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Original Article

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Glycan Synthesis

Expanding glycosaminoglycan chemical space: towards the creation of sulfated analogs, novel polymers and chimeric constructs

Rachel S Lane[2], Kalib St. Ange[3], Behnam Zolghadr[4], Xinyue Liu[5], Christina Schäffer[4], Robert J Linhardt[3,5,6,7], and Paul L DeAngelis[1,2]

[2]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA, [3]Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA, [4]Department of NanoBiotechnology, NanoGlycobiology Unit, Universität für Bodenkultur Wien, Muthgasse 11, A-1190 Vienna, Austria, [5]Department of Biology, [6]Department of Chemical and Biological Engineering, and [7]Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[1]To whom correspondence should be addressed: Tel: 1-405-271-2227 x 61210; Fax: 1-405-271-3092; e-mail: paul-deangelis@ouhsc.edu

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Abstract

Glycosaminoglycans (GAGs) have therapeutic potential in areas ranging from angiogenesis, inflammation, hemostasis and cancer. GAG bioactivity is conferred by intrinsic structural features, such as disaccharide composition, glycosidic linkages and sulfation pattern. Unfortunately, the in vitro enzymatic synthesis of defined GAGs is quite restricted by a limited understanding of current GAG synthases and modifying enzymes. Our work provides insights into GAG-active enzymes through the creation of sulfated oligosaccharides, a new polysaccharide and chimeric polymers. We show that a C6-sulfonated uridine diphospho (UDP)-glucose (Glc) derivative, sulfoquinovose, can be used as an uronic acid donor, but not as a hexosamine donor, to cap hyaluronan (HA) chains by the HA synthase from the microbe *Pasteurella multocida*. However, the two heparosan (HEP) synthases from the same species, PmHS1 and PmHS2, could not employ the UDP-sulfoquinovose under similar conditions. Serendipitously, we found that PmHS2 co-polymerized Glc with glucuronic acid (GlcA), creating a novel HEP-like polymer we named hepbiuronic acid [-4-GlcAβ1-4-Glcα1-]$_n$. In addition, we created chimeric block polymers composed of both HA and HEP segments; in these reactions GlcA-, but not *N*-acetylglucosamine-(GlcNAc), terminated GAG acceptors were recognized by their noncognate synthase for further extension, likely due to the common β-linkage connecting GlcA to GlcNAc in both of these GAGs. Overall, these GAG constructs provide new tools for studying biology and offer potential for future sugar-based therapeutics.

Key words: chemoenzymatic reaction, heparin, heparosan, hyaluronan, sulfation, synthase

Introduction

Glycosaminoglycans (GAGs) are sugar polymers composed of repeating disaccharide units that include a hexosamine. Hyaluronan (HA) and heparosan (HEP) are two examples of GAGs that are composed of the same two monosaccharides, but connected through different glycosidic linkages that give each of the polysaccharides their unique biological effects. HA ([-4-GlcAβ1-3-GlcNAcβ1-]$_n$) has multiple binding partners within the

body, influencing the regulation of proliferation, cellular migration and inflammation (Gaffney et al. 2010). HEP ([-4-GlcAβ1-4-GlcNAc-α1-]$_n$), the unmodified precursor backbone of heparan sulfate (HS) and heparin, on the other hand, has no other known function within the body. *N*-deacetylase/*N*-sulfotransferase initiates the transformation of HEP to HS, paving the way for further modifications by C5-epimerase and *O*-sulfotransferases. These modifications create biologically active products, either HS or the more highly modified heparin. The differences in degree and pattern of sulfation result in a multitude of unique carbohydrate species with hundreds of binding partners within the body (Li et al. 2013).

GAGs that are not naturally sulfated can also benefit from precise sulfation patterns. For example, HA, when sulfated at the C6 hydroxyl of GlcNAc, inhibits the pro-angiogenic growth factor $VEGF_{165a}$, but not the $VEGF_{165b}$ isoform (Lim et al. 2015). Inhibition of the former growth factor isoform could be useful in cancer treatments by denying tumors a blood supply.

Unfortunately, producing defined artificial versions of sulfated GAGs has proven somewhat difficult and generally affords low yields of the target (DeAngelis et al. 2013). The various modification enzymes and their isoforms that normally convert HEP into HS/heparin in the Golgi cannot be precisely manipulated in the laboratory. The resulting products are often heterogeneous and the procedures are not always extremely reproducible as one type of modification often influences the ability of a subsequent enzyme to catalyze the next step(s). Chemical syntheses for sulfating HA or HEP also lack specificity and precision; precautions must be taken to not over-sulfate the polymers as artificial GAG molecules can have deadly effects in humans, as observed with over-sulfated chondroitin in the contaminated heparin crisis of 2008 (Guerrini et al. 2008).

New methods to precisely engineer sulfated GAGs would be immensely beneficial. Specific sulfation techniques would allow investigators to more fully understand protein/GAG interactions and design molecules that express higher bio-selectivity, precisely targeting one binding partner within the body instead of interacting with, or activating several binding partners simultaneously. For example, the potent anticoagulant heparin could be more widely employed for various diseases including inflammation and cancer, but the risk of a fatal hemorrhagic side effect is currently too great.

GAG synthases or polymerases are types of glycosyltransferases that polymerize the repeating disaccharide chains in various organisms including animals and certain pathogenic microbes that use similar polymers for extracellular capsules that camouflage against host defenses (DeAngelis 2002a, 2002b). The microbial enzymes, especially from the Gram-negative bacterium *Pasteurella multocida*, have very robust behavior in vitro and have been harnessed to make defined oligosaccharides, quasi-monodisperse polysaccharides and analog-containing polymers (DeAngelis et al. 2003; Jing and DeAngelis 2004; Sismey-Ragatz et al. 2007). The *Pasteurella* enzymes are bifunctional glycosyltransferases that have two independent active sites that extend GAG chains with either a hexosamine or an uronic acid monosaccharide unit. Each site contains substrate-binding pockets for both the uridine diphospho (UDP)-sugar donor and the oligosaccharide acceptor; the latter interacts with the nascent GAG chain.

Here we have investigated new GAG synthase-based chemoenzymatic methods for creating sulfated and chimeric GAGs as well as discovered a novel HEP-like analog that we have named "hepbiuronic acid" (HBA) or "hepbiuronan."

Results

Pasteurella HA synthase utilizes UDP-Sqv as a substrate for its uronic acid-transferase donor site

HA is not naturally sulfated, but sulfated versions potentially offer therapeutic utility. We tested whether a novel precursor, a UDP-glucose (UDP-Glc) analog with C6 sulfonation called UDP-sulfoquinovose (UDP-Sqv), could create precisely sulfated HA. This sugar nucleotide is a naturally occurring donor found in some plants and bacteria used in the biosynthesis of glycolipids or S-layer glycoproteins, respectively (Benning 1998; Meyer et al. 2011). In theory, a sulfate installed on a sugar during enzymatic synthesis of the chain allows exact placement in the GAG polymer. In contrast, post-polymerization chemical or enzymatic sulfation of the sugar currently does not have the positional control needed and/or the required catalysts.

