



# The 3-*O*-sulfation of heparan sulfate modulates protein binding and lyase degradation

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Humans express seven heparan sulfate (HS) 3-*O*-sulfotransferases that differ in substrate specificity and tissue expression. Although genetic studies have indicated that 3-*O*-sulfated HS modulates many biological processes, ligand requirements for proteins engaging with HS modified by 3-*O*-sulfate (3-OS) have been difficult to determine. In particular, the context in which the 3-OS group needs to be presented for binding is largely unknown. We describe herein a modular synthetic approach that can provide structurally diverse HS oligosaccharides with and without 3-OS. The methodology was employed to prepare 27 hexasaccharides that were printed as a glycan microarray to examine ligand requirements of a wide range of HS-binding proteins. The binding selectivity of antithrombin-III (AT-III) compared well with anti-Factor Xa activity supporting robustness of the array technology. Many of the other examined HS-binding proteins required an IdoA2S-GlcNS3S6S sequon for binding but exhibited variable dependence for the 2-OS and 6-OS moieties, and a GlcA or IdoA2S residue neighboring the central GlcNS3S. The HS oligosaccharides were also examined as inhibitors of cell entry by herpes simplex virus type 1, which, surprisingly, showed a lack of dependence of 3-OS, indicating that, instead of glycoprotein D (gD), they competitively bind to gB and gC. The compounds were also used to examine substrate specificities of heparin lyases, which are enzymes used for depolymerization of HS/heparin for sequence determination and production of therapeutic heparins. It was found that cleavage by lyase II is influenced by 3-OS, while digestion by lyase I is only affected by 2-OS. Lyase III exhibited sensitivity to both 3-OS and 2-OS.

3-*O*-sulfation | glycan microarray | anti-Factor Xa | herpes simplex virus 1 | heparin lyases

Heparan sulfates (HSs) are highly sulfated polysaccharides that reside on the surface and in the extracellular matrix of virtually all cells of multicellular organisms (1, 2). A large number of proteins, including blood coagulation factors, growth factors and morphogens, chemokines and cytokines, proteins involved in complement activation, and cell adhesion and signaling proteins can bind to HS, resulting in conformational changes, stabilization of receptor–ligand complexes, protein oligomerization, sequestration, and protection against degradation (3, 4). These molecular recognition events regulate many physiological processes, including embryogenesis, angiogenesis, blood coagulation, and inflammation. These interactions are also important for many disease processes such as cancer, viral and bacterial infections, neurological disorders, and a number of genetic diseases (1–4).

The biosynthesis of HS starts with the assembly of a protein-bound polymer composed of alternating *N*-acetyl glucosamine (GlcNAc) and glucuronic acid (GlcA) residues. Discrete regions of this polymer are modified by *N*-deacetylase/*N*-sulfotransferases to replace *N*-acetyl by *N*-sulfate moieties. Subsequently, the regions of *N*-sulfation are further modified by a C-5 epimerase that converts

GlcA into iduronic acid (IdoA), followed by *O*-sulfation by iduronosyl 2-*O*-sulfotransferase (2-OST), glucosaminyl 6-*O*-sulfotransferases (6-OST), and 3-*O*-sulfotransferases (3-OST) (5).

HS modifications are often incomplete, resulting in at least 20 different HS disaccharide moieties, which can be combined in different manners, creating considerable structural diversity (4, 6). The way these disaccharides are arranged is not random but dictated by the substrate specificities of the HS biosynthetic enzymes. These enzymes are present in multiple isoforms, each having unique substrate specificity. It has been hypothesized that, by regulating the expression of the isoforms of HS biosynthetic enzymes, cells can create unique HS epitopes (4, 7). The so-called “HS sulfate code hypothesis” is based on the notion that such epitopes can recruit specific HS-binding proteins, thereby mediating multiple biological and disease processes.

