter HA fragments to elute from the gel. Supplemental Figure 4B shows that approximately 25 discrete bands can be seen within the stained area for the 30 kDa HA standard, corresponding to the 75 disaccharide average length, and larger/smaller fragments differing in length by one disaccharide.

Validation of the electrophoretic methods for molecular mass analysis of polydisperse HA samples

Because HA isolated from tissues or biological fluids is always polydisperse in molecular mass, it is important to test whether new methods for molecular mass analysis can achieve correct characterization of the molecular mass averages and distribution. We have previously shown that HA with an average molecular mass of more than a few hundred kilodaltons can be successfully analyzed by electrophoresis on 0.5% agarose in TAE buffer. In order to test the validity of the new protocols for lower molecular mass HA, we analyzed four polydisperse HA samples with viscosity-average (close to weight-average) molecular masses of 112, 59, 37, and 22 kDa. Figure 6A shows the result for electrophoresis of the

three largest HA samples on a 3% agarose gel run in TBE buffer, and Figure 6B shows the corresponding result for all four samples on a 4–20% polyacrylamide gel in TBE buffer. On the agarose gel, the polydisperse HA samples show the presence of HA ranging in size from about 300 kDa to 10 kDa. On the polyacrylamide gel, the same samples show the presence of a broad distribution of HA fragments extending almost to the bottom of the gel (corresponding to HA of approximately 11 disaccharides).

Densitometric profiles for the four polydisperse HA samples are shown in Figure 7. For the 112 kDa and 59 kDa HA samples, 3% agarose gels in TBE buffer were used. For the 37 kDa HA, a 4% agarose gel in TBE was used. For the 22 kDa HA, a 4–20% polyacrylamide gel in TBE was used. The gel composition choice was driven by the need to ensure that all of the HA in a given sample migrated according to its molecular mass, such that the linear portion of the calibration profile for the gel applied. If a gel had been chosen, for which some of the HA were too large to be adequately separated, a peak or shoulder in the densitometric profile would be observed at the high molecular mass side (similar to the void volume peak in a gel filtration experiment). Conversely, if some of the HA were too small to be adequately sieved by the gel, a peak or shoulder on the low molecular mass side of the profile would be seen (similar to the total volume peak in gel filtration). Our choices of appropriate gel compositions for the four test samples do not show such artifacts.

In order to test the validity of the molecular mass analysis of HA by these electrophoretic methods, the weight-average and number-average molecular masses were calculated for each sample from the densitometric profiles (Table 1). (The method for this calculation is provided as supplementary material in the accompanying paper of Cowman et al.) The weight-average molecular mass should be close to the viscosity-average molecular mass value provided by the manufacturer. The results indicate good agreement, when the proper gel composition is used for each sample. Thus for the 112 kDa HA sample, 2–3% agarose gels gave reasonable values for weight-average molecular mass (104–115 kDa), although the profile of the sample on a 2% gel showed some evidence of compaction on the low mass side, due to incomplete separation. Gels with tighter matrices (4% agarose or 4–20% polyacrylamide) did not properly separate the high mass components, and underestimated the average mass. For the 59 kDa HA sample, 3–4% agarose gels were best (weight-average molecular mass of 59–63 kDa), while a 2% gel showed poor separation of the low mass components, and the polyacrylamide gel matrix was unable to properly separate the high mass components of this sample. For the 37 kDa HA sample, 3–4% agarose and the 4–20% gradient polyacrylamide gel gave equivalent results (weight-average molecular mass of 28–30 kDa). For the smallest HA sample of 22 kDa, only the polyacrylamide gel matrix was able to separate the sample, and allow determination of its weight-average molecular mass (20 kDa).

Application of gel electrophoresis to electrophoretic mobility shift assays

The routine use of electrophoresis to analyze the molecular mass of HA requires that the HA be free of bound proteins that could affect the HA mobility. This phenomenon suggests an alternate use of electrophoresis to study HA-protein complexes. The availability of chemoenzymatically-synthesized HA samples with low degree of polydispersity makes it particularly easy to monitor electrophoretic mobility shifts due to protein binding. As an example of this application, we investigated the ability of a recombinant HA binding protein (rHuHABP: G1-IGD-G2 domains of human aggrecan) to bind HA and shift its electrophoretic mobility. Using HA of 162 kDa, the aggrecan rHuHABP caused a substantial shift of the HA at molar ratios of 5:1 and 10:1 rHuHABP:HA (Fig 8A). At the 10:1 ratio, the 162 kDa HA migrated equivalently to free HA with a molecular weight of approximately 400–500 kDa. There was no evidence of cooperativity in the binding of the aggrecan rHuHABP to HA, which would have resulted in the coexistence of free HA and

