

Defined megadalton hyaluronan polymer standards

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Abstract

The utility of polymer standards for the calibration of average molecular mass estimates often is limited by the polydispersity—the breadth of the size distribution—of the standard. Here monodisperse synthetic hyaluronan (or hyaluronic acid [HA]) complexes in the approximately 1- to 8-megadalton (MDa) range were prepared in two steps. First, synchronized stoichiometrically controlled in vitro reactions yielded linear narrow size distribution biotinylated HA chains. Second, streptavidin protein was added at substoichiometric levels to prepare a series of complexes with one, two, three, or four HA chains per streptavidin molecule. The dendritic-like molecules approximate the mobility of natural linear HA chains on agarose gels, making the complexes useful as defined size standards for high-molecular weight HA preparations.

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Hyaluronan (or hyaluronic acid [HA])², a polysaccharide composed of β 4-glucuronic acid- β 3-*N*-acetylglucosamine disaccharide repeats, is essential for mammals [1–3] and is employed as a virulence factor in certain pathogenic bacteria to infect their animal hosts [4]. The determination of the size of HA molecules is important for basic researchers because some behaviors of vertebrate cells, including proliferation, mobility, and apoptosis, appear to depend on the chain length [5–9]. In addition, HA-based therapeutic products and medical devices need to be scrutinized rigorously in the quality control process [10,11].

Based on the growing HA biology literature and the number of emerging therapeutic applications, the need for HA size analyses will continue to grow. Traditionally, HA standards to calibrate molecular weight determinations were prepared by gel filtration chromatographic fractionation of natural HA extracts (and/or their fragmented derivatives) followed by light scattering, gel electrophoresis, and/or viscometric measurements to assess the size distribution [12,13]. These reagents were useful but suffer from rather wide size distributions (polydispersity), resulting in smeared bands on electrophoretic gels and broad peaks on gel filtration profiles.

HA synthases are the glycosyltransferases responsible for catalyzing the polymerization of HA polymer chains in vertebrates and certain pathogenic microbes in vivo [14,15]. The monosaccharide units from UDP-GlcNAc and UDP-GlcA are transferred sequentially in an alternating fashion to produce the disaccharide repeats of the heteropolysaccharide. Recombinant derivatives of one HA synthase, PmHAS from the gram-negative bacterium *Pasteurella multocida* type A [16], have proved to be very use-

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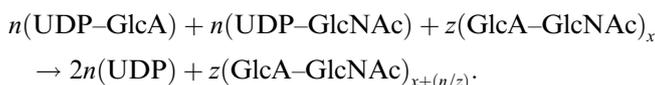
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² *Abbreviations used:* HA, hyaluronan (or hyaluronic acid); GlcA, glucuronic acid; PmHAS, *Pasteurella multocida* HA synthase; MDa, megadaltons; BuOH, *n*-butanol; AcOH, acetic acid; EtOH, ethanol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SEC-MALLS, size exclusion chromatography multiangle laser light scattering; M_w , weight average molecular mass; M_n , number average molecular mass; NHS, *N*-hydroxysuccinimide.

ful for chemoenzymatic syntheses of both oligosaccharides [17] and polysaccharides [18,19].

In 2004, the PmHAS was employed in synchronized, stoichiometrically controlled polymerization reactions *in vitro* to produce monodisperse HA polysaccharide preparations [18]. Reaction synchronization is achieved by providing the HA synthase with an oligosaccharide acceptor to bypass the slow polymer initiation step *in vitro*. All HA chains are elongated in parallel and thus reach the same length, yielding a narrow size distribution population. The synthase will add all available UDP-sugar precursors to the nonreducing termini of acceptors in a nonprocessive fashion as in the following equation:



Therefore, size control is also possible. For example, if there are many termini (i.e., z is large), then a limited amount of UDP-sugars will be distributed among many molecules and thus result in many short polymer chain extensions. Conversely, if there are few termini (i.e., z is small), then the limited amount of UDP-sugars will be distributed among few molecules and thus result in long polymer chain extensions.

The observed upper size limit of synthetic monodisperse HA chain production by the above method, however, was approximately 2.5 megadaltons (MDa) before the size dis-

tribution started to broaden. We speculate that the greater viscosity of the reaction mixture and/or the increased frequency of unsynchronized *de novo* synthesis under high UDP-sugar concentration conditions were the most likely roots of this empirical upper limit.