Vertebrates express seven 3-OST isozymes in a cell- and tissue-selective manner. It is the largest family of HS-modifying enzymes, indicating that, in concert with other sulfotransferases, they have an ability to create unique epitopes for recruitment of specific proteins (8). A prototypic example of a protein requiring binding to 3-*O*-sulfated HS is antithrombin-III (AT-III) to confer anticoagulant activity. The pentasaccharide GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S has been identified as high-affinity ligand for AT-III (9). Removal of the sulfate at C-3 of *N*-sulfoglucosamine (GlcNS3S) results in a 10<sup>5</sup>-fold reduction in

## Significance

Although heparan sulfate (HS) modified by a 3-*O*-sulfate (3-OS) has been implicated in many biological processes, there is only a rudimentary understanding of ligand requirements of HS-binding proteins requiring this type of modification. We developed a modular synthetic approach that provided a structurally diverse collection of HS oligosaccharides with and without 3-OS. The compounds were printed as a glycan microarray to determine ligand requirements of HS-binding proteins, which revealed specific HS sequons for binding, and led to the discovery of proteins needing 3-OS for binding. The compounds made it possible to determine receptor requirements of herpes simplex virus, and substrate specificities of heparin lyases. The platform provides an important means to examine the biology of 3-OS–modified HS.

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binding affinity. The context in which the 3-*O*-sulfate (3-OS) is presented is also important, and removal of any other sulfate leads to a substantial reduction in binding affinity. Glycoprotein D (gD) of HSV-1 is another example of an early discovered protein that binds to HS epitopes having a 3-*O*-sulfated GlcNS residue (10). The interaction of gD with HS in concert with other viral envelope proteins is critical for triggering fusion with the host cell surface membrane. Although HS oligosaccharides have been used to probe ligand requirements of gD, the optimal carbohydrate sequence remains to be determined. A number of other proteins are known to require a 3-*O*-sulfated HS epitope for binding and biological activity, and examples include neuropilin-1 (Nrp-1) (11), cyclophilin B (12), stabilin (13), receptor for advanced glycosylation end product (RAGE) (8), and fibroblast growth factor-7 (FGF-7) (14). The context in which the 3-OS group needs to be presented for optimal binding is largely unknown. It is the expectation that many other proteins need a 3-*O*-sulfoglucosamine moiety for binding and biological activity. In this respect, this modification has been implicated in many physiological and disease processes, including cell differentiation, axon guidance and growth of neurons, inflammation, vascular diseases, and tumor progression, yet HS-binding proteins that are involved in these diseases are often not known (8).

There are indications that 3-*O*-sulfation can interfere in the degradation of HS by heparin lyases (15, 16). These enzymes are critical for the analysis of heparin/HS (Hep/HS) by controlled depolymerization into smaller fragments, which can then be more readily analyzed by various methods including mass spectrometry (MS) (17). Furthermore, a mixture of lyases I, II, and III can digest heparin into disaccharides facilitating compositional analysis (18). Lyases are also employed for the production of low molecular weight heparins (LMWHs) with higher anticoagulant activities and improved pharmacokinetic profiles (19). Treatment of heparin with a mixture of lyases I, II, and III results in the formation of “resistant” trisaccharides and tetrasaccharides that usually contain a 3-OS moiety (15). These studies have been performed with heparin, which is structurally less diverse compared to HS, and, as a result, there is limited knowledge of how a 3-*O*-sulfation impacts the degradation of HS by lyases (19).

The rudimentary understanding of ligand requirements of HS-binding proteins requiring a GlcNS3S moiety is, in part, due to the fact that this modification is relatively rare and difficult to detect and analyze. This is compounded by a lack of robust technologies that can establish the importance of a 3-OS moiety for binding and biological activity. It is the expectation that a sufficiently large collection of synthetic HS oligosaccharides with and without a 3-OS will provide a powerful discovery tool to establish ligand requirements of 3-OS-binding proteins. Such a collection of compounds will also be valuable to define substrate specificities of heparin lyases and other HS-processing enzymes.

