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Review

Hyaluronic acid: separation and biological implications

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Abstract

Hyaluronic acid (hyaluronan) is a ubiquitous extracellular matrix component, and present at high concentrations in skin, joints and cornea. In the skin, it is synthesized primarily by dermal fibroblasts and by epidermal keratinocytes. Hyaluronic acid usually exists as a high molecular mass (600 000–1 000 000) and non-sulfated glycosaminoglycan composed of a disaccharide unit of –3GlcNAcβ1–4GlcAβ1–. Hyaluronic acid has been widely used not only for osteoarthritis and ophthalmology but also for cosmetics for skin care. To examine the biological activities of hyaluronic acid, we have to accurately determine the quantity and molecular masses in biological samples. We review recent development in the analysis of hyaluronic acid having various molecular sizes using electrophoretic and chromatographic techniques. Recently, interactions between hyaluronic acid oligomers and hyaluronic acid-binding proteins have attracted the interest for understanding the biological functions. We show some interesting reports on biological interactions of hyaluronic acid and its oligomers with some proteins.

Keywords: Reviews; Hyaluronic acid

Contents

1. Introduction

Hyaluronic acid (hyaluronan, HA), a high molecular mass polysaccharide, was discovered by Meyer and Palmer in 1934 in the vitreous humor of cattle eyes [1]. This polysaccharide is most frequently referred as "hyaluronan", because HA exists as polyanion form and not as the free acid form. However, the name "hyaluronic acid" is often used in pharmaceutical area, and we use both words in the present review.

HA is generally of very high molecular mass but can also exist as small fragments or oligosaccharides. HA is a linear, and unbranched polymer, which is composed of a simple repeating disaccharide. The disaccharide consists of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) linked through a β1-4 glycosidic bond. The disaccharides are linked through β1-3 bonds to form the HA chain. Thus, HA is a quite homogeneous polymer, but distributions of the molecular size are of wide range (10^5–10^7). Other glycosaminoglycans such as chondroitin 4- and

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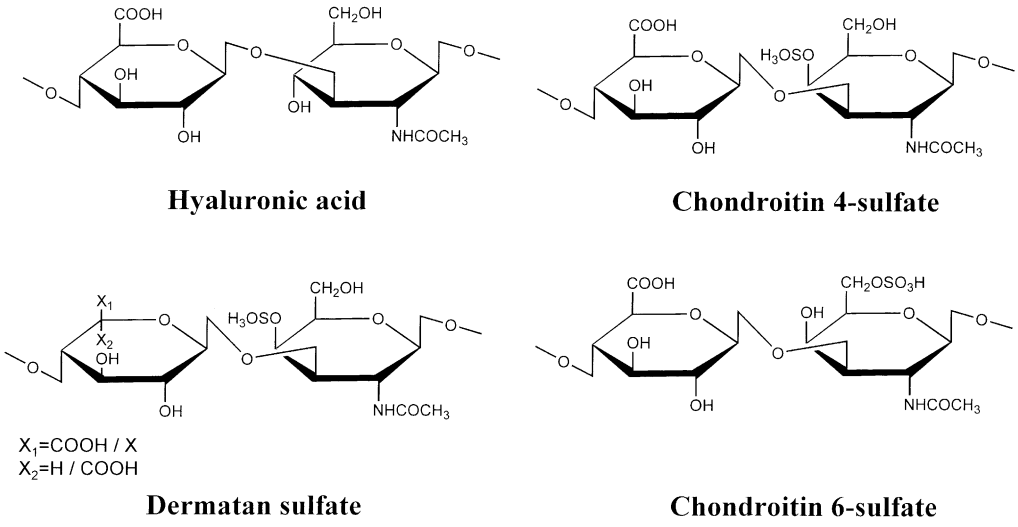

Fig. 1. List of basic structures of glycosaminoglycans.

6-sulfate are also present along with HA. Fig. 1 shows the list of basic structures of a few typical glycosaminoglycans.

Most glycosaminoglycans are synthesized in the Golgi network within the cell and are attached to proteins. In contrast, HA is synthesized at the plasma membrane and is not associated with any core protein [2]. Studies have revealed that HA is synthesized at the inner face of the cell membrane. Outside of the cell, HA can form a complex with hyaluronan-binding proteins including members of the lectican family, such as aggrecan or versican.

Although HA is widely distributed in the intercellular matrix of mammalian connective tissues, it is obtained from bovine vitreous humor, rooster combs, and umbilical cords, and also produced by some bacteria such as *Streptococcus zooepidemicus*. There is an excellent web page (http://www.glycoforum.gr.jp/science/hyaluronan) for the introduction on biology of HA. In a recent review, Vinios et al. [3] reported a review article on the analysis of glycosaminoglycans: assessment of physiological and pathological states of connective tissues. Readers should also refer to the book on a comprehensive and up-to-date collection of preparations and analytical methods for the in-depth analysis of proteoglycans [4].

