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SEC-MALLS ANALYSIS OF HYALURONAN SIZE DISTRIBUTIONS MADE BY MEMBRANE-BOUND HYALURONAN SYNTHASE

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Abstract

SEC-MALLS analyses of *E. coli* membranes expressing *Streptococcus equisimilis* hyaluronan synthase (seHAS) demonstrated an inherent artifact (10–100 MDa) that co-eluted with HA, and skewed the apparent weight-average mass of HA to erroneously high values. Briefly heating samples to 65–75°C eliminated this artifact and increased the yield of recovered HA, due to the release of HA chains that were attached to membrane-bound HAS. Inclusion of alkaline phosphatase, which removed UDP produced during the reaction, improved the linearity of HA synthesis - even at high substrate utilization. Surprisingly, addition of EDTA, to chelate Mg²⁺ ions, did not completely stop the HAS reaction at 30°C or at 4°C. The best conditions for stopping the reaction without altering SEC-MALLS profiles of the product HA were to chill samples on ice in the presence of both EDTA and UDP. Even with excess substrate, the maximum size of product HA decreased as the enzyme concentration increased. Therefore, the maximum HA size made by HAS was determined by extrapolation to zero enzyme concentration. Using the above conditions, membrane-bound seHAS synthesized a cohort of HA products that steadily increased in weight-average molar mass, reaching a final maximal steady-state size of 4–6 MDa within 2–4 hours.

Keywords

streptococcal; hyaluronan synthase; light scattering; size distribution; membranes; molar mass

Introduction

In order to understand the mechanisms by which HAS¹ enzymes can regulate HA product size, it is necessary to characterize the HA size distributions created by HASs [1] in isolated membranes, intact cells, and tissue samples. Although several groups have reported the average HA masses made by various wildtype or mutant HAS proteins in membranes or cells [2–7], these studies have generally used either gel filtration [8] or gel electrophoresis techniques [9,10] to assess apparent HA size. One of the best current techniques for determining the molecular masses of heterogeneous polymers, such as HA, is size exclusion chromatography coupled to multiangle laser light scattering (SEC-MALLS). The SEC-MALLS technique provides accurate and informative data regarding the size distribution of polydisperse HA samples without the need for calibration standards [11–14]. Although many investigators have used light scattering techniques to study HA since the 1950s [15–21], we found no reports in

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¹Abbreviations: HA, hyaluronic acid, hyaluronate, hyaluronan; HAS, HA synthase; seHAS, *Streptococcus equisimilis* HAS; PBS, phosphate buffered saline; SEC-MALLS, size exclusion chromatography - multiangle laser light scattering.

the literature in which MALLS has been utilized to analyze the size distribution of HA products made by membrane-bound HAS.

The three human HAS isozymes (HAS1, HAS2, and HAS3) make different HA size distributions in crude membranes *in vitro* [2,3], and in live *Xenopus laevis* embryos, HAS1 and HAS2 make different size HA [4]. Based on these reports, it is possible under different physiological conditions *in vivo* that HASs can make HA molecules of different masses that in turn have different size-specific biological functions. These exciting and important possibilities were first recognized when HA oligosaccharides were shown to be angiogenic [22]. Thus, it would be very useful and relevant to use SEC-MALLS to characterize HA size distributions in biologically relevant samples. Here, we report the identification of, and experimental solutions to, several problems and potential artifacts arising from how HA samples made by membranes containing HAS are processed for SEC-MALLS analysis.