In both the basic science and industrial quality control laboratories, a need for HA standards covering the approximately 2- to 8-MDa range is apparent. Here we present an approach to prepare multivalent HA complexes employing biotin/avidin technology. The new dendritic-like molecules approximate linear HA chains based on electrophoretic migration, and thus the complexes are useful as standards for gels.

Materials and methods

All materials were the highest grade available from either Fisher or Sigma unless otherwise noted. The sugar contents of the various HA derivatives in Fig. 1 were measured by the carbazole assay with a GlcA standard [20].

Biotin-HA polymer preparation

Amino-HA₄ was prepared by reductive amination of HA₄, the HA tetrasaccharide derived from extensive testicular hyaluronidase digestion of streptococcal HA [17], with ammonium ion. The dry HA₄ sugar was dissolved in anhydrous methanol (0.71 mg/ml [w/v] or 0.93 mM final) under

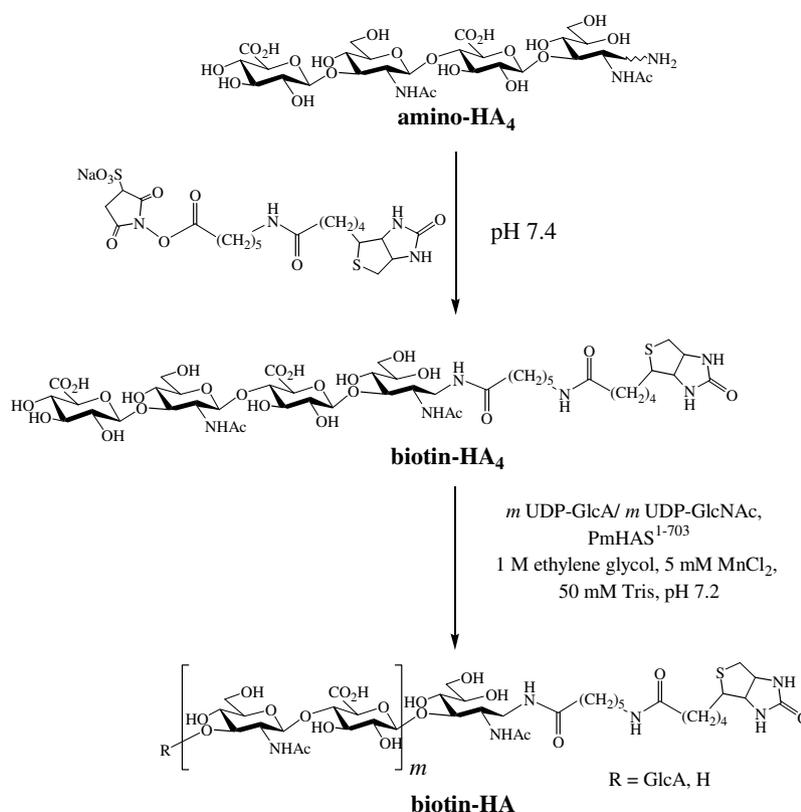


Fig. 1. Schema of monodisperse biotin-HA synthesis. Reductive amination created a HA oligosaccharide, amino-HA₄, that was derivatized with a biotin group. The biotin-HA₄ serves as the acceptor for synchronized, stoichiometrically controlled synthesis of monodisperse biotin-HA polymers.

sonication. After the addition of solid ammonium acetate and NaBH₃CN (final 1 M and 0.1 M, respectively), the mixture was heated to reflux (~70–80 °C). Typically after overnight reaction, thin-layer chromatographic analysis (silica, BuOH/AcOH/H₂O, 1:1:1, v/v/v, with detection by naphthoresorcinol reagent) [17] showed complete consumption of the starting material. The reaction was quenched by the slow addition of 20% AcOH (note: carry out in fume hood due to toxic gases). The solvent was evaporated in vacuo and the residue was dissolved in 0.2 M ammonium formate for desalting by gel filtration chromatography on a P-2 resin column (Bio-Rad, Hercules, CA, USA) in the same volatile buffer. The fractions containing the target molecule were pooled and lyophilized. The volatile salts were removed by two more cycles of dissolving in water and lyophilization. Flash silica gel column chromatography (silica gel 60, Merck, BuOH/AcOH/H₂O, 1:1:1, v/v/v) was employed for further purification. The structure of the amino-HA₄ product was confirmed by matrix-assisted laser desorption time-of-flight mass spectrometry analysis (calculated 777.68, found 777.26) [17].