HA participates in a hydrated network with collagen fibers (e.g. vitreous humor), where it acts as an organizing core in the intercellular matrix, connecting together and spatially distributing the cartilage glycoconjugates to form complex intercellular aggregates [5]. HA is also a constituent of the pericellular coat of unfertilized eggs, and a variety of other cells [6].

Alterations in HA metabolism, distribution and function have been documented in many diseases, e.g. arthritis, immune and inflammatory disorders, pulmonary and vascular diseases, and cancer [7,8]. One of the most successful medical applications of HA is the use of sodium hyaluronate for the treatment of osteoarthritis [9]. Because the biological functions of HA are closely related to the molecular mass, two types of HA having different molecular masses were used to examine the effect on osteoarthritis and traumatic arthritis [10]. Application of HA in ophthalmology is another medical application and widely used clinically as an ophthalmic substance worldwide.

Biological functions of small HA oligomers have also attracted the interest in relation to their molecular masses. West et al. [11] reported that low molecular mass HA showed angiogenesis activity. Xu et al. [12] examined the expression of heat shock protein 72 of synovial cells in the presence of HA oligosaccharides having various sizes, and found that treatment of the cells with HA tetrasaccaride up-regulated expression of heat shock protein 72 and the corresponding mRNA after exposure to hyperthermia resulting suppression of apoptosis of the cells. HA shows a crucial role in dynamic cellular processes such as embryonic development, tissue regeneration, and tumorigenesis [13,14].

In the present review, we describe recent development in the analysis of HA having various molecular sizes using electrophoretic and chromatographic techniques in the first two sections, then introduce some topics on HA analysis in relation to its biological interactions with some proteins.

2. Analysis of HA

2.1. Analysis of HA using electrophoresis methods

Electrophoresis on cellulose acetate membrane has been a common method for qualitative and semiquantitative analysis of glycosaminoglycan mixtures. The advantage of this method is its simplicity, and the ability to process several samples at the same time. Because of the simplicity and the low cost of analysis, this method is still the first choice for the analysis of clinical samples [15,16]. Electrophoresis us-

ing nitrocellulose membrane was also reported, and applied to the analysis of glycosaminoglycans extracted and purified from bovine aorta and bovine lung [17]. An electrophoretic method using an agarose gel electrophoresis was reported for the determination of molecular mass distribution of HA [18]. HA was separated by electrophoresis on a 0.5% agarose gel, followed by detection of HA with a cationic dye (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine). The method was applied to analyze HA in normal human knee joint synovial fluid and owl monkey vitreous HA. The results showed that both samples showed narrow molecular mass distributions of $6–7\times10^6$ and $5–6\times10^6$, respectively. The method was further developed for the visualization of nonradiolabeled glycosaminoglycans resolved by agarose gel electrophoresis using staining with toluidine blue followed by Stains-All procedure. This method, which could detect as little as 10 ng of a single species, was used to stain a few micrograms of a complex polysaccharide mixture, and applied to the analysis of the complex glycosaminoglycans (i.e. heparin, heparan sulfate, chondroitin/dermatan sulfate) and nonsulfated polyanions (i.e. HA) [19].

HA preparations having different molecular masses were quantitatively determined by capillary electrophoresis in a bare fused-silica capillary using a slightly acidic buffer (pH 4.0). Calibration curves showed good linearity with similar slopes from 0.01 to 3.3 mg/ml for HA samples having different molecular mass distributions. The lower limit of detection was ≈1 μg/ml [20]. The same report describes that HA samples having different molecular masses are observed at different migration times in a buffer containing pullulan as sieving matrix. The method is useful for determination of the amounts and molecular mass of HA in pharmaceutical preparations.

A simple method using capillary electrophoresis was proposed for the determination of molecular mass of chemically modified HA [21]. Chemical modification of HA such as sulfonation and acetylation affords novel biological activities such as anti-coagulant effect [22,23]. However, HA often accompanies considerable degradation during chemical modifications to yield products having low-molecular masses. HA sulfate esters were synthesized according to the method reported by Nagasawa et al. [24]. Longer reaction time produced smaller sulfated HA products (HAPS). The results are shown in Fig. 2.

The small oligomers of sulfate ester were well separated at least into 20 disaccharide units, but the separation of larger species became worse. In the first step for determination of their molecular masses, the standard curve was constructed from the relationship between electrophoretic mobilities of each HAPS oligomer and the molecular mass. Then, the mobility of an unknown sample was extrapolated to the standard curve, and the molecular mass of each HAPS product could be calculated. The results are shown in Table 1.