Although synthetic approaches to prepare HS oligosaccharides have progressed (7, 20), the preparation of a sufficiently large collection of compounds is still challenging, and careful consideration should be given to compounds selection. A literature survey indicates that GlcNS3S moieties can be flanked by different types of uronic acids, and typical sequences include GlcA-GlcNS3S-GlcA (13, 21), IdoA-GlcNS3S-GlcA (22), IdoA2S-GlcNS3S-GlcA (23–25), GlcA-GlcNS3S-IdoA (13, 22, 23), IdoA-GlcNS3S-IdoA (22), IdoA2S-GlcNS3S-IdoA (25), GlcA-GlcNS3S-IdoA2S (13, 23, 26–28), and IdoA2S-GlcNS3S-IdoA2S (24, 29, 30) (Fig. 1A). In these sequences, the GlcNS3S moieties can be further modified by a sulfate ester at C-6. Such a modification is preferentially installed by specific isozymes, and, for example, it is known that 3-OST-1 prefers substrate having 6-OS on GlcNS, whereas 3-OST-3 has a higher activity for compounds with a hydroxyl at this position (24). To corroborate the importance of 3-OS for binding, it is also important to prepare structural counterparts without such a functionality.

Based on these considerations, 27 synthetic HS oligosaccharides were designed based on nine different core trisaccharides encompassing all relevant uronic acid modifications (Fig. 1B and C). The central GlcNAc moiety of the nine templates is modified by either a 3-OS, 6-OS, or 3,6-OS and extended at the reducing and nonreducing end by GlcNS6S and GlcA-GlcNS6S, respectively to give sufficiently large set of compounds for binding and enzymology studies. It is expected that, after hit identification, the constant regions can be further optimized in a systematic manner to identify the optimal ligand.