We tested the ability of PmHAS, the *P. multocida* HA synthase (DeAngelis 1999), to extend defined 2-aminoacridone (AMAC)-tagged HA oligosaccharide acceptors terminated at the nonreducing termini with either GlcA (A) or GlcNAc (N) using the UDP-Sqv donor. For example, a dye-tagged GlcNAc-terminated HA pentasaccharide (HA5, NANAN-AMAC) was extended by UDP-Sqv as evidenced both by gel electrophoresis (Figure 1A, lane 4) and by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) (Figure 1B, bottom panel). When extended with UDP-Sqv, the new oligosaccharide migrates faster than the original pentasaccharide due to its increased negative charge (similar to when HA5 is extended with the natural sugar unit, glucuronic acid; Figure 1A, lane 2). When the same UDP-Sqv was added to reactions with the GlcA-terminated hexasaccharide HA6 (ANANAN-AMAC), the addition of only a simple Glc unit was observed, as determined by gels and confirmed by MALDI-ToF MS analysis (Figure 1A, lane 5; Figure 1C, bottom panel). The source of the Glc was hypothesized to be due to the presence of contaminating UDP-Glc in the UDP-Sqv preparation (the former is the precursor of the latter donor in the chemoenzymatic process (Meyer et al. 2011); these reactions used 20-M excess donor over acceptor). To confirm this hypothesis, we tested pure UDP-Glc donor; in these reactions, the extension of HA6 produced the identical heptasaccharide product with the same slower migration (due to the addition of an uncharged sugar) (Figure 1A, lane 6), as well as some octasaccharide with two Glc units.

Co-polymerization trials with UDP-Sqv and either UDP-GlcA or UDP-GlcNAc were attempted under various reaction conditions (varying additives, metals, etc.) with PmHAS, but were unsuccessful. Mixtures of UDP-Glc with UDP-GlcA or UDP-GlcNAc also did not enable polymerization. These results suggest that both Sqv- and Glc-capped acceptors are poor substrates for elongation by the PmHAS enzyme due to the catalyst's intrinsic substrate selectivity.

Pasteurella HEP synthases do not utilize UDP-Sqv as a donor substrate, but do use UDP-Glc

HEP is the precursor of HS and highly sulfated heparin. Precisely modifying HEP with unique sulfation patterns could provide new, more selective heparinoids with targeted biological activity. Again, we reacted the UDP-Sqv donor with HEP oligosaccharides and the synthase enzymes to test if we could obtain a heparinoid with a specific C6 sulfonate. UDP-Sqv was tested with PmHS-G, an engineered promiscuous catalyst composed of domains of both PmHS1 and PmHS2 isozymes (Otto et al. 2012), and HEP acceptors that were

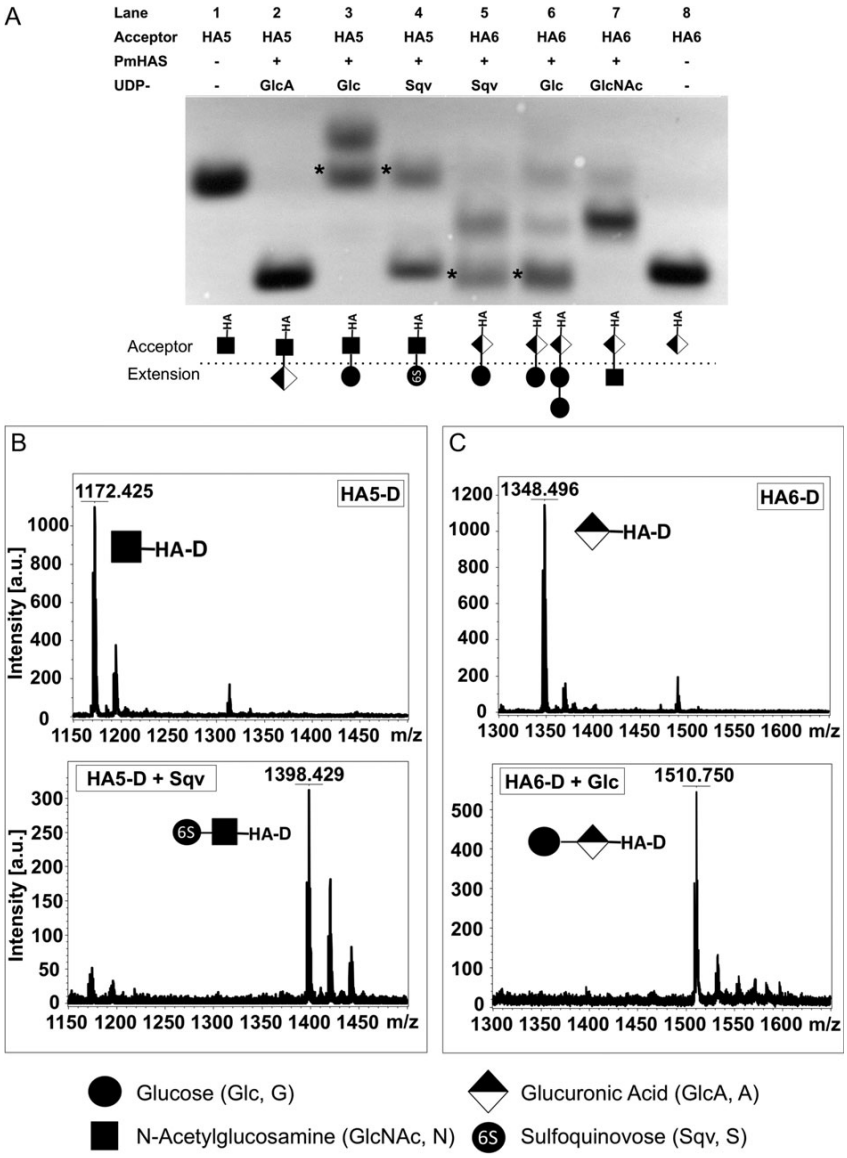

Fig. 1. PmHAS uses UDP-Sqv as an uronic acid donor, but not as a hexosamine, to extend HA. Gel electrophoresis (21% polyacrylamide gel electrophoresis, PAGE) and MALDI-ToF MS were employed to identify if UDP-Sqv is incorporated by PmHAS using AMAC-tagged HA5 (GlcNAc-terminated) or HA6 (GlcA-terminated) acceptors. (Panel **A**) PmHAS can extend HA5 with the UDP-Sqv or UDP-Glc donor (difference in migration pattern is due to difference in charge status; lane 4 vs. lane 3, respectively). For convenience, the unmodified acceptor band in reactions is marked with an *asterisk* (*) on the left side, and the schematic of the prominent product(s) is shown below the lanes. On the other hand, PmHAS could only transfer a Glc unit onto HA6; the new band in the UDP-Sqv reaction is actually due to the contaminating UDP-Glc in the preparation as the new products in lanes 5 and 6 are identical. (Panel **B**) MALDI-ToF MS confirmation of HA5-AMAC extension with a single Sqv unit (corresponds to lane 4 of gel; *D* = AMAC dye). Expected masses (Da): HA5 = 1172.54; HA5 + Sqv = 1398.54. (Panel **C**) MALDI-ToF MS confirmation of HA6-AMAC extension with a single Glc unit (corresponds to lane 5 of gel). Expected masses: HA6 = 1348.54; HA6 + Glc = 1510.54; HA6 + Sqv = 1574.54 (*note*: this latter species was *not* observed here). (a.u., arbitrary units).

linked with a benzaldehyde at the reducing end to enable AMAC fluorescent tagging and either GlcA- (ANA-BnAl; HEP3) *or* GlcNAc-terminated (NANA-BnAl; HEP4). The only products observed were Glc-capped oligomers (Figure 2) that did not contain the predicted Sqv unit. Again, the presence of UDP-Glc was postulated to be due to contamination of the UDP-Sqv preparation. The pure natural UDP-Glc donor extended the acceptors into the same products detected in the UDP-Sqv reactions, but more quickly due to the higher concentration of the actual functional substrate (Figure 2A, B; Supplementary data, Figure S1).