The results were compared with those obtained by size exclusion chromatography using Sephadex G-100 as sieving material. The method using conventional size-exclusion chromatography requires at least several hours. The sample amount required for separation was more than 10 mg. Furthermore, standard samples having known molecular mass distributions such as dextran should be previously separated to prepare a standard curve to determine the relationship between molecular masses and elution volume. However, the analysis by capillary electrophoresis was completed at least within 1 h. The sample amount required for CE analysis was less than 100 μg/ml of aqueous solution. HAPS preparation having high-molecular masses showed large difference between both methods. But low-molecular mass products showed similar values. It should be noticed that standard

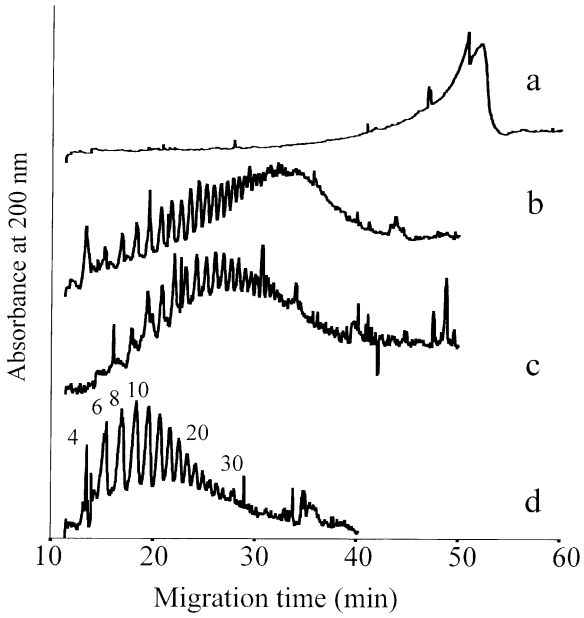

Fig. 2. CE profiles of HAPS preparations. The samples for (a)–(d) were prepared by changing the reaction times in sulfation for 1 h (HAPS1), 6 h (HAPS6), 24 h (HAPS24), and 48 h (HAPS48), respectively. Analytical conditions: capillary, fused silica capillary coated with dimethypolysiloxane (57 cm, 100 μm I.D., effective length of 50 cm); buffer, 50 m*M* Tris-borate (pH 8.5) containing 10% polyethyleneglycol having molecular mass of 70 000; applied voltage, 15 kV; detection, UV absorption at 200 nm; The sample solutions was introduced for 10 s by pressure method. Reproduced from Ref. [21] under permission by the publisher.

Table 1
Average molecular masses of HAPS preparations determined by capillary electrophoresis and gel filtration chromatography

HAPS preparations	Present method (capillary electrophoresis)		Gel filtration chromatograph
	Mobility	M_w	
HAPS1	9.87×10^{-5}	18 680	40 000
HAPS6	9.53×10^{-5}	11 480	12 000
HAPS24	8.68×10^{-5}	8650	9000
HAPS48	5.78×10^{-5}	3880	4500

HAPS1, HAPS6, HAPS24, and HAPS48 were prepared by changing reaction time, for 1, 6, 24, and 48 h, respectively. Gel filtration chromatography was performed using Sephadex G-100 as sieving material. Reproduced from Ref. [21] under permission from the publisher.

samples having defined molecular masses are necessary for size exclusion chromatography. However, the extrapolation method of mobilities using capillary electrophoresis does not require standard samples of the known molecular masses and allows direct determination of the molecular masses of polymer samples.

Karamanos and Hjerpe [4,25] presented protocols for the analysis of unsaturated disaccharides after digestion of GAGs with specific lyases. Derivatization of the disaccharides derived from hyaluronan with 2-aminoacridone (AMAC) and detection by laser-induced fluorescence achieved at least 100 times higher sensitivity than that obtained by UV detection at 232 nm of underivatized oligosaccharides [4,25].

Fluorophore-assisted carbohydrate electrophoresis (FACE), originally developed for the analysis of glycoprotein-derived carbohydrates [26,27], was applied to the determination of disaccharide composition of hyaluronan and chondroitin/dermatan sulfate [28,29]. The digestion products with hyaluronidase/chondroitinase were labeled with AMAC. The method was applied to the analysis of total hyaluronan and chondroitin sulfate composition from cartilage [30]. The FACE protocols allowed the analysis of hyaluronan and chondroitin/dermatan sulfate of as little as 25 μg of wet weight or 1–2 μg of dry weight of cartilage tissue. Such sensitivity made it possible to analyze even topographical profiles of glycosaminoglycan compositions in tissues. FACE analyses of the fine structure of chondroitin sulfate chains in purified human aggrecan and in rat chondrosarcoma tissue were compared with the previously published results to validate the FACE technique.