HA heavily complexed with protein, in agreement with the findings of Morgelin et al. [71, 72]. In contrast, the G1 domain of versican is reported to bind small HA fragments in a cooperative manner [67, 68]. Proteinase K digestion of the HA-HABP complex returned the HA mobility to its original position. We further investigated the possibility that boiling could dissociate and precipitate HABP from HA, using 70 kDa HA. Boiling of the HA-HABP complex successfully returned the HA mobility to its original position in a 4–20% polyacrylamide gel (Fig 8B), indicating that this might be an effective way to disrupt the HA-HABP complex interaction and allow a proper size estimate of the HA in tissue samples. Further studies will be required to establish the broad utility of this approach.

Discussion

The average molecular mass of HA in normal tissues is usually found to be greater than 1000 kDa, but lower molecular mass HA is believed to occur in certain pathological conditions. Lower molecular mass HA is proposed to serve as a danger signal, via its size-dependent binding to cell surface proteins such as CD44, TLR2, and TLR4. In early reports of the signaling capability of lower molecular mass HA, the size of the HA was estimated by 0.5% agarose gel electrophoresis, and was reported to be less than 200 kDa. That size estimate was at the limit of applicability of the electrophoretic method. Since that time, a number of observations of lower molecular mass HA have been made, using agarose gels of varied concentration. The applicability of the particular electrophoretic conditions to the determination of HA molecular mass had not been firmly established. In the present report, we provide a detailed analysis of the conditions under which HA of different sizes can be accurately determined.

HA with a molecular weight entirely above 200 kDa should be analyzed on 0.5% agarose in TAE buffer, using the method previously described. The most suitable electrophoretic system for the analysis of HA with molecular mass falling predominantly within the range of approximately 80–1500 kDa is 1.0–2.0% agarose in TAE or (preferably) TBE buffer. Agarose gels of 3.0–4.0% concentration in TBE buffer are well suited to the analysis of HA within the range of 10–500 kDa. HA with even lower molecular mass, predominantly in the range of approximately 5–100 kDa, is preferably analyzed on a 4–20% gradient polyacrylamide gel in TBE buffer. Each of these methods has now been validated by analysis of polydisperse HA samples, for which the weight-average (or viscosity-average) molecular weight was independently determined.

As an example application of the new electrophoretic methods, an electrophoretic mobility shift assay (EMSA) of the binding of a recombinant human HA-binding protein to nearly monodisperse 160 kDa HA was performed. This experiment also points to the importance of purifying HA from all binding proteins prior to molecular mass analysis by gel electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Electrophoretic separation of 58–1510 kDa HA standards on agarose as a function of buffer composition and agarose concentration. The gel buffer and running buffer were Tris-acetate-EDTA (A–D) or Tris-borate-EDTA (E–H). The agarose concentration was 0.5% (A, E); 1.0% (B, F); 1.5% (C, G); or 2.0% (D, H). The HA standards were Select-HA™ standards of 58, 68, and 81 kDa, Select-HA™ LoLadder (495, 310, 214, 110, 30 kDa) and Select-HA™ HiLadder (1510, 1090, 966, 572, and 495 kDa). The order of Select-HA™ sample loading in gel lanes was variable.

Figure 2.

Dependence of HA standard electrophoretic mobility on the agarose gel concentration in (A) Tris-acetate-EDTA (TAE) buffer, or (B) Tris-borate-EDTA (TBE) buffer. Mobility was normalized to that of the 30 kDa standard. Calibration curves correspond to (top to bottom) agarose gel concentrations of (○) 0.5%, (◆) 1.0%, (□) 1.5%, (▲) 2.0%, (△) 3.0%, and (●) 4.0%.

Figure 3.

Calibration graph (A) and densitometric profiles (B) of HA standards electrophoretically separated on 3.0% agarose in TBE buffer. The calibration relation between $\log M$ (M in Da) and pixel position was approximately linear ($y = -0.0015x + 5.73$; $R^2 = 0.996$) for HA ranging in size from 495 kDa to 9 kDa.