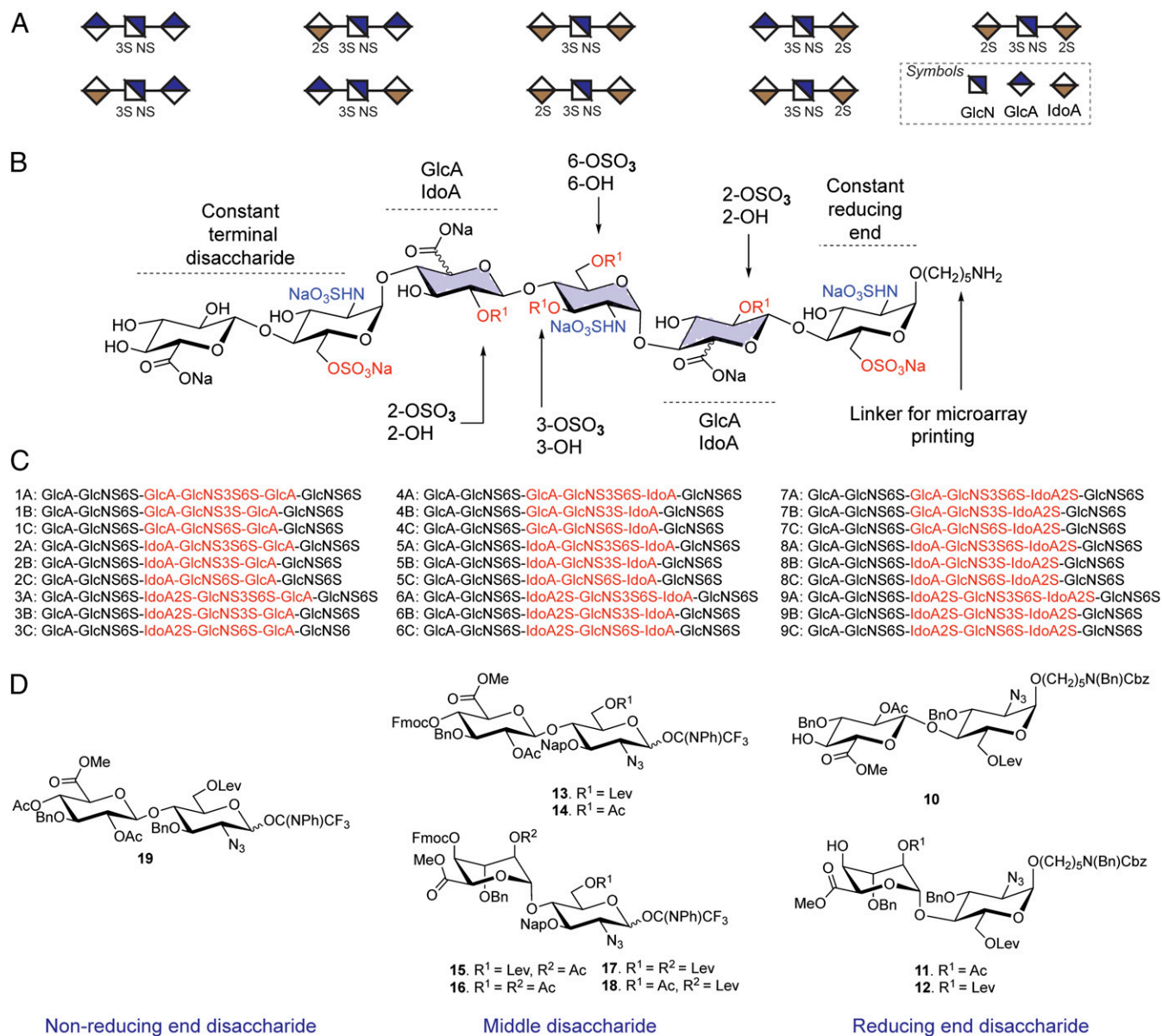
## Results

**Synthesis of Focused HS Library with and without 3-OS.** The preparation of a library of 27 structurally diverse HS hexasaccharides with or without a 3-OS represents a considerable synthetic challenge. Several laboratories have successfully prepared HS oligosaccharides, and, although elegant, these approaches are mainly focused on the preparation of compounds composed of the same repeating unit (7). Efforts to prepare 3-*O*-sulfated derivatives have almost exclusively focused on the preparation of the AT-III pentasaccharide (9). Enzyme-mediated synthesis of HS oligosaccharides requires significantly fewer steps but cannot provide a wide range or well-defined structures due to the promiscuity of the biosynthetic enzymes (31). As a result, structure–activity relationship (SAR) studies have employed small numbers or unfocused sets of HS oligosaccharides that cannot properly probe ligand selectivities of HS-binding proteins.

Previously, we reported a modular synthetic approach for the facile synthesis of a wide array of HS oligosaccharides (32, 33). It employs a set of disaccharide building blocks that resemble differently sulfated disaccharide moieties found in HS that repeatedly can be employed for the synthesis of panels of oligosaccharides.

Here, we substantially expand the capabilities of the modular strategy to include compounds having a 3-OS and envisage that the hexasaccharides **1A** to **1C** through **9A** to **9C** (Fig. 1C) could readily be prepared from disaccharide building blocks **10** to **19** (Fig. 1D). In a parallel combinatorial manner, compounds **10**, **11**, and **12** can be coupled with disaccharide donors **13** to **18** to provide a collection of tetrasaccharides. The 9-fluorenylmethyl carbonate (Fmoc) protecting group of the latter compounds can be selectively removed by a hindered base (34) to give glycosyl acceptors that can be further glycosylated with glycosyl donor **19** to give properly protected hexasaccharides differing in the pattern of GlcA and IdoA residues and modifications by levulinoyl (Lev) esters and naphthylmethyl (Nap) ether. The latter protecting groups can selectively be removed by treatment with hydrazine acetate and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), respectively, to reveal hydroxyls that can then be converted into sulfate esters. Next, the azides can be reduced to amines which can be sulfated, and global deprotection will then give the targeted library of HS oligosaccharides. In this strategy, the Nap ether serves a dual role and either can selectively be removed in presence of benzyl ethers to afford a site for sulfation or can function as a permanent protecting group and be removed during global deprotection to provide sequences lacking a 3-OS ester.

The disaccharides **10** to **19** were prepared (*SI Appendix, Schemes S1 and S2*) on a large scale from properly protected 2-azido-2-deoxy-glucopyranoside acceptors (see **S1–S4** in *SI Appendix*), thioethyl glycosyl donors (see **S5** in *SI Appendix*), and thioethyl idosyl donor (see **S6** and **S7** in *SI Appendix*). The preparation of HS hexasaccharides **1A** to **1C** (Fig. 2) is described in detail to demonstrate in which way the modular disaccharides can be employed to prepare a library of HS oligosaccharides having GlcNS modified by 3,6-OS (**1A**), 3-OS (**1B**), or 6-OS (**1C**). Thus, a triflic acid-mediated glycosylation of glycosyl donors **13** and **14** with acceptor **10** gave tetrasaccharides **20** and **21**, respectively, as only the  $\alpha$ -anomer. The anomeric configuration of