Fig. 3 shows FACE analysis of AMAC-derivatized products from partial digestion of a constant amount of hyaluronan for a constant time with serially diluted testicular hyaluronidase [29]. This enzyme is a hydrolase having endo-hexosaminidase specificity, and gives a ladder of oligomers that differ by one disaccharide unit. The partial digests at the lower enzyme concentrations (lanes 4 and 5) reveal a ladder of oligosaccharides consisting from more than 50 disaccharides.

As the enzyme concentration increases, the primary end products, tetra- and hexa-saccharides (HA_4 and HA_6) increase. Interestingly, they no longer migrate on the basis of molecular size and show inversion in mobility, with HA_6 overlapping HA_8 and HA_4 migrating at the position of HA_{14}.

Another microanalysis of HA oligomers using polyacrylamide gel electrophoresis was also reported. Using a mini-slab gel, HA oligomers of five to more than 50 repeating disaccharide units were separated into discrete ladder-like bands in a short electrophoresis time of 45 min. Using a combined Alcian blue and silver staining protocol, the detection limit was less than 1 ng per band for 11 repeating disaccharide units, showing 50 times higher sensitivity than that reported previously [31].

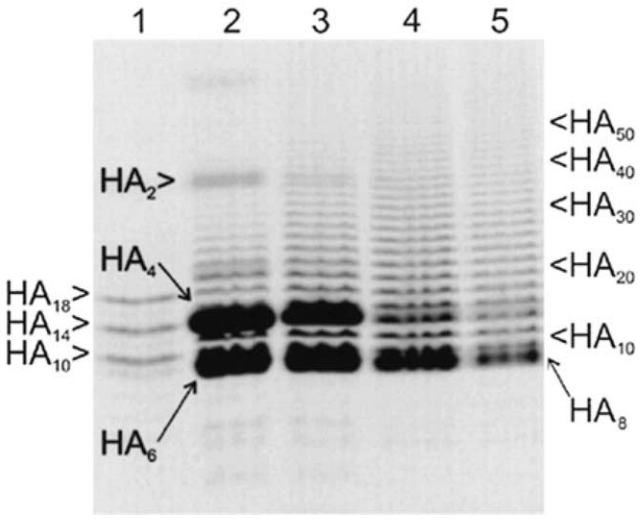

Fig. 3. FACE analyses of AMAC-derivatized products from partial digestion of a constant amount of hyaluronan (100 μg) for 4 h at 37 °C with 1:3 serial dilutions of testicular hyaluronidase starting at 1000 U/ml (lanes 2–5). The relative positions of the saturated hyaluronan oligomers containing 1 (HA_2), 2 (HA_4), 3 (HA_6), 4 (HA_8), 5 (HA_{10}), 10 (HA_{20}), 15 (HA_{30}), 20 (HA_{40}), and 25 (HA_{50}) disaccharides are indicated. Lane 1 contains a standard mixture of three purified, AMAC-derivatized hyaluronan oligomers (HA_{10}, HA_{14}, and HA_{18}) used to index the ladder. Reproduced from Ref. [29] under permission by the publisher.

2.2. Analysis of HA using chromatography techniques

Glycosaminoglycans, including HA and chondroitin/dermatan sulfates are converted into unsaturated disaccharides through the action of chondroitin sulfate lyases (Fig. 4).

These unsaturated disaccharides are analyzed by HPLC with pre- and postcolumn derivatization. All the unsaturated non-sulfated, mono- and di-sulfated disaccharides, derived from chondroitin sulfate, dermatan sulfate, and HA, were successfully resolved and detected using HPLC by fluorometric detection after derivatization with dansylhydrazine [32]. This method was found superior to others, because unsaturated disaccharides could be separated with good resolution in about 50 min using an isocratic mode with higher sensitivity (≈50 pmol, 20–30 ng). Calibration curve showed good linearity from 50 to 500 pmol. The method for the determination of HA and chondroitin/dermatan sulfates in the tissue sections on a glass slide, which were prepared by histological technique, was established, and applied to the analysis of glycosaminoglycans in porcine skin [33]. Using this technique, effects of HA on the proliferation and chondroitin sulfate synthesis of chondrocytes embedded in collagen gels were examined [34]. Sensitivity using CE or HPLC in detection of the unsaturated disaccharides was compared using effusions from human malignant mesothelioma [35]. Much lower detection limit (attomole level) by the CE method allowed the analysis of HA content in serum.