Amino-HA₄ was reacted with a 10-fold molar excess of the 22.4-Å spacer version of an activated biotin ester, sulfo-*N*-hydroxysuccinimide-LC-biotin (Pierce, Rockford, IL, USA), in 60% dimethyl sulfoxide (DMSO), 0.1 M potassium phosphate, and 0.15 M NaCl (pH 7.4) for 2 h at room temperature (Fig. 1). The biotin-HA₄ in the reaction mixture was purified by gel filtration chromatography on P-2 resin as above, followed by flash silica gel column chromatography (*n*-BuOH/EtOH/H₂O, 3:5:2, v/v/v) and a second gel filtration step to remove any residual organics. Mass spectrometry confirmed the identity of the biotin-HA₄ reagent (calculated 1117.13, found 1116.51).

In the synchronized reactions, various ratios of biotin-HA₄/UDP-sugars were polymerized with PmHAS¹⁻⁷⁰³ enzyme, a pure soluble truncation product of PmHAS, in 1 M ethylene glycol, 5 mM MnCl₂, and 50 mM Tris (pH 7.2) for 90 h at 30 °C [18]. The reactions were deproteinized by repeated chloroform extraction. The biotin-HA polymer was recovered by precipitation with three volumes of EtOH.

Avidin/Biotin-HA complexes

The complexes were assembled by adding approximately 0.5 molar equivalents of recombinant streptavidin (Pierce) or hen egg avidin protein with biotin-HA polymers in phosphate-buffered saline (PBS) at room temperature overnight. Both proteins possess four biotin-binding sites, and the use of substoichiometric amounts with respect to the tagged HA polymer allows the formation of all four of the potential complex species (Fig. 2).

Size analysis

The size of HA and its derivatives was analyzed on agarose gels (0.7–1.2%, 1× TAE buffer, 20–40 V) stained with

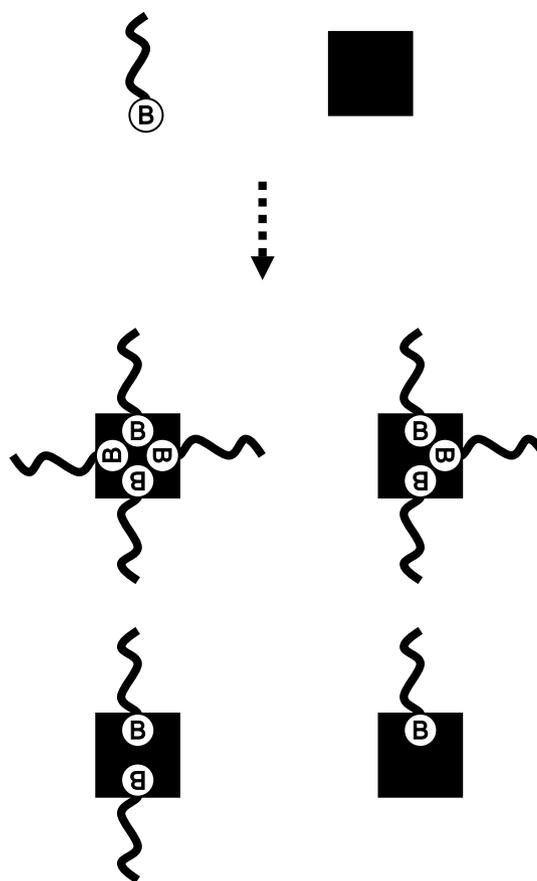


Fig. 2. Schematic model of streptavidin/biotin-HA complexes. The use of substoichiometric amounts of streptavidin (black box) with monodisperse HA (black lines) containing a single biotin tag (white circle) results in the formation of a series of complexes with defined incremental addition of HA chains.

