

Synchronized Chemoenzymatic Synthesis of Monodisperse Hyaluronan Polymers*

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Wei Jing‡ and Paul L. DeAngelis§¶

From the ‡Hyalose LLC, Oklahoma City, Oklahoma 73104 and the §Department of Biochemistry and Molecular Biology, Oklahoma State University Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

The length of the hyaluronan (HA) polysaccharide chain dictates its biological effects in many cellular and tissue systems. Long and short HA polymers often appear to have antagonistic or inverse effects. However, no source of very defined, uniform HA polymers with sizes greater than 10 kDa is currently available. We present a method to produce synthetic HA with very narrow size distributions in the range of ~16 kDa to ~2 MDa. The *Pasteurella* HA synthase enzyme, pmHAS, catalyzes the synthesis of HA polymer utilizing monosaccharides from UDP-sugar precursors. Recombinant pmHAS will also elongate exogenously supplied HA oligosaccharide acceptors *in vitro* in a nonprocessive fashion. As a result of bypassing the slow initiation step *in vitro*, the elongation process is synchronized in the presence of acceptor; thus all of polymer products are very similar in length. In contrast, without the use of an acceptor, the final polymer size range is difficult to predict and the products are more polydisperse. HA polymers of a desired size are constructed by controlling the reaction stoichiometry (*i.e.* molar ratio of precursors and acceptor molecules). The use of modified acceptors allows the synthesis of HA polymers containing tags (*e.g.* fluorescent, radioactive). In this scheme, each molecule has a single foreign moiety at the reducing terminus. Alternatively, the use of radioactive UDP-sugar precursors allows the synthesis of uniformly labeled native HA polymers. Overall, synthetic HA reagents with monodisperse size distributions and defined structures should assist in the elucidation of the numerous roles of HA in health and disease.

Hyaluronan (HA)¹ is a polysaccharide chain composed of repeating β 4GlcUA- β 3GlcNAc disaccharide units with molecular masses generally ranging from $\sim 10^4$ to 10^7 Da in vertebrates and bacteria (1–5). In animals, HA plays structural, recognition, and signaling roles. Certain pathogenic bacteria, namely *Streptococcus* Groups A and C and *Pasteurella multo-*

cida Type A, utilize extracellular HA polysaccharide capsules to avoid host defenses and to increase virulence.

The biological functions and biomedical applications of HA have long been of interest. It is now recognized that the HA of different sizes can have dramatically different effects on cellular behavior and growth (6–10). Vertebrates may be able to control HA size *in vivo* by differential expression of biosynthetic enzymes (11, 12). Currently, the major method to generate HA is extraction from either rooster (chicken) comb or bacterial cultures. These HA preparations not only contain potential components from the original sources that could cause immunogenic, inflammatory, or allergenic responses but are also mixtures of a wide range of molecular masses. To interpret accurately the various biological functions of HA and to synthesize better HA-containing biomedical products, it is necessary to obtain uniform size-defined HA.

HA synthases are the enzymes from vertebrates and microbes that polymerize the HA chain using UDP-sugar nucleotide precursors. The Class II HA synthase, pmHAS from Gram-negative *P. multocida* Type A, uses two separate glycosyltransferase sites to add GlcNAc and GlcUA monosaccharides to the nascent HA chain (13–15). A useful property of pmHAS is that it can extend exogenously provided HA acceptor oligosaccharides *in vitro* (16). This polymerization reaction occurs in a nonprocessive fashion where the enzyme binds, elongates, and releases the growing HA chain in a rapid repetitive fashion (13, 17). In this report, we utilize the acceptor elongation activity for the chemoenzymatic synthesis of a variety of HA polymers with narrow size distributions.