A variety of methods have been described for assessing the molecular mass distribution of HA in synovial fluid and other fluids, using viscometry [36], high-performance

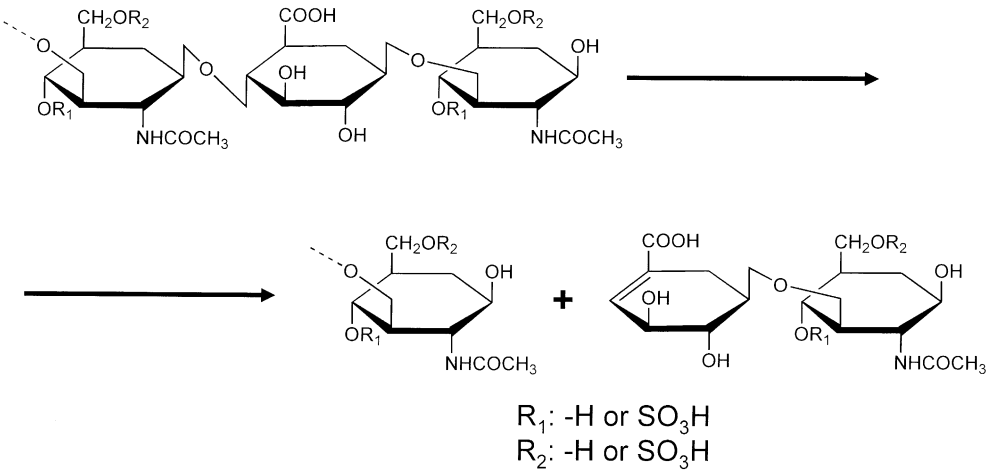

Fig. 4. The action of chondroitin sulfate lyase on glycosaminoglycans.

gel permeation chromatography (GPC) [37,38], capillary electrophoresis [20], gel electrophoresis [18] and low-angle laser-light scattering [39]. Centrifugal precipitation chromatography has been applied to the separation of HA molecular species. All HA samples were fractionated into two distinct fractions regardless of their different molecular masses of 20 000–130 000. Although the separated products could not be characterized because no suitable analytical method was available for identification, centrifugal precipitation chromatography will be an alternative for separation of HA preparations [40].

A method for estimation of molecular size of HA using size exclusion chromatography requires a range of standards of known molecular mass to generate a relationship between retention volume and log of weight average molecular mass (log(M_w)). However, this is not a viable option for molecular mass determination of HA preparations, since HA standards having well-defined molecular masses are not available. Calibration using other polysaccharide standards of similar structures seems questionable due to the unique solution conformation of HA [36]. Characterization of the molecular parameters of HA samples from different origins and in ovine synovial fluid was accomplished using a multi-angle laser light scattering (MALLS) detector coupled to a gel permeation chromatography (GPC) system [41]. The weight average molecular mass (M_w) and number average molecular mass (M_n) values obtained for six of the seven preparations were in good agreement with the reported values. The method was applied to determine the molecular characteristics of the endogenous HA in normal ovine synovial fluids. The effects of freezing and thawing synovial fluid upon molecular mass of HA were also investigated. Freezing and thawing of ovine synovial fluid affect the molecular parameters (M_n, M_w, M_z) of HA and caused decrease of molecular mass of HA as shown in Table 2.

As mentioned in the electrophoresis section, fully *O*-sulfated glycosaminoglycans exhibit interesting biological activities such as anticoagulant and anti-proliferative actions [42,43]. Molecular size of these chemically modified HA products was examined by high-performance size-exclusion chromatography with conductivity detection [44]. Analysis was performed on a TSK gel G3000SWXL HPSEC column with borate buffer as eluent. Conductivity detection of *O*-sulfated HA allowed a highly sensitive detection in the picogram range.

As indicated above, a number of papers reported that fragments of HA have different properties compared to the intact molecule. Therefore, it is important to use well-characterized and highly purified oligosaccharides in cell biological and structural studies so that erroneous results are avoided. HA oligomers of defined size were purified using a combination of size exclusion and anion-exchange chromatography after digestion of HA with testicular hyaluronidase. After

Table 2
Effect of freeze–thawing on synovial fluid hyaluronan molecular parameters

Fluid[a]	$M_n \times 10^6$ Dalton	% Change	$M_w \times 10^6$ Dalton	% Change	$M_z \times 10^6$ Dalton	% Change
Fresh, diluted	1.00	–	1.33	–	1.43	–
Diluted, freeze, thaw	1.00	–	1.19	−8.46	1.30	−9.09
Freeze, thaw, diluted	0.85	−19.8	1.00	−23.1	1.07	−25.2

[a] The sample was collected from synovial fluid from right joint of sheep. M_n = number average molecular mass. M_w = weight average molecular mass. M_z = *Z*-average molecular mass. Reproduced and modified from Ref. [41].

separation of HA oligomers, the fractions were further purified using a DEAE-5PW column. HA oligomers ranging from tetrasaccharides to 34-mers were successfully separated [45]. Large scale preparation of HA oligosaccharides from 4-mer to 52-mer were also reported by other group [46]. HA oligomers prepared after partial digestion with bovine testicular hyaluronidase were purified by anion-exchange chromatography on a Dowex 1×2 column. Gram or mg scale of size-uniformed HA oligosaccharides were obtained from 200 g of rooster comb HA. The purity and size of each HA oligosaccharides were confirmed by HPLC, FACE, and ESI–MS.