Materials and Methods

Materials

Uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc), uridine 5'-diphosphoglucuronic acid [UDP-GlcUA), uridine 5'-diphosphate (UDP) and Stainsall were from Sigma-Aldrich Corporation (St. Louis, MO). Uridine 5'-diphospho-[¹⁴C]glucuronic acid (UDP-[¹⁴C]GlcUA) was from Perkin Elmer Life and Analytical Sciences (Boston, MA). Calf intestinal alkaline phosphatase, molecular biology grade, was from EMD Biosciences, Inc. (La Jolla, CA). Agarose (GenePure LE) was from ISC BioExpress (Kaysville, UT). Commercial HA preparations used as standards were from Genzyme (Boston, MA) or Lifecore (Chaska, MN). Select-HA and Mega-HA standards of well defined size were obtained from Hyalose (Oklahoma City, OK). Size exclusion chromatography used either TSK-GEL G6000PW_{xl} columns from TOSOH Bioscience LLC (Montgomeryville, PA) or PL Aquagel-OH 60 columns from Polymer Laboratories, Inc. (Amherst, MA). The DAWN DSP Laser Photometer and the OptiLab DSP Interferometric Refractometer were from Wyatt Technology Corporation (Santa Barbara, CA). The auto-sampler was a Waters 717plus from Waters Corp. (Milford, MA).

HA Synthesis

Membranes were prepared and assayed for HAS activity as described by Tlapak-Simmons *et al.* [23]. HAS activity was assessed by measuring HA production either by utilization of UDP-[¹⁴C]GlcUA or by integration of refractive index values obtained by SEC-MALLS analysis of non-radiolabeled samples. Final volumes for the radioactive and MALLS assay samples were 0.1 and 0.5–1.0 mL, respectively. Membranes were suspended by brief sonication at 0°C and added to 2 ml microcentrifuge tubes to a final concentration of 0.01 – 0.02 mg/ml total membrane protein in assay buffer containing 18.75 mM sodium phosphate and 6.25 mM potassium phosphate, pH 7.0, 75 mM NaCl, 2% (v/v) glycerol, 0.1mM EDTA, 1.0 mM DTT, 0.1 mM PMSF, 1.0 μM Pepstatin, and 2 μM Leupeptin. UDP-GlcUA and UDP-GlcNAc were added to 1 mM final concentration, the mixture incubated at 30°C for 10 min, and calf intestinal alkaline phosphatase was added to a final concentration of 0.02 U/μL. For the radiolabel assays, UDP-[¹⁴C]GlcUA was also added to 0.7 μM. The synthesis reactions were then initiated by the addition of MgCl₂ to a final concentration of 20 mM and incubation for up to 8 hr at 30°C in a vibrating Taitec mixer (San Jose, CA). Unless noted otherwise, synthesis was terminated by adding EDTA and UDP to final concentrations of 40 mM and 10 mM, respectively, and chilling on ice for ~20 min and then heating in a 100°C bath (sand) to inactivate the phosphatase. The same heat treatment was used for MALLS samples. The duration of heating was 1 min per 0.1 mL volume and the final temperature of treated samples was 65–75°C. Radio-labeled samples were quenched by adding SDS to 2% and then analyzed by paper chromatography

[23]. If SEC-MALLS samples were stored at 4°C for more than a few hours, then the heat treatment was repeated prior to injection.

SEC-MALLS

Analyses were performed by chromatographic separation of samples using either one TSK-Gel G6000PW_{XL} column (TOSOH-BIOSEP) or one or two (in series) PLaquagel-OH60 (Polymer Labs) columns at 22°C at a flow rate of 0.4 – 0.5 ml/min in 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 0.05% sodium azide. MALLS analysis of a sample was performed continuously on the column eluate, as it passed through a DAWN DSP Laser Photometer in series with an OPTILAB DSP Interferometric Refractometer (both from Wyatt Technologies, Inc). Data were analyzed using Astra v4.73, a dn/dc value of 0.153 (24), an A₂ value of 0.0023 (25), and either 1st order Zimm or 2nd order Berry fits, depending on the size of the HA being analyzed. In our experience, 1st order Zimm fits had less uncertainty in analysis of data for HA of < 2 MDa, whereas 2nd order Berry analysis of data for HA of > 2 MDa gave better fits. Samples (200 µl) in microcentrifuge tubes were incubated in a 100°C bath for 2 min prior to injection; the solution temperature after 2 min was ~65°C, as determined with a thermistor. Column performance was monitored by the routine analysis of a commercial HA used as a reference standard. Regular washing of the column with 0.1% SDS in water (2 ml) restored column performance and HA fractionation, and minimized artifacts that were probably due to accumulation of lipid on the column. In our experience, high HA concentrations should also be avoided when using SEC-MALLS, since artificially high mass values may occur, possibly due to chain entanglement. Unlike gel electrophoresis techniques, however, MALLS inherently provides concentration data so that one can easily monitor samples and stay below an appropriate concentration to minimize concentration-dependent artifacts (*e.g.* we routinely inject samples that are ≤ 0.05 mg/ml HA).