The incorporation of UDP-Glc by PmHS-G was a novel finding that we further explored. We incubated the enzyme with UDP-Glc and either UDP-GlcA *or* UDP-GlcNAc to test if this catalyst could afford other polymeric structures. Surprisingly, [Glc/GlcA] co-polymers were obtained with UDP-Glc and UDP-GlcA using HEP acceptors, as monitored by gel electrophoresis (Figure 3, lane 3). We confirmed this

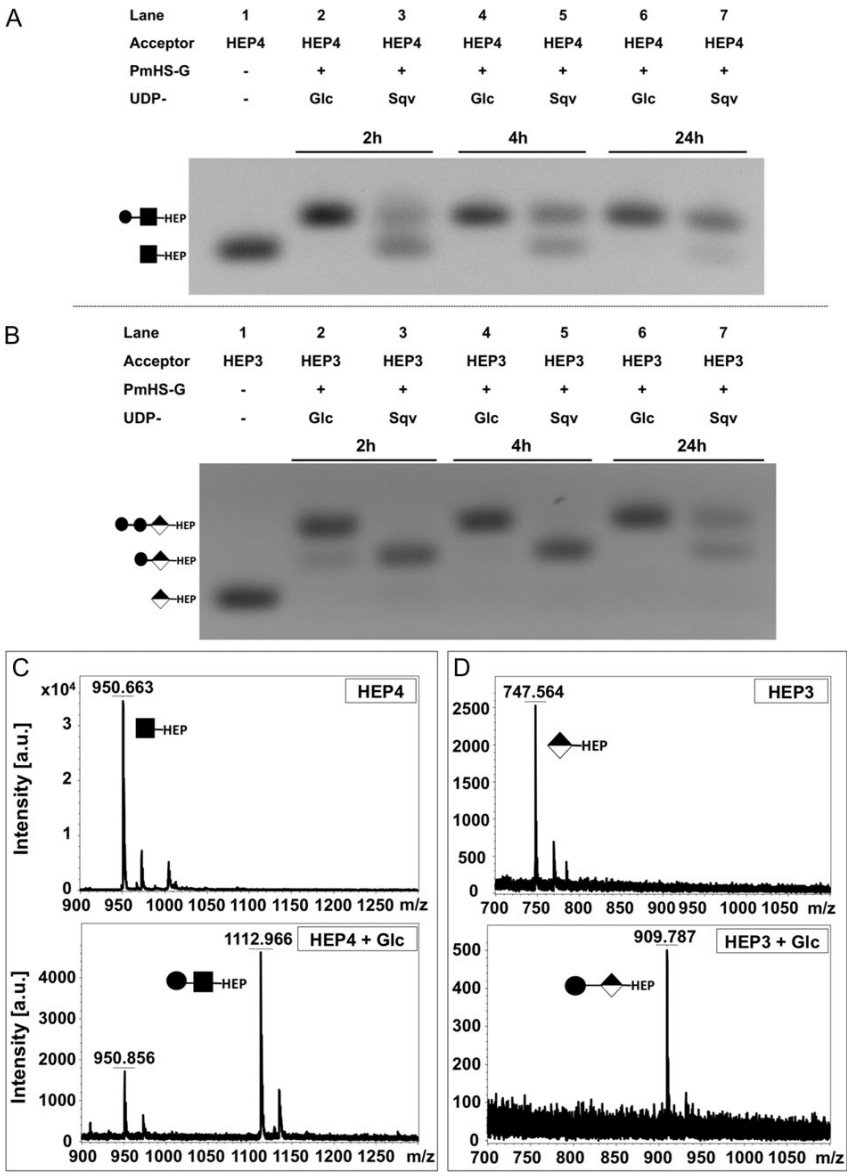

Fig. 2. PmHS-G uses UDP-Glc, but Not UDP-Sqv, to extend HEP. The use of UDP-Sqv as a potential donor by the PmHS-G, a chimeric enzyme composed of both PmHS1 and PmHS2 segments, was tested with AMAC-tagged HEP acceptors via PAGE gels (as in Fig. 1). HEP3 terminates with GlcA, while HEP4 terminates with GlcNAc. (Panel **A**) PmHS-G extends HEP4 with one sugar unit from UDP-Glc, but does not use UDP-Sqv itself. (Panel **B**) PmHS-G extends HEP3 with the UDP-Glc contaminant in UDP-Sqv preparations to add 1 or 2 sugar units, but does not use UDP-Sqv itself. (Panels **C** and **D**) MALDI-ToF MS confirmation that only UDP-Glc is utilized as a donor to extend GlcA- and GlcNAc-terminated HEP acceptors, respectively. Expected masses: HEP4 = 950.6; HEP4 + Glc = 1112.6; HEP4 + Sqv = 1176.6; HEP3 = 747.6; HEP3 + Glc = 909.6; HEP3 + Sqv = 973.6 (*note*: the two potential Sqv-extended species were *not* observed here).

finding by MALDI-ToF MS (Figure 4A); a ladder of polymers with the predicted disaccharide repeat was observed in the products. In parallel controls, a potential [Glc/GlcNAc] polymer was not observed when using UDP-GlcNAc and UDP-Glc (Figure 3, lane 3 vs. 6).

While both PmHS1 and PmHS2 (the natural sources of the synthetic PmHS-G component sequences) are capable of extending oligomers with a single monosaccharide derived from the UDP-Glc donor, only PmHS2 is capable of making a longer [Glc/GlcA] co-polymer chain via repetitive additions. These results are consistent with the previous findings of the PmHS1 enzyme's more rigid substrate selectivity (Otto et al. 2012) suggesting that the Glc-capped acceptors are poor substrates for elongation by this isozyme. In contrast, PmHS2 was shown to be more flexible in its polymerization of various C2 HexNAc sugar analogs.

Elucidation of the structure of HBA

We named this novel [GlcA-Glc] co-polymer "HBA" (or "hepbiuronan" for the ionized version in neutral aqueous solution) based on its sugar composition, its anticipated structural similarity to the

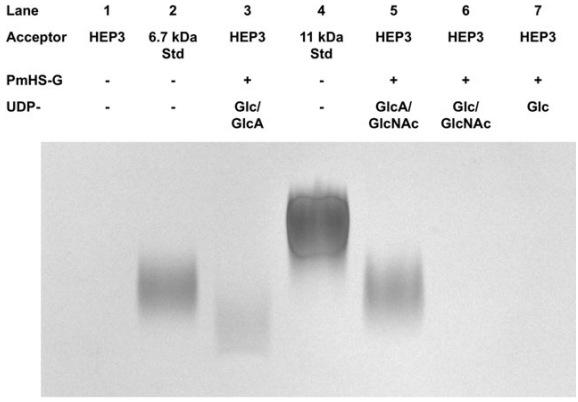

Fig. 3. PmHS-G can create co-polymers utilizing UDP-GlcA and UDP-Glc. Electrophoretic monitoring (12% PAGE with Alcian Blue staining) of PmHS-G mediated polymerization of the natural HEP disaccharide repeat (GlcA/GlcNAc) vs. the novel repeat: GlcA/Glc (HBA) using a HEP3 acceptor. PmHS-G is capable of creating co-polymers of GlcA and Glc (lane 3), but *not* of GlcNAc and Glc (lane 6) or from Glc alone (lane 7). Two HEP standards (*Std*) are shown for reference.

natural HEP molecule and the nomenclature precedent of the *Streptococcus pneumoniae* Type 3 capsular polysaccharide (cellubiuronic acid = [-3-GlcA-β1-4-Glcβ1-]$_n$). The predicted difference between HBA and the natural HEP carbohydrate is the substitution of the NAc group with a hydroxyl (-OH) at the C2 position.