EXPERIMENTAL PROCEDURES

Acceptor Preparation—All of the reagents were the highest grade available from either Sigma or Fisher unless otherwise noted. The tetrasaccharide HA4, the starting acceptor for the synthesis of longer polymers, was generated by exhaustive degradation of streptococcal HA polymer with ovine testicular hyaluronidase Type V and purified by extensive chloroform extraction, ultrafiltration, and size exclusion chromatography on P2 (Bio-Rad) resin with 0.2 M ammonium formate buffer. The volatile salts were removed by repeated lyophilization from water. The HA4 molecule was converted into a fluorescent derivative in two steps. First, an amino-HA4 derivative was prepared by reductive amination of HA4 (12 mM) with sodium cyanoborohydride (70 mM) and excess diaminoethane (200 mM) in 0.1 M borate buffer, pH 8.5, 1 mM CuCl₂ at 37 °C for 2 days. The amino-HA4 product was purified on P2 resin. Second, a fluorescent acceptor was prepared by derivatizing amino-HA4 with the *N*-hydroxysuccinimide ester of Oregon Green™ 488 (3-fold molar excess; Molecular Probes, Eugene, OR) in 50% dimethyl sulfoxide, 100 mM Hepes buffer, pH 8.5. The major isomer of fluor-HA4 was purified by preparative normal-phase thin layer chromatography (2:1:1 *n*-butanol/acetic acid/water and silica, Whatman). The identities of HA4, amino-HA4, and fluor-HA4 were verified by virtue of the agreement of their expected and experimental masses (775, 819, and 1213 Da, respectively) as assessed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry in negative mode (17).

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¶ To whom correspondence should be addressed. Tel.: 405-271-2227; Fax: 405-271-3092; E-mail: paul-deangelis@ouhsc.edu.

¹ The abbreviations used are: HA, hyaluronan or hyaluronic acid; pmHAS, *Pasteurella multocida* HA synthase; GlcUA, glucuronic acid; SEC-MALLS, size exclusion chromatography multi-angle laser light scattering; *M_n*, number average molecular mass; *M_w*, weight average molecular mass; MDa, million Daltons.

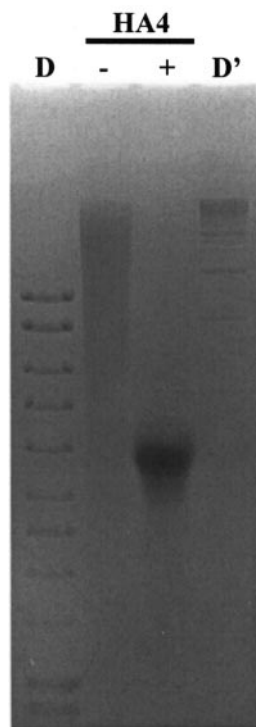


FIG. 1. Gel analysis of *in vitro* HA synthesis products in the presence or absence of HA4 acceptor. A matched set of reactions (100 μ l each) containing 12 μ M pmHAS, 30 mM UDP-GlcNAc, 30 mM UDP-GlcUA, and either 38 μ M HA4 acceptor (+) or no acceptor (-) was incubated for 48 h. A portion (0.2 μ l) of the reactions was analyzed on a 0.7% agarose gel and Stains-All detection. For comparison, DNA standards were run (D, Bioline DNA HyperLadder, top to bottom -10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.8, 0.5 kb; D', Invitrogen high-*M_w* DNA ladder, top band 48.5 kb). A smaller, narrow size distribution HA polymer is formed by pmHAS in the presence of HA4 as seen by the faster migrating tight gel band.

Catalyst Preparation and *in Vitro* Synthesis—The catalyst, pmHAS¹⁻⁷⁰³, is a soluble purified *Escherichia coli* derived recombinant protein (13). The enzyme in the octyl thioglucoside cell extracts was purified by chromatography on Toyopearl Red AF resin (Tosoh) using salt elution (50 mM Hepes, pH 7.2, 1 M ethylene glycol (an enzyme stabilizer) with 0–1.5 M NaCl gradient in 1 h) (17). The fractions containing the target protein ($\geq 90\%$ pure by SDS-PAGE/Coomassie Blue-staining) were concentrated and exchanged into 1 M ethylene glycol, 50 mM Tris, pH 7.2, by ultrafiltration with an Amicon spin unit (Millipore). The syntheses in general contained pmHAS¹⁻⁷⁰³, UDP-GlcNAc, UDP-GlcUA, 5 mM MnCl₂, 1 M ethylene glycol, and 50 mM Tris, pH 7.2. Reactions were incubated at 30 °C for 2–72 h.