Agarose gel electrophoresis was performed essentially as described by Lee and Cowman (9) using 0.8% (w/v) gels and ~3 µg HA per lane (estimated from MALLS results). Samples were precipitated with 3 volumes of ethanol at –20°C, and redissolved in the running buffer, which contained 1 mM EDTA, 40 mM trishydroxymethylamino methane, 100 mM sodium acetate, pH 7.6. Electrophoresis was at 80V for 2.5–3 hr and the gels were stained overnight with 0.005% Stainsall in 50% ethanol. Gels were destained by washing and exposure to light. Digital images were captured as jpeg files using a FluoroChem FC from Alpha Innotech Corp (San Leandro, CA).

Results

Our initial goal was to utilize SEC-MALLS to determine the rate of HA synthesis and the rate of HA product size increase by recombinant seHAS in membranes. Since MALLS techniques are notoriously sensitive to debris, protein aggregation, and other light scattering artifacts, most investigators have not tried to assess HAS function in native membranes using this technique. In fact, we could find no reports in the PubMed database in which light scattering had been used to analyze HA made by any HAS in membrane preparations. Given that MALLS is one of the best methods to assess HA molar mass and size distributions, we sought to eliminate potential artifacts in order to use this technique to obtain reliable data about HAS function in membranes.

Elimination of an artifact associated with SEC-MALLS analysis of membranes

The first problem we encountered was the discovery of large molecular mass material that was associated with the use of membrane suspensions. This material had an apparent mass of 10 – 100 MDa by MALLS, even though it eluted at a SEC position corresponding to HA of 300 – 500 kDa, and gave rise to increasing, rather than decreasing, apparent HA mass as the

chromatographic elution progressed (light scattering profiles in Fig. 1A). However, we discovered that this artifact could be eliminated or minimized by brief heating of membrane samples in a 100°C bath until the temperature was 65–75°C. The high molar mass material in heat-treated samples was HA, since it was not present in samples lacking UDP-sugars and was completely eliminated by treatment with *Streptomyces* hyaluronidase (not shown). The utility of the heat treatment step was greatest if the treatment was just prior to sample analysis (*i.e.* within a few hours). Samples that were heat treated, stored for days at 4°C and then analyzed, showed increasing reappearance of the artifact noted above (not shown).

Using this simple heat treatment protocol, we also investigated whether the addition of membranes containing HAS (without UDP-sugars) to highly purified commercial HA of different M_w had any effect on the SEC-MALLS analysis of the HA (Table 1). Without heat treatment, the presence of exogenous membranes interfered with the analysis as expected from Fig 1 (not shown), but the brief heat treatment allowed the acquisition of data that were essentially as reproducible and accurate as that obtained in the absence of membranes. The assessments of weight-average molar masses were only a few percent different for the heat-treated samples in the presence of membranes. There were no statistically significant differences between the pure HA samples (without membranes and not heated) and those analyzed in the presence of membranes after heat treatment. Importantly, the heat treatment in the presence of added membranes also had no effect on the experimentally determined M_w values for HA preparations with a wide range of different size distributions. Thus, the presence of membranes *per se*, does not preclude the ability to obtain high quality data on HA molar mass distributions by SEC-MALLS.