Figure 4.

Electrophoretic separation of HA standards on a 4–20% gradient polyacrylamide gel in TBE buffer. HA samples were (lane 1) LoLadder™ (495, 310, 214, 110, 30 kDa HA), (lane 2) fractionated HA digest fragments 19–25 disaccharides in length (7.6–10 kDa); (lane 3) polydisperse HA digest fragments with profile peak at 36 disaccharides in length (14.4 kDa); (lanes 4–6) Select-HA™ of 58, 68, and 81 kDa.

Figure 5.

Calibration graph (A) and densitometric profiles (B) of HA standards electrophoretically separated on a 4–20% gradient polyacrylamide gel in TBE buffer. The calibration relation between $\log M$ (M in Da) and pixel position was approximately linear ($y = -0.0012x + 5.28$; $R^2 = 0.995$ for HA ranging in size from 110 kDa to 8 kDa, but larger HA standards (red box on calibration graph) deviated from the calibration equation.

Figure 6.

Electrophoretic separation of polydisperse HA samples and HA standards on A) 3% agarose gel in TBE, or B) 4–20% gradient polyacrylamide gel in TBE. A) HA samples were as follows (left to right): polydisperse HA samples with average molecular weights of 112, 59, and 37 kDa, Select-HA™ standards of 58, 68, and 81 kDa, Select-HA™ LoLadder (495, 310, 214, 110, 30 kDa HA) mixed with 7.6–10 kDa HA fragments. B) HA samples were as follows (left to right): Select-HA™ LoLadder (495, 310, 214, 110, 30 kDa HA), Select-HA™ standards of 81, 68, and 58 kDa; polydisperse HA samples with average molecular weights of 112, 59, 37, and 22 kDa.

Figure 7.

Densitometric profiles of polydisperse HA samples electrophoretically separated on agarose or polyacrylamide gels. The viscosity-average molecular mass for each sample is indicated above the corresponding profile. The gel type used was as follows: 3% agarose for 112 and 59 kDa HA, 4% agarose for 37 kDa HA, 4–20% polyacrylamide for 22 kDa HA. Absorbance data were scaled to the same maximum height in each profile for facile comparison.

Figure 8.

Electrophoretic mobility shift assay (EMSA) for HA binding protein (rHuHABP; rHu aggrecan G1-IGD-G2) interaction with 162 kDa (A) and 70 kDa (B) Select-HA™. A): a 1.5% agarose gel was electrophoresed in TAE buffer. Lane 1, rHuHABP; lane 2, HA; lane 4, rHuHABP:HA = 1:1 mole ratio; lane 5, rHuHABP:HA = 5:1 mole ratio; lane 6, rHuHABP:HA = 10:1 mole ratio; lane 8, Select-HA™ LoLadder standards. B): a 4–20 % polyacrylamide gel was electrophoresed in TBE buffer to test the effect of boiling. Lane 1, rHuHABP:HA = 2.3:1 mole ratio, after boiling; lane 2, rHuHABP : HA = 2.3:1 mole ratio, no boiling; lane 3, rHuHABP; lane 4, HA + Select-HA™ LoLadder standards.

Table 1

Calculated Average Molecular Mass for Polydisperse HA Samples Determined by Gel Electrophoresis

M_v (from supplier)	2% agarose	3% agarose	4% agarose	4–20% polyacrylamide
112 kDa	M _w 115 kDa M _n 51 kDa n = 1	M _w 104 ± 4 kDa M _n 63 ± 1 kDa n = 3	M _w 92 ± 6 kDa M _n 72 ± 7 kDa n = 3	M _w 92 kDa M _n 59 kDa n = 1
59 kDa	M _w 68 kDa M _n 39 kDa n = 1	M _w 63 ± 2 kDa M _n 40 ± 1 kDa n = 3	M _w 59 ± 1 kDa M _n 43 ± 3 kDa n = 3	M _w 56 kDa M _n 23 kDa n = 1
37 kDa		M _w 30 ± 3 kDa M _n 23 ± 1 kDa n = 2	M _w 30 ± 1 kDa M _n 23 ± 1 kDa n = 2	M _w 28 kDa M _n 14 kDa n = 1
22 kDa				M _w 20 kDa M _n 10 kDa n = 1