3. Interaction of HA and HA oligomers with hyaluronic acid-binding proteins in biological systems

There are a huge number of papers on the biological interactions between HA and CD44, a hyaluronan-binding protein. CD44, a distinct class of carbohydrate-binding molecule, is expressed on a variety of cell types, including leukocytes, fibroblast cells, endothelial cells, and epithelial cells. Its best characterized ligand is probably HA [47], whereas other types of ligands have also been described [48]. However, recent development in separation sciences has allowed resolution of HA polymers having up to 200 monosaccharides [49]. To understand the functions of HA in biological interactions, it is essential to determine the minimum number of disaccharide units forming a unit of the three-dimensional structure required for expression of biological function. There are a number of papers reporting contribution of HA oligomers to express biological functions through interaction with other biological molecules such as CD44, heat shock protein and hyaluronidase [50–52].

Recently, we developed a method for the screening of post-translational modification of proteins with carbohydrates using capillary electrophoresis [53]. The method allows classifying a complex mixture of carbohydrate chains.

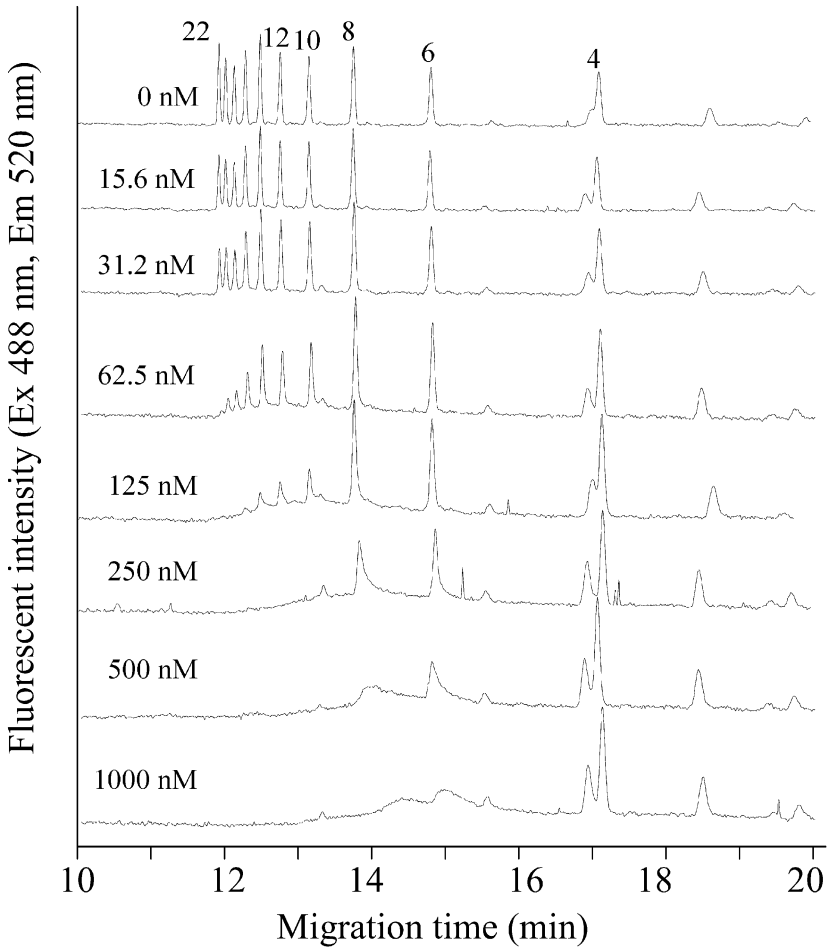

Fig. 5. Affinity capillary electrophoresis of interaction between AMAC-labeled hyaluronic acid oligomers and hyaluronic acid-binding protein. Analytical conditions: capillary, fused-silica capillary coated with dimethypolysiloxane (60 cm, 50 μm I.D., effective length of 50 cm); buffer, 100 m*M* Tris-acetate (pH 7.5) containing 0.1 m*M* $CaCl_2$, 0.5% polyethyleneglycol having molecular mass of 70 000; applied voltage, 25 kV. Fluorescence detection was performed with a 520 nm light filter for emission by irradiating with an argon laser-induced 488 nm light. The sample solutions was introduced for 10 s (1 p.s.i.). Kinoshita et al., unpublished results.

In the initial step, the mixture of fluorescently labeled carbohydrates is analyzed by capillary electrophoresis using a buffer in the absence of a carbohydrate-binding protein. When a component carbohydrate in the mixture shows affinity to the carbohydrate-binding protein, mobility of the carbohydrate changes with the concentrations of the the carbohydrate-binding protein. Kinetic data such as association constant of each carbohydrate can also be obtained by measuring mobilities at different concentrations of the carbohydrate-binding protein. Using this technique, we examined the binding between HA oligosaccharides and hyaluronan-binding protein (HABP) obtained from bovine nasal septum cartilage [54]. The results are shown in Fig. 5.