Heat treatment increases HA yield by releasing membrane-bound HA chains

We also found that additional HA was obtained (*e.g.* >100% more depending on the initial synthesis period), based on the mass of HA calculated from refractive index values (Fig. 1B), after the brief heating step to minimize the large mass artifact. With the exception of the enzyme from *Pasteurella multocida*, which is nonprocessive [26], all HASs appear to retain and tightly bind their growing HA chains until they are released. Once an HA chain is released, it is “completed” in that it can apparently no longer be bound and extended by another HAS. Thus, although the HA chain release process may determine the distribution of product HA sizes made by a HAS, the conditions that facilitate or hinder HA chain release from HASs have not been identified.

Based on previous HAS studies, one would expect that very early during a synthesis reaction, virtually all HA chains would be at various stages of elongation and still bound to HAS; very little of the total HA would be free. In contrast, at much later stages of a synthesis reaction only a small fraction of total HA would still be bound to HAS and thus membrane-associated, and a large fraction would be free HA chains that had continuously accumulated as they were released. These expectations were tested and verified by comparing the total HA yield for parallel samples, analyzed with and without the heat treatment, as a function of incubation time during the synthesis reaction (Fig. 2A). For samples taken early during the reaction period, the HA yield was greatly increased by heating (*e.g.* 217% at 15 min), whereas at later reaction times the increase was very modest (*e.g.* 17% at 4 hr). Overall HA recovery, assessed by MALLS, steadily increased with time, whether the analysis was of heated or untreated samples (Fig. 2B and 2C). The biphasic increase and then decrease in the enhanced recovery of HA by the heat treatment (Fig. 2A) is expected as HA chains are initiated and enzyme-bound, but later released by the active membrane-bound HAS molecules in the sample.

Additional experiments in which samples were heated or centrifuged to pellet membranes confirmed that the heat treatment released HA from membranes (not shown). The finding that the HAS-bound HA is not recovered in SEC-MALLS analysis unless samples are heated, is

most likely due to the binding (and thus loss) of membranes containing attached HA to the column support. As noted in Methods, this accumulated material is readily removed by washing the column periodically with SDS. The kinetic profile obtained for the heated samples was also a better estimate of the continuous steady synthesis of HA, than the kinetic profile of the untreated samples (Fig. 2C). These latter unheated samples showed a pronounced apparent kinetic lag, which actually reflected the failure to recover the HA bound to HAS in these samples.

Stopping the HAS reaction to allow stable storage of samples

Unexpected results were also obtained in kinetic analyses of membrane-bound HAS to determine how HA size changed with time of synthesis and how long it took to achieve a steady-state size distribution. Such analyses require conditions that quickly terminate the enzyme reaction, such as decreasing the temperature or chelating the required Mg^{+2} ions. HAS assays that are to be analyzed by paper chromatography are normally terminated by addition of SDS to a final concentration of 2% (w/v), but this approach cannot easily be used when performing SEC-MALLS analyses, due to interference caused by the light scattering of SDS micelles. Thus, our goal was to assess other possible conditions to stop the HA synthase reaction, in order to validate subsequent kinetic studies.

Unfortunately, freezing samples at $-20^{\circ}C$ to stop the enzyme reaction was not useful, because this treatment generated a high molar mass artifact, which subsequent heat treatment did not remove (not shown). Many investigators might reasonably assume that HA synthesis will be negligible at $4^{\circ}C$ or in the presence of chelators and use such conditions to study HA size distributions or the kinetics of HA accumulation. We tested these conditions for their ability to give stable samples; that is, stored samples with no time-dependent changes in HA amount or size distribution after “quenching” the reaction. At $30^{\circ}C$, a normal assay temperature, EDTA (in large molar excess over Mg^{+2} ions) did not stop the synthase reaction (Fig. 3A). HAS-containing membranes in the presence of UDP-sugar substrates also could not just be chilled and stored at $4^{\circ}C$ to stop HA synthesis, and more surprisingly, seHAS was still active at $4^{\circ}C$ in the presence of EDTA (Fig. 3B). Under the latter conditions, the continuous slow synthesis of HA occurred over a period of days (Fig. 3C). However, HAS activity was successfully quenched in the presence of both EDTA and UDP at $4^{\circ}C$ (Figs. 3B and 3C).