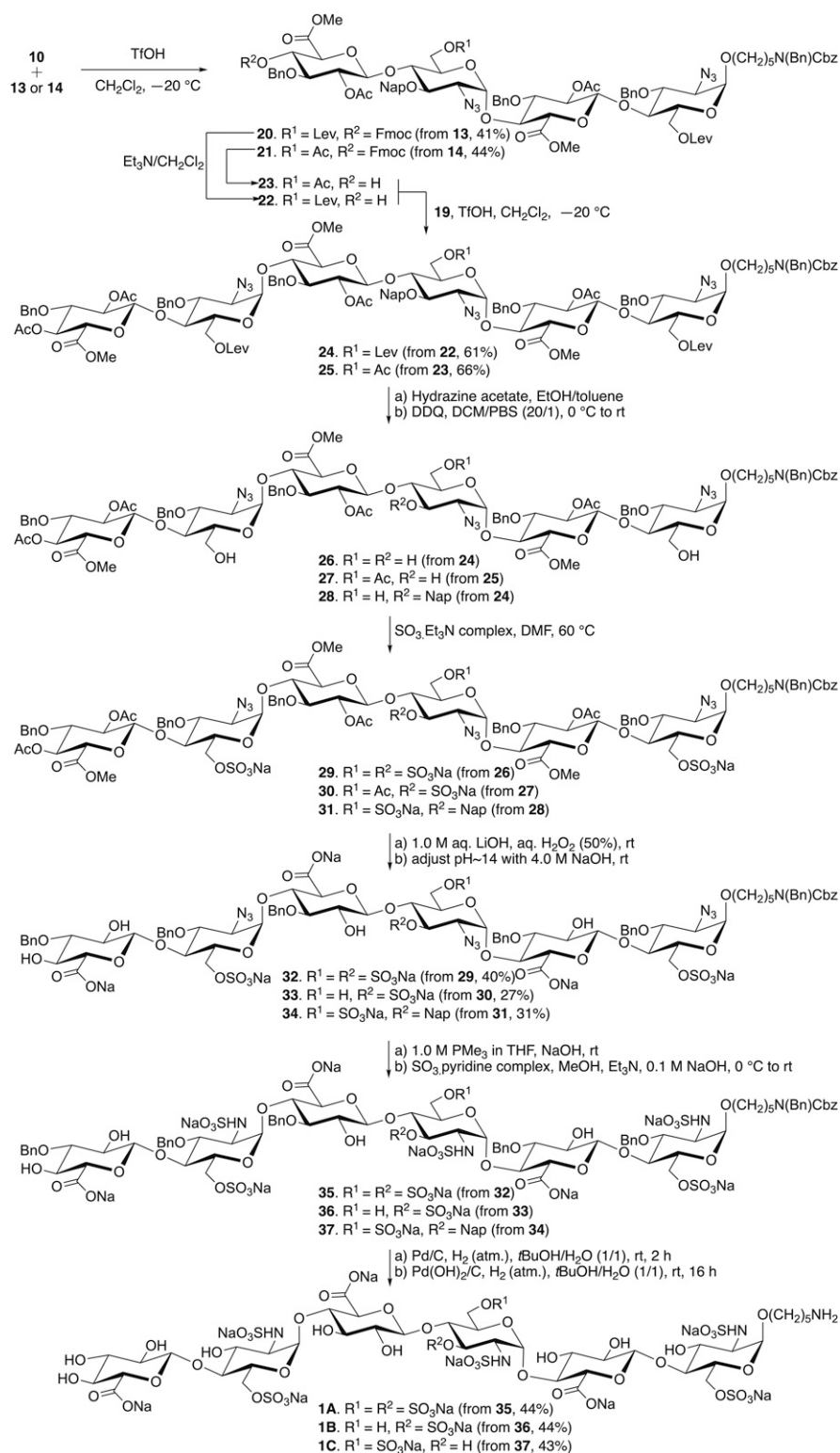
**Fig. 1.** (A) Identified substructures having 3-Os-bearing glucosamine. (B) General structure of target HS hexasaccharides, featuring structurally diverse sequence (shaded pyranose rings), site of sulfation (text in red), uronic acid composition (wavy bond at C-5 carboxylic acid), constant reducing end GlcN and nonreducing end disaccharide, and an anomeric linker for fabrication of HS arrays. (C) Hexasaccharides numbering and backbone composition, variable core trisaccharide in red color; NS, *N*-sulfate; 2S: 2-Os; 3S, 3-Os; 6S, 6-Os. (D) Disaccharide building blocks comprising acceptors **10** to **12** and donors **13** to **19** for modular assembly of hexasaccharides.