All the AMAC derivatives of HA oligomers up to 22 mer were well resolved and detected with high sensitivity at 10^{-6} *M* level in the absence of HABP. Addition of HABP into the running buffer caused decrease of peak intensities of HA oligomers. Large oligomers more clearly decreased their peak intensities than the smaller ones. At 250 n*M* concentration of HABP, oligomers other than tetramer, hexamer and octamer were not observed in the electropherogram. Finally, peaks of hexamer and octamer were disappeared at 1000 n*M* of HABP.

Inhibitory actions of chemically modified HA oligomers on blood coagulation and hyaluronidase activity were examined. The results are summarized in Table 3. HA was partially depolymerized on a large-scale quantity using bacterial hyaluronidase. The hyaluro-oligosaccharide mixture was purified to the size-unified oligosaccharide ranging from 4- to 20-mer. The purified HA oligomers converted to fully O-sulfated HA oligomers under mild conditions using pyridine-sulfurtrioxide in *N*,*N*-dimethylformamide [55].

The activity increases according to the size of *O*-sulfated HA oligomers for larger oligomers than HA 10-mer. The small *O*-sulfated HA 4–8 mers did not show significant inhibition on hyaluronidase. These results were well correlated with the unusual electrophoretic behavior in the analysis of free HA oligomers [49]. During the studies on high-resolution separation of HA using capillary

Table 3
Inhibition effect of O-sulfated HA oligomers on blood coagulation and hyaluronidase

HA oligomer	Inhibition effect	
	Blood coagulation (U/mg)	Hyaluronidase IC_{50} (μg/ml)
4	0.5>	20>
6	0.5>	20>
8	0.5>	20>
10	0.91	10.7
12	1.39	7.41
14	1.82	8.59
16	2.20	4.02
18	3.08	3.35
20	2.52	3.54

The data are from Ref. [55] under permission from the publisher.

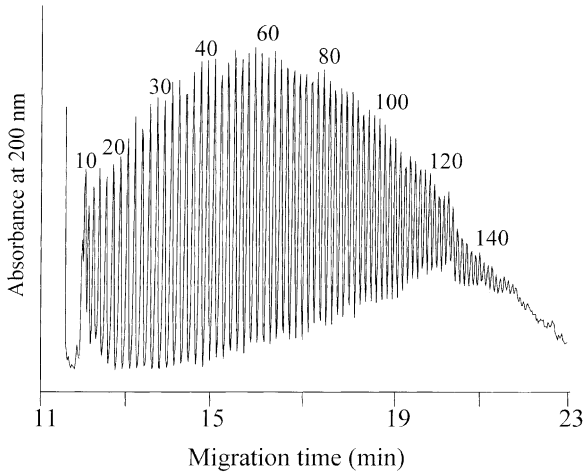

Fig. 6. Capillary electrophoresis of hyaluronic acid oligomers in a buffer containing neutral polymer. Analytical conditions: capillary, fused-silica capillary coated with dimethypolysiloxane (27 cm, 100 μm I.D., effective length of 20 cm); buffer, 50 m*M* Tris-borate (pH 8.5) containing 10% polyethyleneglycol having molecular mass of 70 000; applied voltage, 8 kV; detection, UV absorption at 200 nm; The sample solutions was introduced for 10 s by pressure method. Reproduced from Ref. [49] under permission by the publisher.

electrophoresis, we found that small oligomers of HA showed migration order reversal [49], and these unusual behaviors are in good correlation with biological functions of HA oligomers as described above. For optimization of separation of HA oligomers, several types of capillaries were examined, and capillaries with their inner surfaces chemically modified with (50% phenyl)methylpolysiloxane and dimethylpolysiloxane showed good separations of HA oligomers. A typical example of separation of HA oligomers is shown in Fig. 6.

Presence of a neutral polymer in the running buffer enhanced the resolution of molecular species. We found that the running buffer containing polyethyleneglycol (PEG70000) could resolve HA oligomers as shown in Fig. 6. Polymers composed of more than 80 disaccharide units exhibited excellent resolution. Oligomers larger than the dodecamer migrated in the order of their molecular masses, and polymers (molecular masses: ∼20 000) having at least 50 disaccharide units, were clearly distinguished. However, oligomers smaller than the octamer migrated in the reverse order of their molecular masses, and the octamer (i.e. $(GlcA\beta1\text{-}3GlcNAc\beta1\text{-}4)_4$) was observed at the earliest migration times [56].

Relationship between electrophoretic mobility and gel concentrations are shown in Fig. 7a. The slope increases in proportion with molecular sizes.