Accumulating UDP product decreases the rate of synthesis and the apparent HA product size distributions made by HAS

The streptococcal and eukaryotic HASs, whose activities have been characterized kinetically, are all inhibited by exogenous UDP [26–28]. This was, in fact, the rationale for including UDP in the above enzyme quench procedure. Since the UDP concentration steadily increases as this product is generated by utilization of the two UDP-sugars during HA synthesis, the rate of HA synthesis will decrease progressively (Fig. 4). Although this decreased synthesis rate is sometimes attributed to depletion of the substrates, it can also be due to the build-up of UDP because the addition of alkaline phosphatase, which hydrolyzes nucleotides, substantially restored the kinetic linearity of these reactions.

The apparent HA product size decreases as the ratio of enzyme to substrate increases

In addition to HA-product size being dependent on the duration of synthesis, we found a very significant decrease in HA size with increasing HAS concentration (Fig. 5A). The same result was observed whether the analysis was by MALLS or gel electrophoresis. Even at higher substrate concentrations (*e.g.* 4 mM each UDP-sugar), there was an inverse correlation between the amount of membrane protein used and HA size (not shown). Extrapolation of these curves to zero-HAS content (Fig 5B) eliminated any artifact due to the amount of HAS used, and gave

the theoretical maximum HA mass that could be made by that particular HAS under the experimental conditions.

We finally used the kinetic quenching conditions noted above, inclusion of alkaline phosphatase, and SEC-MALLS to assess the time-dependent changes in HA-product size distribution (Fig 6A). As expected the weight-average molar mass of HA products increased with time, reflecting HA chain growth. A steady-state HA size distribution was obtained after 2–4 hours. The same result was confirmed in experiments to assess changes in HA size by agarose gel electrophoresis (Fig. 6B). These results were also very consistent with those in Fig. 2A, which showed that increased HA recovery, by heating to dissociate HA from HAS, occurred for up to ~2 hours. The results indicate that each HAS may be associated with a growing HA chain for more than an hour before the HA chain is released and the enzyme initiates synthesis of a new HA chain. The HA products made in subsequent rounds of chain synthesis are distributed as a smear trailing below the major band (as in Fig 6B, lane 6). In three different experiments, the percent of total HA that was in the tailing region increased from 27 ± 4 at 30 min to 43 ± 1 at 480 min.

Discussion

We have developed procedures to use SEC-MALLS to study the molar mass distributions of HA made by HA synthases in membranes. This approach has not been reported previously, due to the extreme sensitivity of MALLS to contaminating particles and debris. Nonetheless, there is a great interest in accurate analysis of HA size in biologically relevant samples. Several findings should be noted in terms of compromising or confounding effects in trying to use MALLS for these analyses.

First, any analyses would not have been possible without the ability of a simple heat treatment to eliminate the membrane-related large mass artifact (Fig 1). A second surprising potential artifact in performing kinetic and product size analyses was the finding that addition of excess EDTA alone, to chelate Mg^{+2} ions, was insufficient to terminate the synthase reaction, even at 4°C. Thus, the residual low activity of HAS enzymes at low temperature and in the presence of chelators, such as EDTA, may compromise the results of studies that utilize these conditions, rather than SDS, to quench the reaction. Since freezing membrane-containing samples could not be used due to additional artifacts, we ultimately determined that the best way to stop the HA synthase reaction was to add UDP and EDTA, and chill the samples on ice. SEC-MALLS profiles and HA product size distributions were stable at 4°C in the presence of both UDP and EDTA.