Stains-All dye (0.005% [w/v] in EtOH) [13]. Approximately 0.3–1.0 µg of HA was loaded per lane. Defined DNA (HyperLadder, Boline, Boston, MA, USA) and HA (Select-HA Hi-Ladder, Hyalose LLC, Oklahoma City, OK, USA) were also run as standards. Duplicate gels of all samples were run for measurement of the migration distances.

Size exclusion chromatography multiangle laser light scattering (SEC-MALLS) analysis was employed to determine the absolute molecular masses of the various HA products [18]. Polymers (2.5–12.0 µg mass, 50 µl injection) were separated on PL Aquagel-OH 30 (8 µm), -OH 40, -OH 50, and -OH 60 (15 µm) columns (7.5 × 300 mm, Polymer Laboratories, Amherst, MA, USA) in tandem. The columns were eluted with 50 mM sodium phosphate and 150 mM NaCl (pH 7.0) at 0.5 ml/min. MALLS analysis of the eluant was performed by a DAWN DSP Laser Photometer in series with an OptiLab DSP Interferometric Refractometer (632.8 nm, Wyatt Technology, Santa Barbara, CA, USA). The ASTRA software package was used to determine the absolute average molecular mass using a *dn/dc* coefficient of 0.153 for HA as determined by Wyatt Technology. Polydispersity was calculated as weight aver-

age molecular mass/number average molecular mass (M_w/M_n). Duplicate runs of all molecules were performed, and the values were averaged.

Computational modeling of polymers

Molecular models of HA polymers were constructed from internal coordinates for 4C_1 sugar ring puckering with the glycosidic dihedral angles (O5-C1-Ox-Cx and C1-Ox-Cx-C[x-1]) set to $(-68.1^\circ, 128.0^\circ)$ and $(-71.1^\circ, 112.2^\circ)$ at the $\beta(1,3)$ and $\beta(1,4)$ linkages, respectively. Before converting to Cartesian coordinates, the dihedrals at every linkage were randomly offset using angles extracted from a Gaussian distribution with a standard deviation of 18° and truncated at 1.7 standard deviations according to previously derived solution conformation and dynamics [21]. In the case of the biotin–HA/streptavidin complexes, the individual chains were started from the relevant number of biotin-binding sites in a streptavidin homotetramer model derived from X-ray crystallography. Models where distant parts of the same chain or different chains were found to be closer than 1 nm were rejected (to avoid chain overlap and simulate excluded volume effects). The process was repeated until 1000 random conformations that did not violate the

excluded volume criteria for models of total molecular mass of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 MDa, and each of the stoichiometric complexes, had been produced. The average radii of gyration (R_g) were calculated for each of the models (Supplementary Tables 1–4). All software was written in-house using the C programming language. Plots of $\log R_g$ against $\log M$ were constructed and yielded straight lines that were parallel to one another (Supplementary Fig. 1). This finding indicates that there is a simple scaling factor between the R_g values for different stoichiometric complexes at particular molecular weights, which was inferred directly from the plots.

Results and discussion

The PmHAS enzyme elongates acceptor molecules at the nonreducing terminus, thereby allowing the reducing terminus to be modified prior to the enzymatic extension [22]. A biotin group was added to an HA tetrasaccharide derivative with a free amino group (Fig. 1). We observed that a significant spacer between the biotin group and the saccharide was required for efficient elongation with PmHAS catalyst; we did not see efficient extension with NHS–biotin reagents without a spacer (data not shown).