The slope is called retardation coefficient (K_r) according to the following equation [57]:

$$\log(\mu) = \log(\mu_0) - K_r(T) \quad (1)$$

where μ is mobility in polymer solution, μ_0 mobility in free solution (i.e. zero concentration of sieving matrix), K_r

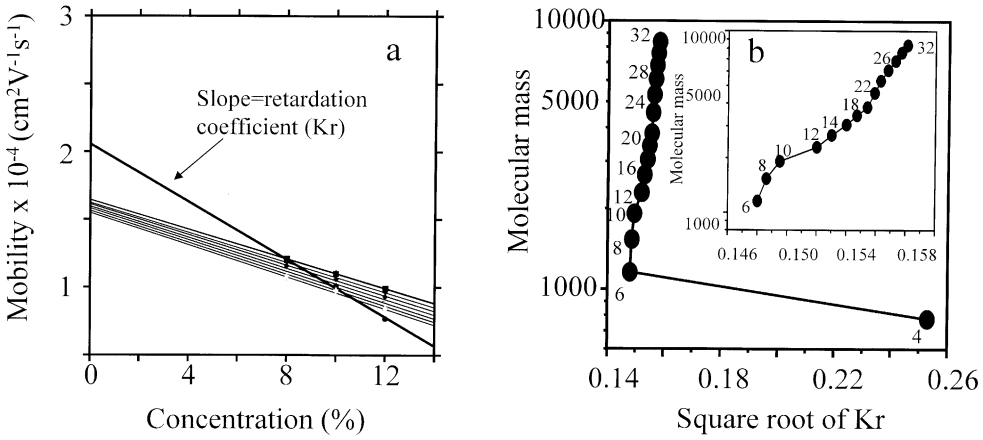

Fig. 7. Relationship between electrophoretic mobility and gel concentration in the electophoresis of hyaluronic acid oligomers. (a) Relationship between the mobility of HA oligomers and the gel concentration. (b) Relationship between the square root of K_r values and the molecular mass of HA oligomers. Reproduced from Ref. [56] under permission by the publisher.

retardation coefficient, and T concentration of sieving matrix. K_r is also expressed in the following equation:

$$K_r = \pi\tau(r + R)^2 \times 10^{-16} \quad (2)$$

where τ is matrix fiber length (cm/g), r fiber radius, and R molecule's radius (nm). Relationship between square root of K_r values and molecular masses of HA oligomer is shown in Fig. 7b. Although these equations can be applied to spherical molecules such as proteins, Fig. 7b means that the tetrasaccharide behaves as an extraordinary large molecule.

Although HA is present as a high-molecular-weight polymer, numerous studies have established that only short oligosaccharides are necessary for the recognition and binding by hyaluadherins [58]. At least a decasaccharide unit is essential for the binding with aggrecan core and link protein, while a hexasaccharide unit is large enough to bind with the cell surface HA receptor [59–61]. In the recent article [12] (as indicated in section 1 of the present review), effect of hyaluronan oligosaccharides was examined on the expression of heat shock protein 72. Treatment of K562 cells with HA tetrasaccharides up-regulated expression of heat shock protein 72 after exposure to hyperthermia, and the results indicated that a certain size of oligosaccharides, i.e. the tetrasaccharides of HA, up-regulates expression of heat shock protein 72 and suppresses cell death.

4. Conclusion

In the present manuscript, we review analyses of hyaluronic acid/hyaluronan by electrophoretic and chromatographic methods. Analysis of HA of high-molecular mass (i.e. native hyaluronan) is still a challenging target. On the contrary, recent development in high-resolution separation technology with high-sensitive detection method have improved the resolution of HA oligomers, and HA polymers composed of a few hundred disaccharide units can be resolved.

Recent development in separation of HA molecular species has revealed novel biological roles of HA. Especially, various interesting biological interactions of HA oligomers with hyaluronan-binding proteins have been reported. Such studies will be a useful tool for understanding of native hyaluronan molecules.

Macromolecular species of HA are considered to form complex matrix by collaboration with collage and other cell-matrix materials. To investigate the conjugated state of HA and its metabolic alterations in physiological state, we have to establish a new research strategy allowing analysis of macromolecular HA molecular species in high-sensitivity and high-resolution.

5. Nomenclature

AMAC	2-aminoacridone
ESI-MS	electrospray-ionization mass spectrometry
FACE	fluorophore-assisted carbohydrate electrophoresis
GAG	glycosaminoglycan
GlcA	glucuronic acid
GlcNAc	*N*-acetylyglucosamine
HA	hyaluronan/hyaluronic acid
HABP	hyaluronan-binding protein
HAPS	polysulfate of hyaluronic acid
K_r	retardation coefficient
PEG	polyethylene glycol

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