A third surprising finding was related to the amount of HAS assayed, which has been overlooked by most investigators, including our group, in past studies. As the HAS concentration increased, the HA-product size decreased (Fig. 5A). Since one needs to use an amount of HAS activity that gives a good signal, this effect may sometimes be unavoidable. This negative correlation of product size with increasing HAS/substrate ratio might be explained by the competition among individual HAS molecules for precursors to sustain elongation of their HA chains at a certain rate. Although the overall rate of HA synthesis (*e.g.* glycoside bonds made per sec) may be nearly identical between two samples with slightly different HAS concentrations and the same high UDP-sugar concentrations, the sample with fewer active HAS molecules is able to make longer HA chains on average than the one with more HASs. Whatever the reason, this counter-intuitive effect results in an inverse linear relationship (Fig. 5B) between observed weight-average HA mass and HAS protein concentration, at least at lower HAS concentrations.

One reason for this above effect may be related to the fact that polysaccharide synthases are unusual and rare enzymes. They have not been extensively studied, compared to nucleic acid polymerases that use a template or to enzymes that catalyze a typical one or two substrate reaction and then release a product after each round of catalysis. The Class I HASs act on the product of each previous cycle and this new substrate is always different because it is one sugar longer than the previous product/substrate. Another possible confounding effect is that at higher HAS concentration, growing HA chains on different membrane fragments may be more likely to interact (*e.g.* forming nested helices) and consequently slow down the rate of chain elongation. In this situation, a HAS molecule might have to translocate an HA cluster, whose inertial resistance would be greater than that of a single HA chain [5].

A fourth problem was that during the relatively long times required to achieve steady-state HA size distributions, HA synthesis was not always linear. This was solved, however, by including alkaline phosphatase in the HAS reactions, which were then linear for at least 8 h, even when >50% of substrate was converted to HA (Fig. 5). Kinetic inhibition is greatly delayed, because the continuous hydrolysis of UDP by the phosphatase prevents its build-up. When inhibition finally occurs in the presence of alkaline phosphatase, it is because the substrates are depleted to a much greater extent - to the point where substrate concentrations drop to < K_m values and enzyme velocity is substrate-limited. The significance of including alkaline phosphatase when HA size is to be studied by MALLS or electrophoresis is two-fold. First, HA chain elongation can continue longer without feedback inhibition due to UDP buildup, and second, without addition of phosphatase a maximal steady-state HA size distribution may not occur before HA synthesis is inhibited.

Based on the above considerations for appropriate experimental conditions, we have adopted the following approach to determine the largest HA product size that can be made by a HAS. (i) Alkaline phosphatase is routinely included to eliminate feedback inhibition by UDP and enhance linearity of HA synthesis. (ii) Samples are quenched by adding UDP and EDTA to final concentrations of 10 mM and 40 mM, respectively, chilling on ice, and then heating briefly to inactivate the phosphatase. (iii) The stored samples are heated to 65–75 °C just prior to injection for SEC-MALLS analysis. (iv) The time-dependence for the increase in average HA size is determined to ensure that a steady-state size distribution is obtained. (v) The kinetics of HA product size increase is determined for 3–4 different concentrations of HAS-containing membranes in the presence of sufficient alkaline phosphatase to maintain linearity of HA synthesis. (vi) Finally, the M_w (or M_n) values obtained from SEC-MALLS analyses of the 3–4 samples are plotted versus membrane protein (HAS content does not need to be known), and the zero-intercept then calculated to determine the maximum M_w of HA products that can be made by the HAS or HAS variants being studied. A key point is that in an analysis of HA size, investigators need to consider and separately assess both the rate of HA synthesis (*i.e.* the total amount of sugar incorporated into HA products) and the rate of HA product size increase.