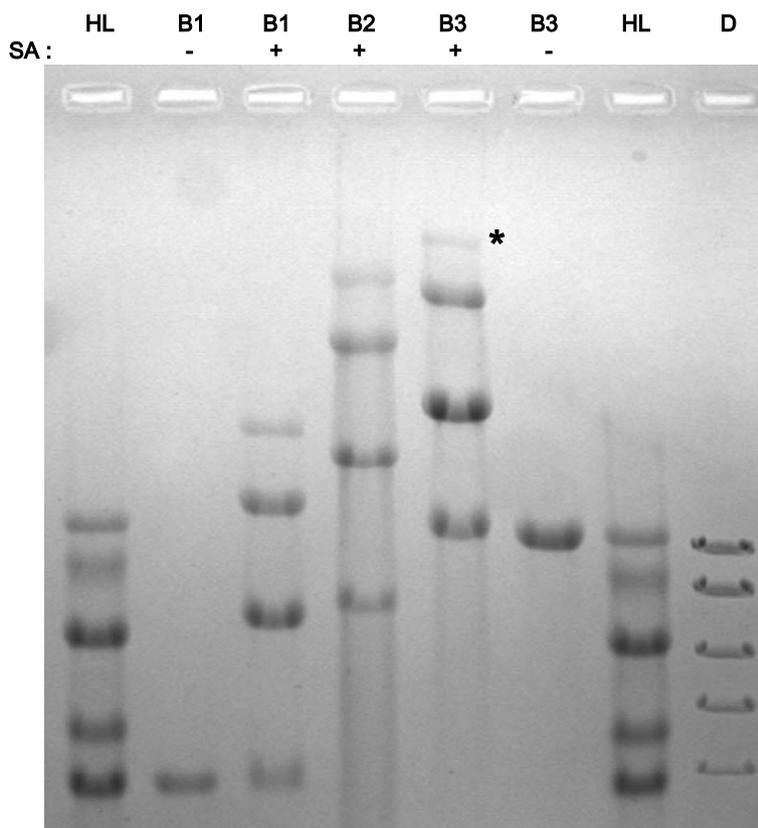


Fig. 3. Gel analysis of biotin–HA/streptavidin complexes. Several biotin–HA polymer preparations (B1, B2, and B3 with average M_w values of 525, 1080, and 1520 kDa, respectively, ~ 0.3 $\mu\text{g}/\text{lane}$) and their streptavidin (SA, ~ 0.7 – 1.0 $\mu\text{g}/\text{lane}$) complexes were analyzed by agarose gel (0.8%) electrophoresis. As standards, the Select-HA Hi-Ladder (HL: 1510, 1090, 970, 570, and 495 kDa from top to bottom) and the DNA HyperLadder (D: 10, 8, 6, 5, 4, and 3 kb from top to bottom) are shown. A series of higher molecular weight forms corresponding to complexes with one, two, three, or four HA chains are observed. An asterisk (*) marks the 6-MDa band here, but 8- and 6-MDa species have also been made using a 2-MDa biotin–HA polymer (not shown).

The biotin–HA chains in the range of approximately 0.5–2.0 MDa were very monodisperse based on their migration on gels as tight bands as in Fig. 3. The SEC–MALLS profiles yielded polydispersity values ranging from 1.02 to 1.06; for reference, the value for an ideal polymer is 1.

Monodisperse biotinylated HA chains were then mixed with substoichiometric amounts of either avidin or streptavidin. Species corresponding to a polypeptide with one, two, three, or four HA polymer chains (schematically represented in Fig. 2) were observed by gel electrophoresis (Fig. 3). The latter bacterial protein gave the sharpest bands (the egg protein is intrinsically more heterogeneous due to glycosylation) and thus was employed throughout. In the most commonly employed gel system for HA analyses of Lee and Cowman [13], recombinant streptavidin, a 53-kDa globular protein, slowed the mobility of the biotin–HA chains only slightly.

The aqueous conformation of the large linear HA molecules found in nature is thought to approximate a flexible expanded coil that conforms to a very large spherical domain [2,23]. This expanded conformation is due in part to mutual charge repulsion of the numerous negative charges of the GlcA residues and the substantial hydration of the hydrophilic HA chains. In the streptavidin/biotin–HA complex, all of the HA chains are tethered via the biotin moiety at their reducing termini to the protein residing at the core, but otherwise they should approximate the native HA chain overall.

Experimental validation of this hypothesis is evident in the linearity of plots of the migration distance on gels versus the log of the average molecular mass (an example is shown in Fig. 4) for both the authentic HA polymers and the various species in any particular biotin–HA/streptavidin complex series (i.e., the monomer, dimer, trimer, and tetramer forms from a single biotin–HA preparation).