Analysis of *in Vitro* Synthesized HA—The size of HA was analyzed on agarose gels (0.7–1.2%; 1 \times TAE buffer (40 mM Tris acetate, 2 mM EDTA); 40 V) stained with Stains-All dye (0.005% w/v in ethanol) (18). Approximately 0.5–5 μ g of HA was loaded per lane. For smaller HA polymers (<40 kDa), HA was also analyzed on polyacrylamide gels (15–20%) with acridine orange staining (19). To purify HA for later analysis, pmHAS was removed by chloroform extraction and the HA product was precipitated with three volumes of ethanol and the pellets were redissolved in water. Alternatively, the unincorporated precursor sugars were removed by ultrafiltration (Microcon units, Millipore). The HA concentration was determined by the carbazole assay using a glucuronic acid standard (20).

Size-exclusion chromatography/multi-angle laser light scattering (SEC-MALLS) analysis was employed to determine the absolute molecular masses of HA products. Polymers (2.5–12 μ g mass; 50- μ l injection) were separated on PL Aquagel-OH 30 (8 μ m), -OH 40, -OH 50, -OH 60 (15 μ m) columns (7.5 \times 300 mm, Polymer Laboratories, Amherst, MA) in tandem or alone as required by the size range of the polymers to be analyzed. The columns were eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7, 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). The ASTRA software package was used to deter-

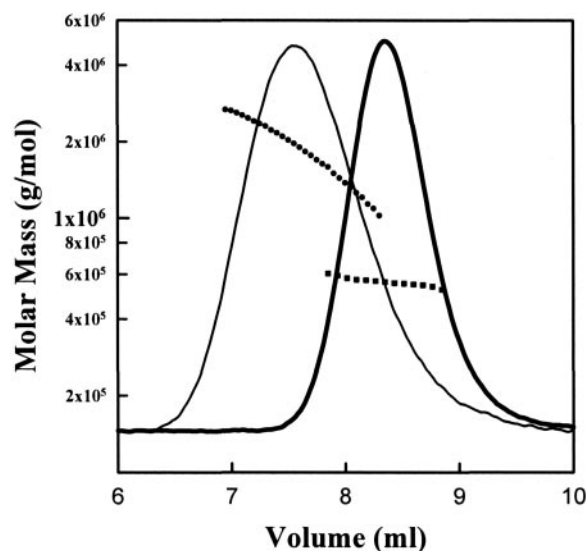
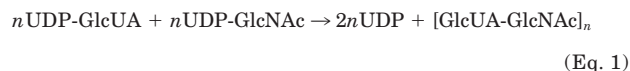


FIG. 2. SEC-MALLS analysis of *in vitro* HA synthesis products in the presence or absence of HA4 acceptor. The refractometer concentration peaks (lines) and the molar mass curves (symbols with corresponding y axis scale) of the matched set of reactions described in Fig. 1 are shown on the same PL Aquagel-OH 60 SEC column profile. A smaller narrow size distribution HA polymer is formed by pmHAS in the presence of HA4 (thick line and squares) as evidenced by its later elution time and flatter molar mass curve in comparison to the reaction without acceptor (thin line and circles).

mine the absolute average molecular mass using a dn/dc coefficient of 0.153 determined by Wyatt Technology. The M_w and polydispersity values are the average of data from at least two SEC-MALLS runs.

RESULTS AND DISCUSSION

Acceptor-mediated Synchronization of Reaction Yields Monodisperse HA Products—Recombinant pmHAS synthesizes HA chains *in vitro* if supplied with both required UDP-sugars (15) according to Equation 1.