The HBA polysaccharide was sensitive to digestion by heparin lyase III (Heparinase III) (Supplementary data, Figure S2), which cuts HEP, but neither HA nor *S. pneumoniae* Type 3 polysaccharide (PnP3). Heparinase III recognizes, at a minimum, an HEP tetrasaccharide, as assessed by X-ray diffraction of substrate/enzyme co-structures, and creates a disaccharide derivative ΔGlcA-β1-4-GlcNAc by cleaving the alpha-linked junction between GlcA and GlcNAc (Hashimoto et al. 2014). Testicular hyaluronidase did not cleave HBA, HEP or PnP3 polymer, but as expected cleaved its native substrate, HA. After treatment of the HBA polysaccharide (synthesized on HEP acceptor ANA-BnAl) with Heparinase III, the products formed were labeled by reductive amination with AMAC and then analyzed using reversed phase high-performance liquid chromatography (HPLC)-electrospray ionization (ESI) MS (Volpi et al. 2014) (Figure 4B). Two major products, eluting at 8.5 and 9.8 min, were identified. The first larger peak had an *m/z* = 530.3, corresponding to ΔUA(1,4)Glc-AMAC (where ΔUA is 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid, the product derived from GlcA) and the second smaller peak had an *m/z* = 571.3, corresponding to ΔUA(1,4)GlcNAc-AMAC. The ratio of the peak areas in the total ion chromatogram was ~10:1. This result is consistent with the HEP acceptor generating one ΔUA(1,4)GlcNAc disaccharide and the HBA polysaccharide extension generating about 10 ΔUA(1,4)Glc disaccharides upon treatment with Heparinase III.

Nuclear magnetic resonance (NMR) spectra collected confirmed the alternating repeat structure of [-4-GlcAβ1-4-Glcα1-]$_n$ for HBA (Figure 5, Table I). The 1D-^{1}H NMR spectrum (Figure 5A) is consistent with this polysaccharide. The small *N*-acetyl signal (1.937 ppm) and minor aromatic signals, observed at 7–8 ppm (not shown), are consistent with the ANA-BnAl acceptor on which the HBA was synthesized. Since the large water peak in the 1D-^{1}H NMR spectrum precludes its complete assignment, we obtained 2D heteronuclear single quantum coherence (HSQC) and total correlated spectroscopy (TOCSY) spectra (Figure 5B and C, respectively). The TOCSY spectrum clearly shows two spin systems associated with a Glc and a GlcA residue. The ^{1}H assignments were afforded using TOCSY and the ^{13}C assignments were obtained using HSQC (Table I). The Glc-GlcA repeating unit was 1→4 linked as evidenced from the chemical shifts for GlcH4 and GlcAH4 observed in Figure 5C. The α-linkage of the Glc residue and the β-linkage of the GlcA residue can clearly be determined by observation of the GlcC1H1 and GlcAC1H1 cross-peaks in Figure 5B. Thus, the NMR assignments support the structure for the hepbiuronic polysaccharide.

Kinetic comparison of UDP-Glc and UDP-GlcNAc co-polymerization with UDP-GlcA

We tested the ability of PmHS-G to co-polymerize UDP-GlcA with UDP-Glc (yielding artificial HBA) vs. with UDP-GlcNAc (yielding natural HEP). A HEP acceptor was extended with saturating UDP-[^{3}H]GlcA (1 mM) and various concentrations of either UDP-Glc or UDP-GlcNAc donor to obtain the apparent K_M (K_{Mapp}) of the synthase's UDP-GlcNAc transferase site and the maximal reaction velocity (V_{max}) utilizing the Michaelis–Menten kinetic model. As determined by the Hanes–Woolf plot, the calculated K_{Mapp} values for both sugars were similar (average ~90 μM vs. ~110 μM for UDP-Glc or UDP-GlcNAc, respectively), suggesting that the absence of

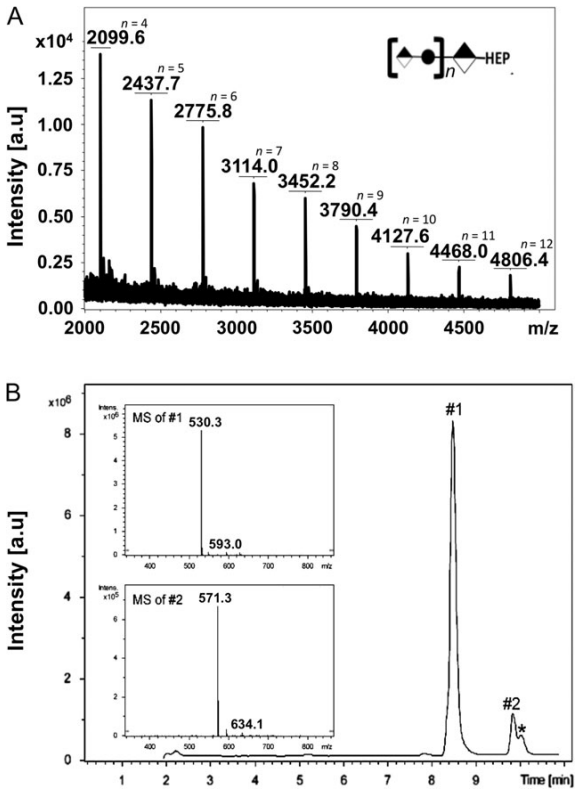

Fig. 4. HBA synthesis and degradation. (Panel **A**) Polymer formation confirmation by MALDI-ToF MS. A ladder of GlcA-Glc disaccharide repeat units is observed. (Panel **B**) RP–HPLC–ESI–MS total ion chromatography of Heparinase III digested HBA polysaccharide (synthesized on HEP acceptor ANA-BnAl). The mass spectra of peak #1 (derived from the disaccharide repeats from the ~5-kDa HBA chain) and peak #2 (derived from the single HEP3-BnAl acceptor per HBA chain) are shown. The peak marked with an *asterisk* (*) was an artifact of AMAC-labeling, observed in other samples.