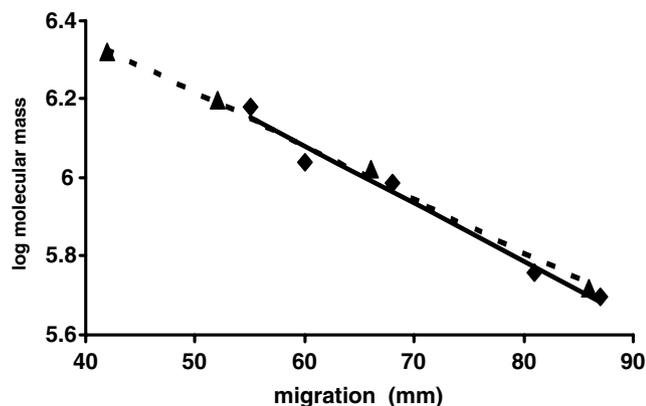


Fig. 4. Relationship of migration versus molecular mass of linear HA and streptavidin/biotin–HA complexes. In this example, the 525-kDa biotin–HA preparation was incubated with streptavidin and then analyzed on 0.8% agarose gel (as in Fig. 3). The mobility of the monodisperse linear HA polymers (\blacklozenge , solid line, Select-HA Hi-Ladder) and the complexes (\blacktriangle , dashed line) were compared. The two trend lines are parallel and correlate well (0.980 and 0.998, respectively), suggesting that linear HA and the dendritic-like complexes have similar aqueous conformations.

There are no linear monodisperse HA standards available for comparison with the very large 3- to 8-MDa complexes, but the lines formed by the 0.5- to 1.5-MDa linear standards extrapolate very well to the higher molecular weight data. Overall, the correlation was very good for all of the complexes in a given series as measured by the R values (range 0.987–0.992, $n = 6$).

However, if one compares complexes with identical total HA masses in different conformations (e.g., a 500-kDa dimer and a 250-kDa tetramer that total to 1 MDa) from different series of complexes (i.e., derived from separate independent HA–biotin preparations), then their migration distances might not be identical depending on the agarose gel percentage employed. The theoretical basis for this non-equivalent behavior may be explained by the predicted differences in geometries of the HA complexes altering their migration mode.

It has been shown that DNA, another linear anionic polymer molecule, exhibits two distinct migration modes during travel through porous gels [24]. For a given pore size, a shorter polymer behaves like a sphere (i.e., the hydrodynamic shape of a rod rotating in three-dimensional space) diffusing through the gel pores in a fashion termed “Rouse-like behavior,” whereas a longer polymer acts like an elongated rod winding through the pores in a snake-like fashion termed “reptational behavior.” For linear HA complexes (i.e., monomer and dimer), the molecules have the potential to execute both modes of locomotion depending on the relative ratio of the polymer length to the pore size. However, the nonlinear streptavidin-tethered HA complexes (i.e., trimer and tetramer) are not able to reptate as deftly through the pores, and thus their migration speed is somewhat hindered relative to that of linear molecules.

Our molecular simulations predict that the nonlinear trimeric and tetrameric complexes are slightly more compact [i.e., their radii of gyration are reduced by approximately 11 and 21%, respectively (Supplementary Fig. 1)] compared with the linear monomeric and dimeric complexes because the central streptavidin molecule constrains the flexible HA molecules and packs more chains into a smaller volume. The branched tetramers also have shorter chains than the monomeric complexes for the same total HA mass and have a more compact globular shape, resulting in an intrinsic reduced tendency to reptate.

Therefore, the opposing effects of molecular compaction and reduced reptational behavior counterbalance to some degree in any given series of HA complexes under our agarose gel electrophoresis conditions. However, the exact magnitude of each phenomenon is expected to vary depending on the electrophoretic parameters, including gel porosity and voltage. Therefore, the biotin–HA complexes may serve as interesting objects for future biophysical studies, but their potential limitations and performance in various electrophoretic systems must also be considered.

The streptavidin/biotin–HA complexes are stable for at least 7 days at 37 °C or for 1 month at 4 °C. The complexes are not disrupted even after approximately 10 freeze–thaw

steps and may be stored in a lyophilized state as well. If desired, HA ladders with streptavidin containing fluorescent, radioactive, or enzyme tags should be possible provided that the tag or catalyst does not contribute too great of a molecular weight or a charge addition to the basic structure.

The understanding of HA biology and the delivery of consistent HA-based therapeutics both truly require that the polymer size be measured. The defined megadalton HA standards now allow the use of commonly available gel apparatuses (e.g., used for DNA separations) to assess the size distributions of various very high-molecular weight HA samples.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.06.009.