However, if a HA-like oligosaccharide ($[\text{GlcUA-GlcNAc}]_x$) is also supplied *in vitro*, the overall incorporation rate was elevated up to ~50–100-fold (16). We suggested that the rate of initiation of a new HA chain *de novo* was slower than the subsequent elongation (*i.e.* repetitive addition of sugars to a nascent HA molecule). The observed stimulation of synthesis by exogenous acceptor appears to operate by bypassing the kinetically slower initiation step, allowing the elongation reaction to predominate as shown in Equation 2.



We performed HA polymerization reactions with purified pmHAS and UDP-sugar precursors under various conditions and analyzed the reaction products by agarose gel electrophoresis and/or size exclusion chromatography with MALLS. We observed that the size distribution of HA products obtained was quite different depending on the presence or the absence of the HA4 acceptor. In summary, the reactions with acceptor produced smaller HA chains with a more narrow size distribution. An example is depicted in Figs. 1 and 2 where the reaction containing HA4 acceptor yielded a HA product with a M_w of 555 kDa and polydispersity (M_w/M_n ; M_n = number average molecular weight) of 1.006, but the parallel reaction without acceptor resulted in a product with a M_w of 1.8 MDa and

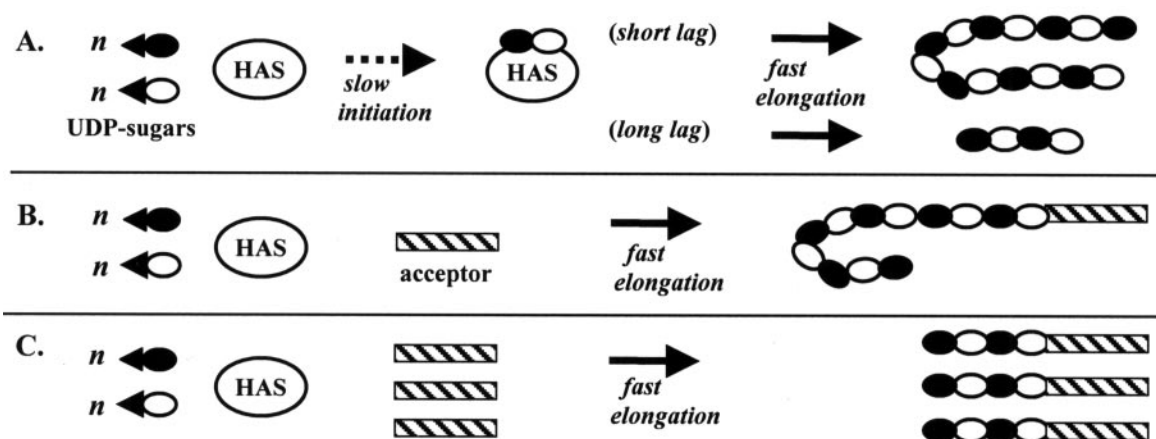
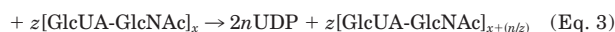


FIG. 3. **Schematic models for acceptor-mediated synchronization and polymer size control.** Panel A depicts the reaction *in vitro* where UDP-sugars (black triangle UDP; small black or white ovals, monosaccharides) are bound to the pmHAS (HAS) and the first glycosidic linkages are formed over a lag period due to this rate-limiting step (*slow initiation*). Once the initial HA chain is started, the subsequent sugars are added rapidly to the nascent polymer (*fast elongation*) by the enzyme. We posit that some chains are initiated before other chains (*short lag versus long lag* period, respectively); thus asynchronous polymerization occurs resulting in a population of HA product molecules with a broad size distribution. Panel B depicts the reaction where the acceptor sugar (striped bar) bypasses the slow initiation step. Thus all of the chains are elongated by the nonprocessive pmHAS in a parallel synchronous fashion resulting in a uniform HA product with a narrow size distribution. Panel C illustrates that if a large amount of acceptor molecules and a finite amount of UDP-sugars are present, the UDP-sugars are distributed among the acceptors to produce shorter polymers than in the case with a smaller quantity of acceptors (resulting in longer chain extensions as shown in Panel 3B). Therefore, it is possible to adjust the molar ratio of acceptor to UDP-sugars to control the ultimate polymer molecular mass.