the newly formed glycosidic bond was confirmed by the  $^3J_{\text{H1,H2}}$  coupling constant (3.9 Hz),  $^1J_{\text{C1,H1}}$  coupling constants (171.0 Hz), and  $^{13}\text{C}$  chemical shifts of C-1 (97.7 ppm). Next, the Fmoc protecting group of **20** and **21** were removed with  $\text{Et}_3\text{N}$  in  $\text{CH}_2\text{Cl}_2$  to give glycosyl acceptors **22** and **23**, which were coupled with **19** in the presence of triflic acid to afford hexasaccharides **24** and **25**, respectively. Next, the Lev esters of **24** and **25** were cleaved with hydrazine acetate in EtOH/toluene, which was followed by oxidative removal of the Nap ether with DDO in a mixture of  $\text{CH}_2\text{Cl}_2$  and phosphate buffered saline (pH 7.4) (PBS) buffer to provide compounds **26** and **27** having a hydroxyl at C-3 of the central glucosamine. Additionally, compound **24** was treated with only hydrazine acetate to provide **28** having C-3 hydroxyl group of the central *N*-sulfated glucosamine (GlcN) moiety still protected as a Nap ether. The free hydroxyls of

compounds **26**, **27**, and **28** were sulfated with sulfur trioxide-triethylamine complex ( $\text{SO}_3\cdot\text{NEt}_3$ ) at elevated temperature (60 °C) for a prolonged reaction time (16 h) to give compounds **29** to **31** in high yields. It was observed that installation of 3-Os using pyridinium sulfur trioxide complex ( $\text{SO}_3\cdot\text{Py}$ ), which is conventionally used for sulfation of hydroxyls, progressed very sluggishly. Next, the methyl and acetyl esters were saponified by first treating the compounds with  $\text{H}_2\text{O}_2$  and LiOH in tetrahydrofuran (THF) followed by NaOH in MeOH to obtain **32**, **33**, and **34**, respectively.

The azides of **32** to **34** were reduced using trimethyl phosphine, and the resulting amines were *N*-sulfated employing an excess of  $\text{SO}_3\cdot\text{Py}$  complex in MeOH in the presence of  $\text{Et}_3\text{N}$  and NaOH to provide **35**, **36**, and **37**, respectively, after C-18 reverse-phase purification using a benchtop column and sodium





**Fig. 2.** An example of the synthesis of hexasaccharides having central GlcNS modified by 3,6-OS (**1A**), 3-OS (**1B**), and 6-OS (**1C**). Synthetic schemes of remaining hexasaccharides are provided in *SI Appendix, Schemes S3–S10*. Reagents: TfOH, trifluoromethanesulfonic acid;  $\text{CH}_2\text{Cl}_2$ , dichloromethane;  $\text{Et}_3\text{N}$ , triethylamine; EtOH, ethanol; DMF, dimethylformamide; LiOH, lithium hydroxide;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; NaOH, sodium hydroxide;  $\text{PMe}_3$ , trimethylphosphine; MeOH, methanol; Pd/C, palladium on carbon;  $\text{H}_2$ , hydrogen gas;  $t\text{BuOH}/\text{H}_2\text{O}$ , tert-butanol/water;  $\text{Pd(OH)}_2/\text{C}$ , palladium hydroxide on carbon.

exchange using Dowex [ $\text{Na}^+$ ] resin. Finally, the target hexasaccharides **1A** to **1C** were obtained by hydrogenation of **35** to **37** using Pd/C in a mixture of  $t\text{BuOH}/\text{H}_2\text{O}$  to cleave the protecting group of the anomeric linker, followed by further

hydrogenation over  $\text{Pd(OH)}_2/\text{C}$  to remove the benzyl and naphthylmethyl ethers, followed by purification by size exclusion chromatography (SEC) over a P-2 column and sodium exchange over Dowex [ $\text{Na}^+$ ] resin. Hexasaccharides **2A** to **2C** through **9A**