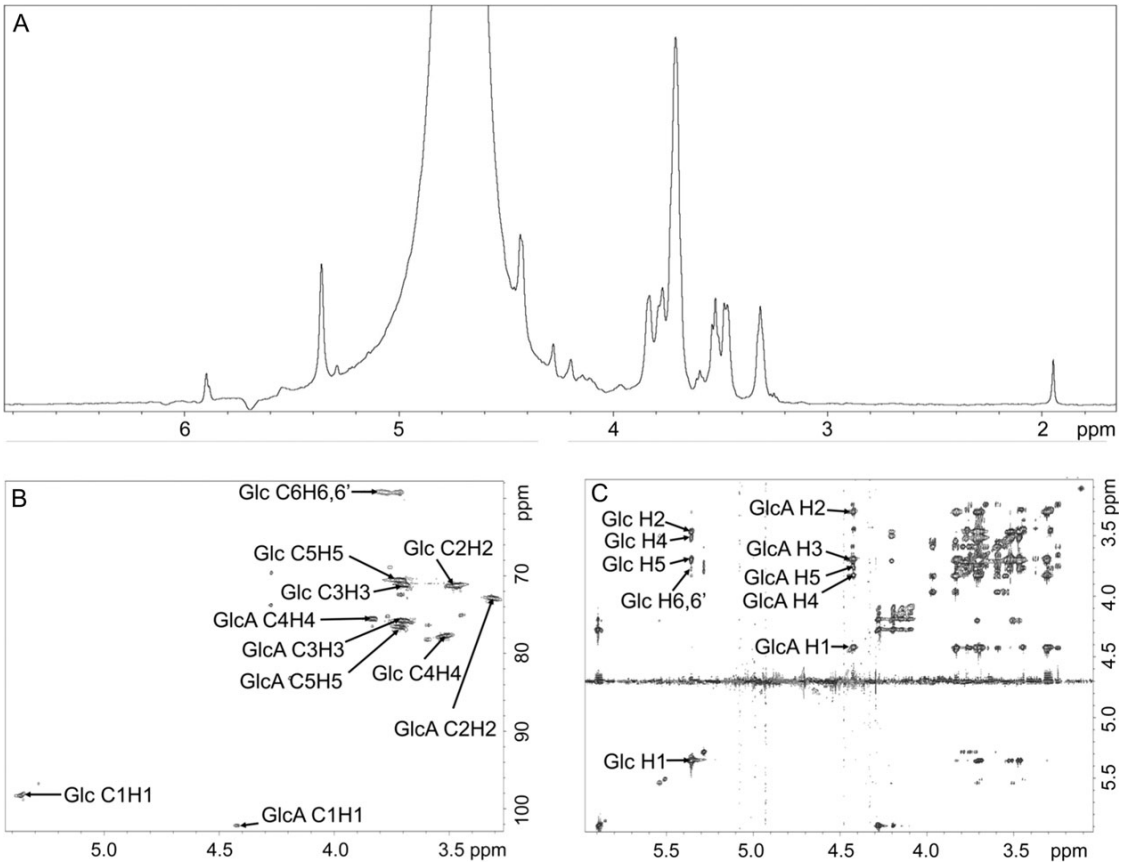

Fig. 5. NMR characterization of HBA. (Panel **A**) 1D-^{1}H NMR experiment of HBA (5-kDa synthesized on the HEP acceptor ANA-BnAl; same sample as in Fig. 4B) performed using a 30° flip angle for 32 scans with a 5 s relaxation delay. (Panel **B**) 2D HSQC-NMR spectrum of HBA with 40 scans in the direct dimension (^{1}H), 380 experiments for the indirect dimension (^{13}C). (Panel **C**) 2D TOCSY-NMR spectrum of HBA with 24 scans in the direct dimension (^{1}H), 950 experiments for the indirect dimension (^{1}H). See Table I for chemical shifts. The data are consistent with the anticipated HBA repeating [-4-GlcAβ1-4-Glc-α1-]$_n$ structure.

Table I. NMR chemical shifts (ppm) of HBA. The ^{1}H assignments were obtained from Figure 5A and C and the ^{13}C assignments rely on Figure 5B

^{1}H			^{13}C		
Position	Glc	GlcA	Position	Glc	GlcA
1	5.358	4.422	1	98.262	102.15
2	3.466	3.309	2	71.170	72.855
3	3.694	3.696	3	71.088	75.746
4	3.518	3.831	4	77.701	75.469
5	3.708	3.707	5	70.511	76.548
6	3.777/3.724	N/A	6	59.184	N/A
Ac	1.937		Ac	21.898	

the NAc moiety in the analog does not detrimentally affect the relative affinity of the enzyme for this donor (Figure 6).

The V_{max} values for co-polymerization, on the other hand, were not equivalent between the two tested UDP-sugar combinations: ~0.22 vs. ~3.4 pmol GlcA transfer/min/pmol enzyme for UDP-Glc compared to UDP-GlcNAc. In the case of a GAG synthase creating chains of disaccharide repeats, for every GlcA that is measured, a second monosaccharide is also added thus the overall enzyme turnover number or k_{cat} is ~0.4/min vs. ~6/min, respectively. This roughly 15-fold slower synthesis rate may partially explain the observed lower HBA product yield in our prototype polysaccharide syntheses; the recovered HBA polymer represented ~1% of total UDP sugars provided, while HEP synthesis consumed ~90–98% of the donors.

Novel chimeric constructs composed of HEP and HA

The HEP synthases and HA synthases use the same two UDP-sugar donors to create two unique GAGs with different glycosidic linkages. We hypothesized that the similarity of one of these sugar structures, namely β-linked GlcA, could allow the noncognate HEP or HA to serve as an acceptor for either HA or HEP synthases, respectively.

Short 8-aminonaphthalane-1,3,4-trisulfonic acid (ANTS)-tagged HA acceptors were incubated with UDP-GlcA, UDP-GlcNAc and the HEP synthase PmHS-G. PmHS-G extended the GlcA-nonreducing termini, but not GlcNAc-nonreducing termini, of HA oligomers with HEP to create HEP-HA chimeric GAG constructs (Figure 7A, lane 5 vs. 3; Figure 8, *note*: the chain's reducing termini is shown on the right by convention). Heparinase III digested these chimeric polymers to the size approximating the original HA acceptor (lane 4). This test was repeated with longer, 12-kDa HA polysaccharides as the acceptor. Again, only GlcA-terminated HA was extended (data not shown). We tested if this chimera-making ability was due to the

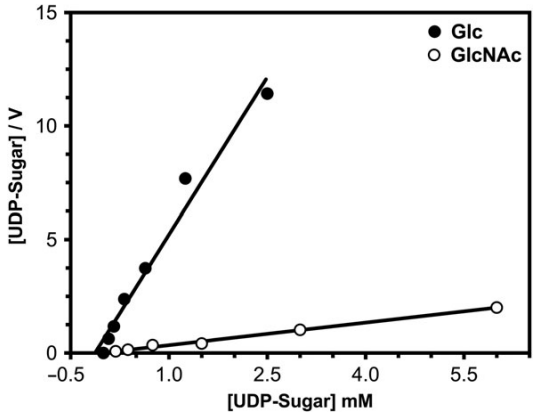

Fig. 6. PmHS-G enzyme kinetics for UDP-Glc and UDP-GlcNAc substrates. The co-polymerization of UDP-[^{3}H]GlcA with either UDP-Glc or UDP-GlcNAc by PmHS-G was examined to compare the utilization of this C2 analog on polymer synthesis against the natural substrate. Hanes–Woolf analysis (representative data set from averaged triplicate assays plotted here) of the polymerization suggests that the sugar donors, UDP-Glc or UDP-GlcNAc, bind with similar affinity to the synthase's α1,4-GlcNAc-transferase active site due to roughly equivalent apparent K_M values (indicated by convergence of both lines at the *x*-axis). However, the V_{max} difference (indicated by the line slopes) between these two reactions was substantial, ~0.22 vs. ~3.4 pmol GlcA monosaccharide transfer/min/pmol protein, for HBA or HEP, respectively, indicating that the artificial polymer is elongated more slowly than the natural GAG under these conditions.

PmHS2 or PmHS1 components of PmHS-G. As with the creation of HBA, the more promiscuous catalyst PmHS2, but not the stringent PmHS1, polymerized GlcA-terminated HA acceptors with HEP (data not shown).

We also tested if production of the alternative chimeric polymer construct, HA-HEP (Figure 8), was possible by using the HA synthase to extend a HEP primer. ANTS-tagged HEP oligomers were incubated with UDP sugars and PmHAS. Again, GlcA-terminated HEP oligomers, but not GlcNAc-terminated ones, were able to be polymerized by PmHAS (Figure 7B, lane 4 vs. 5, respectively). This type of reaction was repeated with 12.5-kDa HEP chains. Again, only GlcA-terminated HEP was extended by PmHAS. This HA extension was digested by testicular hyaluronidase reverting the chimeric product to the size of the original HEP acceptor (data not shown).

Discussion

We describe several methods for synthesizing novel GAGs or GAG-like polymers (Figure 8) that have the potential to more specifically or differentially bind partners and elicit cell signaling. In addition, the observations of tolerated vs. nontolerated reactions with these GAGs provide new structure/function insights into our catalytic tools, which is important as 3D structures are not available for either *Pasteurella* HA or HEP synthases to guide our efforts. The structure of an enzyme similar to PmHAS, the chondroitin polymerase or synthase, KfoC, has been solved (Osawa et al. 2009), but only with bound UDP-sugar precursors, *not* the acceptors or a nascent GAG chain and thus the underlying molecular mechanism(s) remain unknown. Our findings, as detailed later, suggest that the most likely obstacle to GAG analog syntheses is the stringency of the acceptor-binding pockets.