Mw/Mn of 1.17. For reference, the polydispersity value for an ideal monodisperse polymer equals 1.

To explain the findings above, we further hypothesize that polymerization by pmHAS in the presence of an HA acceptor is a synchronized process. Reactions without the acceptor exhibit a lag period interspersed with numerous out of step initiation events that yield a short HA oligosaccharide (Fig. 3A). Once any HA chain is formed, the polymer is elongated rapidly. Other new HA chains that arise later during the lag period are also elongated rapidly, but the size of these younger chains never catches up to the older chains in a reaction with a finite amount of UDP-sugars. In contrast, in reactions containing an acceptor, all of the HA chains are elongated in parallel in a nonprocessive fashion resulting in a more homogenous final polymer population (Fig. 3B). For practical synthesis where there are more acceptor molecules than catalyst molecules, it is critical that processive elongation (*i.e.* no dissociation of the nascent HA chain and the synthase until polymerization is complete) does not occur because disparity would arise when some acceptor chains are elongated before other chains.

Stoichiometric Control of HA Product Size—The two enzymological properties of recombinant pmHAS described above also allow for the control of HA polymer size in chemoenzymatic syntheses. First, as noted above, the rate-limiting step *in vitro* appears to be chain initiation. Therefore, pmHAS will transfer monosaccharides onto the existing HA acceptor chains before substantial *de novo* synthesis. Second, the enzyme polymerizes HA in a rapid nonprocessive fashion *in vitro* (13, 17). Therefore, the amount of HA4 should affect the final size of the HA product when a finite amount of UDP-sugar is present. The synthase will add all of the available UDP-sugar precursors to the nonreducing termini of acceptors as in the Equation 3.



If there are many termini (*i.e.* z is large), a limited amount of UDP-sugars will be distributed among many molecules and thus result in many short polymer chain extensions (Fig. 3C). Conversely, if there are few termini (*i.e.* z is small), the limited amount of UDP-sugars will be distributed among few molecules

and thus result in long polymer chain extensions (Fig. 3B).

To test our speculation, we performed a series of assays utilizing various levels of HA4 with a fixed amount of UDP-sugar and pmHAS (Fig. 4). With this general strategy, we were able to generate HA from 16 kDa to 2 MDa with polydispersity ranging from 1.001 to ~ 1.2 (Fig. 5). By controlling the molar ratio of acceptor to UDP-sugar, it is now possible to select the final HA polymer size desired. Typically, ~ 50 – 70% of the starting UDP-sugars are consumed in the reactions on the basis of HA polysaccharide recovery.

Interestingly, if an intermediate-sized molecular mass HA chain is prepared by this method, the chain may be elongated by simply adding more UDP-sugars to the reaction mixture provided that the active catalyst is present. The resulting polymers migrate as tight bands on gels and appear quite monodisperse throughout the entire reaction time course even after multiple additions of UDP-sugars. The resulting bands with steadily increasing molecular masses indicated that HA polymers larger than oligosaccharides (~ 20 kDa to 1.3 MDa) may also be utilized as starting material for chain elongation by pmHAS (data not shown).

In Vitro Synthesis of Tagged or Labeled HA—Our technology for the production of monodisperse polymers also allows the use of a modified acceptor to synthesize HA polymers containing various types of foreign moieties. The pmHAS adds monosaccharides to the nonreducing terminus of the acceptor chain (16); thus the aldehyde functionality of the reducing end is available for reaction by numerous chemical schemes. An example is shown using fluorescent HA4 acceptor to produce fluorescent monodisperse HA of various sizes (Fig. 6). Similarly, radioactive (*e.g.* ^3H , ^{125}I) or affinity (*e.g.* biotin) tagged HA polymer are possible with the appropriate modified acceptor (data not shown).