References

- [1] J.R. Fraser, T.C. Laurent, U.B. Laurent, Hyaluronan: its nature, distribution, functions, and turnover, *J. Intern. Med.* 242 (1997) 27–33.
- [2] T.C. Laurent, J.R. Fraser, Hyaluronan, *FASEB J.* 6 (1992) 2397–2404.
- [3] J.A. McDonald, T.D. Camenisch, Hyaluronan: genetic insights into the complex biology of a simple polysaccharide, *Glycoconj. J.* 19 (2003) 331–339.
- [4] P.L. DeAngelis, Microbial glycosaminoglycan glycosyltransferases, *Glycobiology* 12 (2002) 9R–16R.
- [5] A.J. Day, G.D. Prestwich, Hyaluronan-binding proteins: tying up the giant, *J. Biol. Chem.* 277 (2002) 4585–4588.
- [6] S. Ghatak, S. Misra, B.P. Toole, Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway, *J. Biol. Chem.* 277 (2002) 38013–38020.
- [7] P.W. Noble, Hyaluronan and its catabolic products in tissue injury and repair, *Matrix Biol.* 21 (2002) 25–29.
- [8] R. Stern, Devising a pathway for hyaluronan catabolism: are we there yet? *Glycobiology* 13 (2003) 105R–115R.
- [9] D.C. West, S. Kumar, The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity, *Exp. Cell Res.* 183 (1989) 179–196.
- [10] D. Uebelhart, J.M. Williams, Effects of hyaluronic acid on cartilage degradation, *Curr. Opin. Rheumatol.* 11 (1989) 427–435.
- [11] K.L. Goa, P. Benfield, Hyaluronic acid: a review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing, *Drugs* 47 (1994) 536–566.
- [12] R.E. Turner, M.K. Cowman, Cationic dye binding by hyaluronate fragments: dependence on hyaluronate chain length, *Arch. Biochem. Biophys.* 237 (1985) 253–260.
- [13] H.G. Lee, M.K. Cowman, An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution, *Anal. Biochem.* 219 (1994) 278–287.
- [14] P.L. DeAngelis, Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses, *Cell. Mol. Life Sci.* 56 (1999) 670–682.
- [15] P.H. Weigel, V.C. Hascall, M. Tammi, Hyaluronan synthases, *J. Biol. Chem.* 272 (1997) 13997–14000.
- [16] P.L. DeAngelis, W. Jing, R.R. Drake, A.M. Achyuthan, Identification and molecular cloning of a unique hyaluronan synthase from *Pasteurella multocida*, *J. Biol. Chem.* 273 (1998) 8454–8458.
- [17] P.L. DeAngelis, L.C. Oatman, D.F. Gay, Rapid chemoenzymatic synthesis of monodisperse hyaluronan oligosaccharides with immobilized enzyme reactors, *J. Biol. Chem.* 278 (2003) 35199–35203.
- [18] W. Jing, P.L. DeAngelis, Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers, *J. Biol. Chem.* 279 (2004) 42345–42349.
- [19] K.J. Williams, K.M. Halkes, J.P. Kamerling, P.L. DeAngelis, Critical elements of oligosaccharide acceptor substrates for the *Pasteurella multocida* hyaluronan synthase, *J. Biol. Chem.* 281 (2006) 5391–5397.
- [20] T. Bitter, H.M. Muir, A modified uronic acid carbazole reaction, *Anal. Biochem.* 4 (1962) 330–334.
- [21] A. Almond, P.L. DeAngelis, C.D. Blundell, Hyaluronan: the local solution conformation determined by NMR and computer modeling is close to a contracted left-handed 4-fold helix, *J. Mol. Biol.* 358 (2006) 1256–1269.
- [22] P.L. DeAngelis, Molecular directionality of polysaccharide polymerization by the *Pasteurella multocida* hyaluronan synthase, *J. Biol. Chem.* 274 (1999) 26557–26562.
- [23] A.J. Day, J.K. Sheehan, Hyaluronan: polysaccharide chaos to protein organisation, *Curr. Opin. Struct. Biol.* 11 (2001) 617–622.
- [24] A. Pluen, P.A. Netti, R.K. Jain, D.A. Berk, Diffusion of macromolecules in agarose gels: comparison of linear and globular configurations, *Biophys. J.* 77 (1999) 542–552.