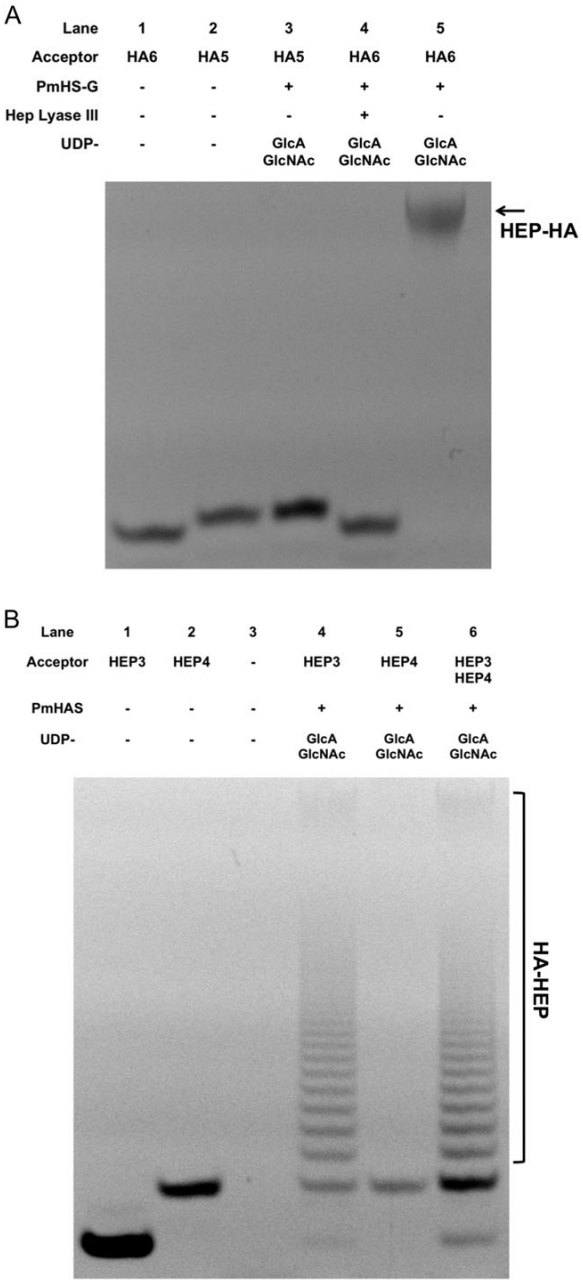

Fig. 7. GlcA-terminated GAG acceptors are extended by noncognate catalysts to form chimeric polymers. To determine if GAG synthases could polymerize noncognate acceptors, oligosaccharides were tagged with ANTS dye (to avoid confusion with any synthesis spawned via de novo initiation from UDP sugars since both PmHS and PmHEP catalysts utilize the same precursors), reacted with UDP sugars and noncognate enzyme, and monitored for a change in migration on 20% PAGE gels. (Panel **A**) HEP synthase (PmHS-G) extended GlcA-terminated (lane 5; product denoted with an *arrow*), but not GlcNAc-terminated, *HA* acceptors (lane 3). The HEP extension was digested by Heparinase III, thus reverting to a small oligosaccharide acceptor (lane 5 vs. 4). (Panel **B**) The HA synthase, PmHAS, extended GlcA-terminated (lane 4 product denoted with a *bracket*), but not GlcNAc-terminated (lane 5), *HEP* acceptors. Subsequent experiments revealed this extension to be hyaluronidase digestible thus proof of an HA chain (data not shown and Supplementary data, Figure S2).

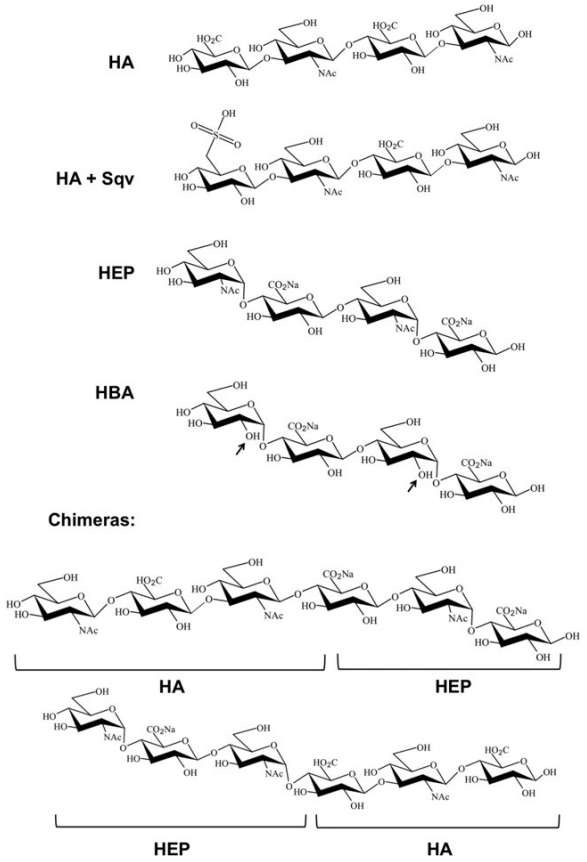

Fig. 8. Schematic structures of natural and synthetic polymers. HA acceptors were extended either with one unit of the C6 sulfated sugar, Sqv or long HEP chains (the chimera HEP-HA) by PmHS-G and PmHS2 (note that for chimeric polymer formation, the HA acceptor must be GlcA-terminated). These HEP synthases also created the novel polysaccharide, HBA ([-4-GlcAβ1-4-Glc-α1-]$_n$) when supplied only UDP-GlcA and UDP-Glc; in comparison to natural HEP, the NAc group is substituted with a hydroxyl (denoted with *arrows*). In addition, GlcA-terminated HEP primers were extended with HA chains by PmHAS to form HA-HEP chimeras.

Use of UDP-sulfated sugars for polymer synthesis

Sulfation plays an essential role in mediating protein interactions with different GAGs. Interestingly, the GAG that is not naturally sulfated, HA, was capped in vitro by PmHAS with a C6-sulfonated Glc donor when used in place of UDP-GlcA (Figure 7). Unfortunately, this novel HA structure was unable to be further extended under our tested conditions with the existing catalyst, suggesting that this unnatural nonreducing terminus is a poor acceptor for the next round of sugar addition. The appearance of Glc addition to the oligomers reacted with UDP-Sqv was initially unexpected; we determined through analytical chromatography that there was some UDP-Glc contaminating the original UDP-Sqv preparations (*data not shown*).

Synthesis and potential utility of HBA

HBA, the new polysaccharide composed of repeating disaccharide units with [-4-GlcAβ1-4-Glcα1-]$_n$ (Figure 8), is not strictly considered a glycos*amino*glycan, since it does not contain a hexosamine. HBA gives structure/function insights into its catalyst, PmHS2. An interesting observation is the use of Glc as the "uronic acid" component of the disaccharide repeat unit by PmHS2. When Glc was used as a "hexosamine" first and then extended by another Glc, substituting for "uronic acid", evidence for a third Glc (thus used as a second "hexosamine") addition was not observed. However, when Glc was used first as an "uronic acid" to extend a GlcNAc-terminated acceptor, no further extension was observed beyond the first Glc. This observation suggests that the C6 carboxylate of GlcA may be important for acceptor binding and a gate-keeper regulating polymerization activity. Without a 3D structure of the HEP synthase enzymes, we are somewhat dependent on these types of empirical observations to make better glycoengineering decisions in the future.

Elucidating the exact etiology of slow HBA synthesis by the HEP synthase is complicated due to the intrinsically nonprocessive nature of this bifunctional enzyme manipulating four distinct substrates (i.e. two UDP-sugar donors and two polymer acceptors with different nonreducing termini) in a repetitive fashion; in-depth analysis of PmHS2's pair of glycosyltransferase domains remains difficult to tease apart at this time. We hypothesize that the catalytic bottleneck for HBA may potentially be due to (i) the monosaccharide unit from UDP-Glc transferring slowly or inefficiently to the GlcA-terminated acceptor, and/or (ii) a Glc-terminated nascent chain acting as a poor acceptor for the UDP-GlcA donor (e.g., a kinetic issue during GlcA transfer and/or the result of lower affinity sugar chain/synthase interaction(s)).