Alternatively, the substitution of all or a portion of the unlabeled UDP-sugars in a chemoenzymatic synthesis reaction with a radioactive precursor (*e.g.* UDP- ^3H GlcUA) is a very useful method to produce labeled HA probes (data not shown). The advantage of this method is that the radioactive HA does not contain any foreign non-sugar moieties that might interfere with biological function or cause mistargeting.

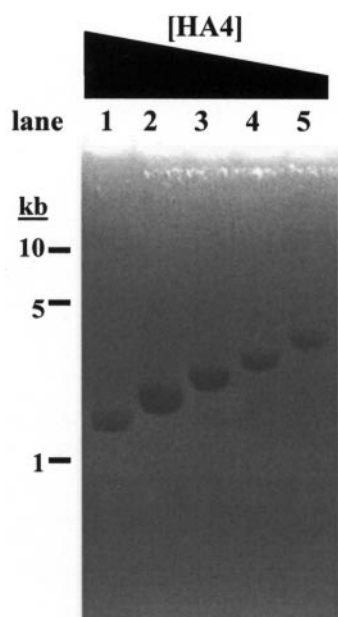


FIG. 4. Control of HA product size by adjusting acceptor/UDP-sugar ratio. Decreasing amounts of acceptor sugar (lanes 1–5, final concentration = 50, 38, 30, 25, or 19 μM HA4) were added to reactions (100 μl , 72 h) containing 8 μM pmHAS, 32 mM UDP-GlcNAc, and 32 mM UDP-GlcUA. Purified synthetic HA (1 μg) was analyzed on a 1.2% agarose gel and Stains-All. The average molecular masses and polydispersity of HA were also determined by SEC-MALLS (M_w and M_w/M_n , respectively, for lane 1, 284 kDa, 1.001; lane 2, 347 kDa, 1.002; lane 3, 424 kDa, 1.004; lane 4, 493 kDa, 1.006; lane 5, 575 kDa, 1.01). The position of certain DNA standards is marked (in kb). The use of higher acceptor/UDP-sugar ratios results in shorter HA chains.

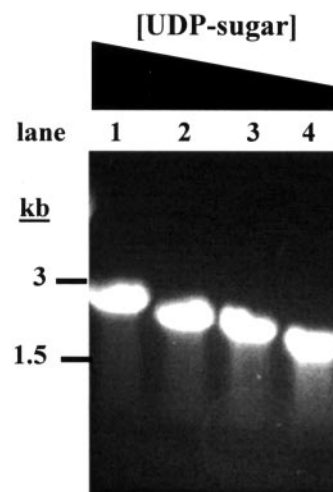


FIG. 6. Synthesis of various monodisperse fluorescent end-labeled HA polymers. A series of parallel reactions (20 μl , 72 h) containing 24 μM pmHAS, 34 μM fluor-HA4, and decreasing amounts of UDP-GlcNAc and UDP-GlcUA (lanes 1–4: final concentration = 32, 25, 20, or 15 mM each) were prepared. Portions of the reactions (1 μl) were analyzed on a 0.7% agarose gel. The signal of the fluorescent tag was detected with long wave UV excitation. The position of certain DNA standards is marked (in kb). The use of higher acceptor/UDP-sugar molar ratios results in shorter HA chains.