The results indicate that 1–2 hours, seHAS molecules release their HA chains and initiate synthesis of new HA chains. However, the subsequent rounds of HA synthesis are not associated with a cohort of similar-sized HA, because the initial synchronization of the enzyme reactions is lost. Samples in which HAS synthesizes multiple rounds of HA chains have a higher percent of HA products that are smaller than the maximum product size (*e.g.* lanes 5 and 6 versus lanes 1–3 in Fig 6B) and distributed over a broad range of sizes, as typically observed for naturally occurring HA samples. The synchronization of HA product sizes observed for seHAS probably occurs because the recombinant enzyme is not associated with endogenous HA, since *E. coli* SURE cells lack the substrate UDP-GlcUA (29).

In this report, we describe some of the unexpected obstacles encountered in kinetic studies to determine simultaneously the rate of HA synthesis and the size distribution of HA made by

membrane preparations expressing a HAS. Many of these potential artifacts will be problematic and could make it difficult to obtain valid results, whether one uses gel electrophoresis, SEC alone or SEC-MALLS to analyze HA size. Fortunately, we found reasonable conditions to minimize these confounding effects, so that meaningful data could be obtained about HA size. Consequently, we and other investigators should now be better able to utilize the power of light scattering analysis to study how HA product size may be regulated by HA synthases.

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Figure 1. Heat treatment of membranes eliminates a large mass artifact and releases HA chains bound to HAS

A. Membranes containing seHAS were incubated with UDP-sugars for 120 min at 30°C as described in Methods, and samples were injected on the SEC-MALLS system with (solid line, filled symbols) or without (dashed line, open symbols) a 2 min, 100°C treatment prior to injection. The lines are the laser light scattering traces, and the individual points are the weight-average molar mass values calculated for each time interval. B. The refractive index traces from the same experiment in A show a 64% increase in recovered HA in the heated sample (solid line), compared to the unheated sample (dashed line).

Figure 2. The relative increase in HA recovery by heat treatment of membranes is dependent on the duration of HA synthesis

Membranes containing HAS were incubated with the UDP-sugar substrates at 30°C and samples were taken, quenched at the indicated times, and analyzed by SEC-MALLS as described in Methods. A. Two separate experiments are shown in which the differences in HA yield are shown between heated (final temperature of 65–75°C) and unheated samples, as a percent increase relative to the untreated samples. B. Total HA yield is shown for samples that were either heat treated (filled circles) or untreated (open circles). C. HA yield for the experiment in panel B is shown for the first 60 min to demonstrate the apparent lag, representing

membrane-bound HA, in HA production for samples that were not heat treated (open circles), whereas the heat treated samples showed linearity (closed circles).

Figure 3. The HAS reaction is not completely stopped by treatment with EDTA or low temperature
 A. Samples containing 0.02 mg/ml membrane protein were allowed to synthesize radiolabeled HA for 15 min, after which EDTA was added to 40 mM and the samples were analyzed immediately (time 0) or stored at 4°C for 1, 3 or 7 days prior to addition of 10 mM UDP to stop the reaction. To verify that the additional incorporated radioactivity was due to synthesis of HA, samples were then treated with *Streptomyces* HA lyase (solid bars) or only buffer (open bars). B. Membranes containing HAS were allowed to synthesize HA in the radio-labeled assay for 10 min at 30°C after which the following additions were made to give the indicated final concentrations: water (control), 40 mM EDTA only, or 40 mM EDTA and 10mM UDP. The samples were then incubated for an additional 50 min at 30°C, quenched by addition of SDS

and processed to determine HA synthesis. C. Parallel nonradioactive assays were processed as described in B, except that samples were not treated with SDS. All samples were quenched by addition of EDTA and UDP to give final concentrations of 40 mM and 10 mM, respectively, and finally chilled on ice. Samples were then treated for 2 min at 100°C and injected for SEC-MALLS analysis. Refractive index traces and the molar mass values calculated from the light scattering data are shown, indicating the relative size and amount of HA made by the control (solid line, black circles), EDTA (long dashed line, white circles), or EDTA and UDP (short dashed line, gray triangles) samples.