Despite its slower synthesis rate and lower reaction yields in the current production protocol, HBA provides a new heparinoid canvas for testing GAG bioactivity specificity because it lacks the C2-amide and instead possesses a C2-hydroxyl. Any HS/heparin-active proteins that require or prefer a NAc or a NS group may not strongly recognize this polymer, but a subset of these roughly 200 distinct proteins may still interact with a sulfated HBA derivative. In the future, HBA should also provide new information about how the various HS-modifying enzymes operate. It is also not yet known if the steps later in the natural biosynthetic pathway will identify HBA as a substrate without other modifications that are usually prerequisites such as those mediated by the *N*-deacetylase/*N*-sulfotransferase.

In addition to the insights above, the HBA polysaccharide itself provides information into the structure/function of Heparinase III, an important HS/heparin-degrading enzyme employed as a profiling tool by both basic researchers and drug manufacturers. This lyase's binding cleft accommodates at least four saccharides of a heparin chain. Our research supports previous findings that amino acid residues in this cleft do not strongly or critically interact with the substrate's NAc moieties (Hashimoto et al. 2014) as this functionality is not present in HBA, a polymer which is sensitive to Heparinase III digestion. Testosteronan [-4-GlcA-α1-4-GlcNAc-α1-]$_n$, a polymer that has the same monosaccharide composition as HEP but different linkages, is *not* recognized by Heparinase III (Otto et al. 2011). The difference between the susceptibility of testosteronan (recalcitrant) and HBA (cleavable) to Heparinase III cleavage suggests that the β1,4 linkage is still important in substrate recognition and digestion, even though it is not actually the targeted cleavage site. However, our results showing that the *S. pneumoniae* Type 3 capsular polymer [-3-GlcA-β1-4-Glcβ1-]$_n$ was not digested by Heparinase III confirms that the β1,4 linkage alone is not sufficient to allow cleavage.

Potential chimeric GAG construct insights and applications

The synthesis of chimeric GAGs increases our understanding of how acceptor structure influences the activity of synthase catalysts; compared to donor recognition and transfer, our knowledge of acceptor usage is more primitive. In a previous report from our group (Tracy et al. 2007), the production of various HA-chondroitin and chondroitin-HA chimeras was reported, but there were only minor differences in the sugar components of this system. Specifically, these two GAGs differ only at the C4 position of the HexNAc (i.e., GalNAc vs. GlcNAc epimers) and the two relevant synthases are quite homologous (~87% identical at the protein level) thus not as great of a leap as the two dissimilar HA/HEP chains (very different glycosidic linkages) and their corresponding catalysts (very disparate amino acid sequences, including different CAZY glycosyltransferase family domains).

In this work, we show that the penultimate linkage, α1,4, (linking the -2 and -3 sugar unit of the GAG chain, where +1 position is the new incoming sugar from UDP-sugar donor) in GlcA-terminated HEP did not prevent the PmHAS enzyme from building onto HEP chains. This observation suggests that the final linkage in the HEP chain, GlcAβ1,4, was similar enough to the GlcAβ1,3 of HA, in the same −1 sugar position, to be extended. On the other hand, HA's β-1,4-linked GlcNAc at the -1 position did not entice PmHS2 to promiscuously add another α1,4-linked GlcNAc, indicating that the acceptor's terminal glycosidic sugar (−1 position) stereochemistry is very important in regulating sugar extensions.

PmHS2, but not PmHS1, was capable of creating HEP-HA chimeras. Although PmHS2 and PmHS1 are 73% identical at the amino acid level, the differences probably confer PmHS2 with increased donor and acceptor flexibility. PmHS1 is only found in *P. multocida* microbes producing Type D capsules (HEP), but PmHS2 exists in microbes with Type A (HA), Type D and Type F (chondroitin) capsules. Furthermore, the gene encoding PmHS2 (*hssB*), unlike PmHS1 and many other GAG genes, is not located in the capsule locus, flanked by other GAG synthases or export genes. This information was originally postulated to play a role in potential capsule switching: allowing microbes to switch from one type of capsule, such as HA in *P. multocida* Type A, to a HEP capsule when stimulated by appropriate environmental conditions (DeAngelis and White 2004).

In an expansion of this hypothesis, PmHS2 may allow synthesis of chimeric sugar capsules, or potentially HBA capsules, to enhance the microbe's infectious potential. *Streptococcus pneumoniae* Type 3 synthesis is regulated by the relative ratio of UDP-Glc to UDP-GlcA: longer polymers are produced at higher rates when UDP-Glc is in excess of UDP-GlcA, but if in too much excess, the rate is reduced (Forsee et al. 2009). It is possible that under stressed conditions or environmental cues, such as relative UDP-sugar availability, PmHS2 activation could give the microbes a more dynamic survival response, providing several options for altering capsular polymer structure thus a wider spectrum of molecular camouflage. In a preliminary test of this hypothesis, we examined the spent artificial culture media of *P. multocida* Type A and Type F (HA or chondroitin capsule, respectively) for the presence of the HBA disaccharide units (via analysis as in Figure 4B), but none was observed. This finding, however, does not rule out the possibility of HBA or chimeric polymer production in vivo.

The creation of HA-HEP or HEP-HA chimeric constructs (Figure 7) has interesting therapeutic implications as well. Sulfating the HEP portion via in vitro enzymatic modification reactions has potential to create a single bio-active molecule capable of triggering both HS and HA signaling pathways. This might be helpful in certain medical conditions, for example, applications requiring $VEGF_{165a}$ targeting. Both sulfated HA and HS bind and down-regulate the activity of this pro-angiogenic protein (Beckouche et al. 2015; Lim et al. 2015). Delivering both compounds simultaneously may provide a synergistic effect. Another translational possibility is that such chimeric constructs can be used as a bioadhesive, tethering cells that express HA receptors/hyaladherins to other cells with HS receptors/binding proteins.

In summary, this study created novel strategies for examining and improving therapeutic GAG production and provided insights into the existing catalytic tools.

Materials and methods

Acceptor, donor and enzyme reagents

All defined HA and HEP oligosaccharides and polysaccharides were gifts from Hyalose, LLC or Caisson Biotech, LLC (both in Oklahoma City, OK), respectively, prepared in general as previously reported (Jing and DeAngelis 2004; Sismey-Ragatz et al. 2007). UDP-Glc was purchased from MP Biomedicals (Santa Ana, CA). UDP-GlcNAc, UDP-GlcA and all other chemicals, unless noted, were purchased from Sigma-Aldrich (St. Louis, MO). UDP-Sulfoquinovose (UDP-Sqv) was prepared by the action of Agl3 enzyme as described previously (Meyer et al. 2011), but then further purified by an additional strong anion exchange treatment. Here, samples were loaded onto a Sepharose Q column (GE; Pittsburg, PA) equilibrated with 50 mM Tris, pH 8.6. The column was washed with 50 mM ammonium acetate (3–6 column volumes, CVs), then 50 mM ammonium acetate with 150 mM NaCl (3–6 CVs). The UDP-Sqv was eluted using a 2 M ammonium formate step (6 CVs). The eluted fraction was lyophilized thrice from water to remove the volatile buffer. PnP3 was obtained from the ATCC (Manassas, VA).

An *Escherichia coli*-derived recombinant truncated (residues 1-703) version of PmHAS was used to make HA products (Pummill et al. 2007). Three different *E. coli*-derived recombinant maltose-binding protein fusions with the *P. multocida* HEP synthase enzymes were used for making HEP: HEP synthase 1 (PmHS1), HEP synthase 2 (PmHS2) and a chimeric protein of these two enzymes called HEP synthase G (PmHS-G) (Sismey-Ragatz et al. 2007; Otto et al. 2012).

Fluorescent tagging of oligosaccharide acceptors and gel electrophoresis

HEP-benzaldehyde (HEP-BnAl) and HA acceptors (typically ~30 μg) with naturally occurring aldehyde reducing ends were tagged with AMAC or ANTS dyes by reductive amination. For AMAC, freeze-dried oligosaccharide samples were incubated with 10 μL of 12.5 mM AMAC in dimethylsulfoxide (DMSO)/acetic acid (17:3, *v/v*) for 10 min at room temperature. An equal volume of 1.25 M $NaBH_3CN$ in water was added to the sample and then incubated overnight at 37°C. For ANTS labeling, 10 μL of 50 mM ANTS in 15% acetic acid was added to the freeze-dried oligosaccharides. After a 10-min incubation at room temperature, 10 μL of fresh 1 M cyanoborohydride in DMSO was added and incubated at 37°C overnight.