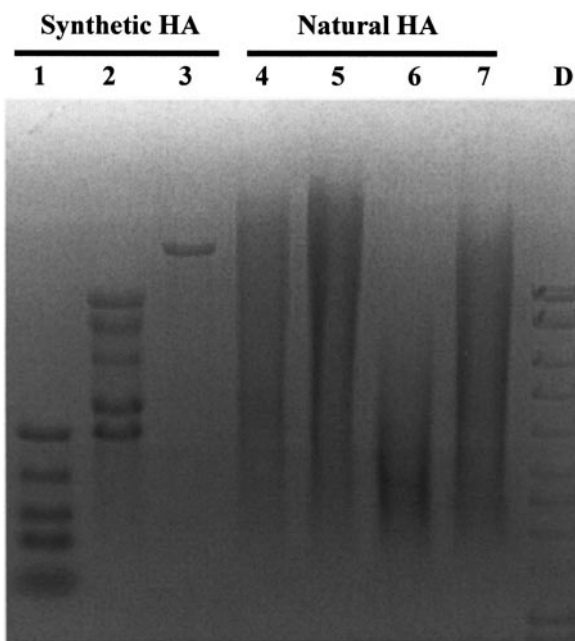


FIG. 5. Comparison of synthetic HA versus natural HA preparations. A variety of HA samples either synthesized by synchronized chemoenzymatic reactions *in vitro* or derived from streptococcal bacteria or chicken sources were analyzed on a 0.7% agarose gel with Stains-All detection. The M_w of each synthetic HA polymer was determined by SEC-MALLS. Lane 1, a mixture of synthetic HA polymers produced in five different reactions (bottom to top, 27, 110, 214, 310 and 495 kDa); lane 2, a mixture of synthetic HA polymers produced in five different reactions (bottom to top, 495, 572, 966, 1090 and 1510 kDa); lane 3, 2.0-MDa synthetic HA; lane 4, rooster comb HA (Sigma); lane 5, streptococcal HA (Sigma); lanes 6 and 7, streptococcal HA (Lifecore); D, DNA HyperLadder. The tight bands of the synthetic HA polymers indicate their relative monodispersity in comparison to extracted HA.

Utility of Synthetic HA—The molecular masses of most commercially available HA preparations are usually in the 10^5 – 10^6 Da range (3). For research requiring smaller HA polymers, degradation via enzymatic (*e.g.* hyaluronidase digestion) or chemical (*e.g.* radicals or oxidation) or physical (*e.g.* ultrasonication) methods are usually employed. However, this process is not always satisfactory because it is time-consuming, the final yield of the targeted HA size is low, and at least one demanding chromatographic step is usually required. We can generate HA as small as ~ 15 kDa with polydispersity (M_w/M_n) around 1.001 with the current synchronized stoichiometrically controlled synthesis technique. If the synthesis of smaller monodisperse HA oligosaccharides (<25 monosaccharide in length or ~ 5 kDa) is required, it is preferable to utilize a pair of reactors with immobilized mutant pmHAS enzymes (a GlcUA transferase and a GlcNAc transferase) operating in an alternating repetitive fashion (17).

High molecular weight HA preparations are commercially available from animal or bacterial sources, but inherent problems including possible contaminants and broad size distributions complicate research. Polydispersities of commercially available HA polymers are commonly higher than 1.5. Indeed, there exists a substantial need for uniform HA in biomedical studies (21). Here we have shown that narrow size distribution, high molecular mass HA (~ 1 – 2 MDa), is also readily prepared by synchronized, stoichiometrically controlled reactions (Fig. 5). Work in progress focuses on extending this product size range upwards.

To determine the exact average molecular mass of HA, MALLS is usually the choice. Yet many researchers need to quickly estimate the molecular mass, but they lack the required instrumentation. The correlation of HA migration on agarose gels with DNA (18) is often used for this purpose. Drawbacks of this method include the following. (i) The original “calibration standard” HA samples were not uniform or monodisperse, and (ii) the migration of HA and DNA on agarose gels changes differentially with alteration of the agarose concentration. A ladder comprised of an assortment of synthetic HA polymers with defined, narrow size distributions (Fig. 5) provides an excellent series of standards for characterizing the size of HA in experimental samples.

In general, our unique technology platform allows the generation of a variety of improved synthetic HA tools with narrow size distributions and defined compositions for elucidating the numerous roles of HA in health and disease. We also expect that similar synchronized, stoichiometrically controlled reactions utilizing the other *Pasteurella* glycosaminoglycan synthases (5) will allow the chemoenzymatic synthesis of monodisperse chondroitin and heparosan polymers in the future.

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