Figure 4. Continuous removal of UDP enhances the linearity of HA synthesis by HAS

Membranes containing seHAS were incubated with radio-labeled UDP-sugars at 30°C for the indicated times either in the presence (■; n=5) or absence (●; n=3) of alkaline phosphatase as described in Methods. Values are the mean $\pm$ SEM and are presented as a percent of the initial UDP-sugar used (*i.e.* incorporated into HA) to emphasize the linearity even at high substrate utilization.

Figure 5. The apparent size of HA products is inversely related to the amount of HAS used
 Different amounts of total membrane protein (containing proportionately different amounts of seHAS), as indicated, were incubated with non-radiolabeled UDP-sugars and alkaline phosphatase at 30°C for 0.5 (●), 1.0 (▲) or 2.0 (■) hours. The reactions were quenched by addition of EDTA and UDP, chilling on ice, and brief heat treatment to inactivate the phosphatase as described in Methods. Samples were then analyzed by SEC-MALLS to assess HA molar mass. Panel A shows the results, with second-order curves fit by regression analysis (cc values = 0.88–0.96), for samples containing up to 0.3 mg/ml membrane protein. Panel B shows the region from ≤ 0.09 mg/ml with straight lines calculated by linear regression analysis (cc values = 0.89–0.95). Theoretical maximum HA molar mass values were calculated by extrapolation of regression lines to zero membrane (HAS) concentration.

Figure 6. Kinetic analysis of HA product size distributions

Membranes containing seHAS were incubated at 30°C with non-radiolabeled substrates and HA product size distributions were determined by SEC-MALLS (A) or agarose gel electrophoresis (B) as described in Methods. A. Weight-average molar masses were determined at the indicated times in 5 independent experiments using 4 different membrane preparations. Values are the mean $\pm$ SEM of at least triplicates. B. Samples in lanes 1–6 were taken at 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 hours, respectively. Lane 7 is a mixture of Select-HA and Mega-HA standard ladders (~ 3 μ g total) ranging from 0.5 MDa to 4.5 MDa as indicated.

Table 1**Effect of heat treatment in the presence of membranes on the size and recovery of commercial HA**

E. coli membranes containing seHAS were added (to a final protein concentration of 0.02 mg/ml) to four different commercial HA solutions (0.075 – 0.1 mg/ml), each with a different weight-average molar mass. No UDP-sugars were added. These membrane-treated samples, as well as a parallel set of control samples not containing membranes, were incubated with gentle mixing at 30°C for 4 hr. UDP and EDTA were added to final concentrations of 10 mM and 40 mM, respectively, and the samples were diluted with running buffer to bring the HA concentration to 0.05 mg/ml and then incubated with gentle rocking overnight at 4°C. Prior to SEC-MALLS analysis, 200 µl of the membrane-treated samples were incubated at 100°C for 2 min. Replicates of these samples, as well as the control samples (200 µl) incubated without membranes were then injected and the weight-average molar mass values calculated.

Commercial Sample	Control Mw (MDa)	Control Mw (MDa) Mean ± SEM	Treated Mw (MDa)	Treated Mw (MDa) Mean ± SEM
HA1	0.23	0.23 ± 0.01	0.22	0.23 ± 0.01
	0.24		0.22	
HA2	0.22	0.66 ± 0.03	0.24	0.67 ± 0.01
	0.69		0.67	
HA3	0.65	1.23 ± 0.03	0.68	1.17 ± 0.03
	0.64		0.66	
HA4	1.26	3.00 ± 0.10	1.20	2.92 ± 0.17
	1.21		1.17	
	3.11		2.99	
	2.98		2.85	
	2.91		2.92	