Labeled oligosaccharides were separated from free dye using GlykoPrep Clean-Up cartridges with acetonitrile for AMAC-labeled

oligos and the APTS buffer (Prozyme, Hayward, CA) for ANTS-labeled products. Yield was estimated by the Beers–Lambert equation with absorbances at 260 nm for AMAC or 354 nm for ANTS (using extinction coefficients 40,000 or 6500 M^{-1} cm^{-1}, respectively).

AMAC-tagged oligosaccharides (~100 pmol) were run on a 21% acrylamide tris acetate EDTA gel (37.5:1 monomer: cross-linker; Bio-Rad, Hercules, CA) with a 4% acrylamide stacking gel in 50 mM Tris base/50 mM Tricine running buffer, pH 8.1–8.3. Gels were run at 60 V for 15–20 min and 300 V for 40–60 min on ice, in the dark. ANTS-tagged polymers were run on 20% acrylamide Tris/Tricine gels at 300 V for 40–60 min on ice, in the dark. Gels were visualized using an Alpha Innotech Corporation (San Leandro, CA) gel imaging system using long-wave ultraviolet excitation.

In addition, untagged polymers (~1 μg/lane) were run on 12% 1× tris borate EDTA PAGE and stained with Alcian blue as described (Ikegami-Kawai and Takahashi 2002).

Single sugar extension and polymerization reactions

In general for single sugar extensions AMAC-, ANTS- (~50–100 pmol) or unlabeled (~1 μg) oligosaccharide acceptors were incubated with a specific UDP sugar, as noted, at 0.2–2 mM, 0.2–2 mg/mL synthase enzyme (type as noted in each experiment), 2–10 mM $MnCl_2$, and 50 mM HEPES, pH 7.2, for 2–20 h at 30°C. HA synthase reactions also included 1 M ethylene glycol as an enzyme activity stabilizer. For polymerization tests, the various oligosaccharide acceptors were incubated with two types of UDP sugars at 2 mM as noted under the same conditions as the single sugar extensions above.

The apparent K_M and V_{max} of the donors for the chimeric HEP synthase, PmHS-G, were obtained in kinetic experiments employing radiolabeled sugar incorporation assays using the same buffer conditions as the FACE tests and a 12.5-kDa HEP acceptor (2 μg) to facilitate isolation of even short polymer extensions during the initial linear phase of the reaction. A constant saturating concentration of UDP-[^{3}H]GlcA (1 mM; 0.34 μCi/assay) was used with a titration of either UDP-Glc (0.08–2.5 mM) or UDP-GlcNAc (0.19–6 mM). Due to the relative slowness of HBA vs. HEP synthesis, co-polymerizations with UDP-Glc required more catalyst and longer reaction times than with UDP-GlcNAc (5 μg PmHS-G, 10-min reaction *or* 1 μg, 6-min, respectively). Descending paper chromatography on Whatman 3 MM (GE) with overnight development in 65:35 (*v*/*v*) ethanol/1 M ammonium acetate, pH 5.5, solvent was utilized to isolate the polymer products (remaining at the origin) from the unincorporated donors. The origin of the chromatogram was then excised, eluted with water for 1 h, and mixed with EconoSafe cocktail (Research Products Intl.; Mt. Prospect, IL) before liquid scintillation counting. The kinetic values were obtained from two independent experiments with averaged duplicate or triplicate assay points and data analysis by Hanes–Woolf plots.

MALDI-ToF MS of GAG products

Post-incubation, reaction mixtures were co-spotted with an equal volume of 6.5 mg/mL 6-aza-2-thyothymine matrix in 50% acetonitrile with 0.1% trifluoroacetic acid and air dried (Otto et al. 2011). An UltraFlex II MALDI instrument (Bruker, Billerica, MA) was used in negative ion mode with laser at 20% power, reflector and 40% detector gain to obtain spectra.

Reversed phase–HPLC–ESI–MS

HBA polysaccharide (~5-kDa polymer synthesized on the trisaccharide HEP acceptor, ANA-BnAl) was digested by recombinant Heparinase III (10 mU) in 100 μL of digestion buffer (50 mM NH_4Ac, 2 mM $CaCl_2$, pH 7.0) at 37°C overnight. The reaction was terminated by eliminating the enzyme by passing through 3-kDa molecular weight cut-off spin columns (Millipore; Billerica, MA). The filter unit was washed twice with 200 μL distilled water and the combined filtrate was lyophilized. The dried samples were AMAC-labeled by adding 10 μL of 0.1 M AMAC in DMSO/acetic acid (17:3, *v*/*v*), incubating at room temperature for 10 min, followed by adding 10 μL of 1 M aqueous sodium cyanoborohydride, and incubating for 1 h at 45°C. A mixture containing two kinds of disaccharide standards (HS 0S standard, Iduron, Manchester, UK) prepared at 12.5 ng/μL was similarly AMAC-labeled and used for each run as an external standard. The resulting samples were centrifuged at 12,000 × *g* for 10 min. Finally, supernatant was collected and stored in a light resistant container at room temperature until analyzed.

LC–MS analyses were performed on an Agilent 1200 LC/MSD Instrument (Agilent Technologies, Inc., Wilmington, DE) equipped with a 6300 ion-trap, a binary pump and a Poroshell 120 EC-C18 column (3 × 50 mm, 2.7 μm; Agilent). Mobile phase A was 50 mM NH_4Ac in water and mobile phase B was methanol. A gradient of 10–35% B for 10 min was run with a flow rate of 250 μL/min at 45°C. The electrospray was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V and a source temperature of 350°C. Mass range of the spectrum was 300–900 *m*/*z*. Nitrogen (8 L/min, 40 psi) was used as drying and nebulizing gas.

NMR spectroscopy

NMR data were obtained on an Advance II 800 MHz spectrometer (Bruker Bio-Spin, Billerica, MA). The HBA sample (~5-kDa polymer by PAGE built on a HEP3-BnAl primer; 0.6 mg) was dissolved in 200 μL of D_2O (99.9 atom%, Sigma) and lyophilized thrice from D_2O for deuterium exchange. After deuterium exchange, the sample was dissolved in 70 μL of D_2O (99.996 atom%, Cambridge Isotopes, Tewksbury, MA) and analysis was performed in an 80-μL micro NMR tube. The 1D-^{1}H NMR experiment of HBA was performed using a 30° flip angle for 32 scans with a 5 s relaxation delay. No water suppression was used. The 2D HSQC-NMR spectrum of the polysaccharide was obtained with 40 scans in the direct dimension (^{1}H), 380 experiments for the indirect dimension (^{13}C). The 2D TOCSY-NMR spectrum of the polysaccharide was obtained with 24 scans in the direct dimension (^{1}H), 950 experiments for the indirect dimension (^{1}H).

Supplementary data

Supplementary data is available at *GLYCOBIOLOGY* online.

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Conflict of interest statement

None declared.

Abbreviations

AMAC, 2-aminoacridone; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DMSO, dimethylsulfoxide; GAGs, glycosaminoglycans; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; HA, hyaluronan; HBA, hepbiuronic acid; HEP, heparosan; Heparinase III, heparin lyase III; HS, heparan sulfate; HSQC, heteronuclear single quantum coherence; MALDI-ToF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PmHAS, *P. multocida* HA synthase; PmHS, *P. multocida* HEP synthase; PnP3, *S. pneumoniae* Type 3 polysaccharide; Sqv, sulfoquinovoseTOCSY, total correlated spectroscopy; UDP, uridine diphospho.

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