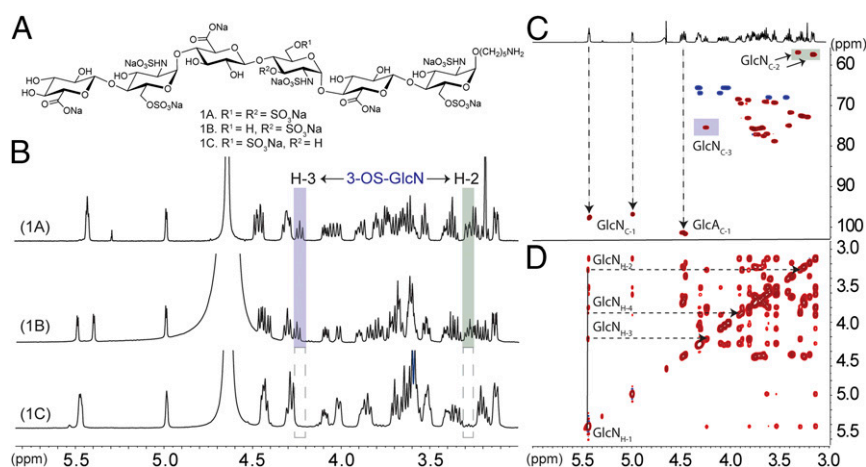
to **9C** were prepared by similar chemical manipulations using disaccharides **10** to **19** (*SI Appendix, Schemes S3–S10*). All final compounds were obtained in quantities ranging from 2 mg to 5 mg. HS hexasaccharides (**1A** to **1C** through **9A** to **9C**) were characterized by nuclear magnetic resonance spectroscopy (NMR) and high-resolution electrospray ionization-mass spectrometry (ESI-MS). The  $^1\text{H}$  NMR spectrum was fully assigned by one-dimensional (1D) and 2D NMR experiments ( $^1\text{H}$ - $^1\text{H}$  correlation spectroscopy [COSY],  $^1\text{H}$ - $^1\text{H}$  total COSY [TOCSY], and  $^1\text{H}$ - $^{13}\text{C}$  heteronuclear single quantum coherence spectroscopy [HSQC]; Fig. 3). The sites of sulfation were confirmed by downfield shifts of ring protons ( $\sim 0.5$  ppm for  $\text{GlcN}_{\text{H-6}}$  and  $\text{IdoA}_{\text{H-2}}$ , and  $\sim 0.7$  ppm for  $\text{GlcN}_{\text{H-3}}$ ) and by downfield shift of ring carbons ( $\sim 4$  ppm for  $\text{GlcN}_{\text{C-6}}$  and  $\text{IdoA}_{\text{C-2}}$ , and  $\sim 7$  ppm for  $\text{GlcN}_{\text{C-3}}$ ). Furthermore, the presence of 3-OS results in additional downfield shift of 0.2 ppm of central  $\text{GlcN}_{\text{H-2}}$  for *N*-sulfation (Fig. 3C).

**Developing an HS Oligosaccharide Array to Uncover the Importance of 3-O-Sulfation for Protein Binding Selectivities.** The synthetic HS hexasaccharides (**1A** to **1C** through **9A** to **9C**) are functionalized with an aminopentyl anomeric linker, which made it possible to develop a microarray by noncontact piezoelectric printing on *N*-hydroxysuccinamide (NHS)-activated glass slide (33). First, we examined binding selectivities of AT-III, which belongs to the serine protease inhibitor superfamily (serpins) that inactivates several enzymes of the coagulation pathway including Factor-IXa, Factor-Xa, and thrombin (35). Its enzymatic activity is greatly enhanced by binding to a 3-OS-modified pentasaccharide sequence (GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S) embedded within the heparin and HS (26). A synthetic version of this pentasaccharide (Fondaparinux) is clinically used for the prevention of deep vein thrombosis in surgical patients and for the treatment of pulmonary embolisms (9). SAR studies have shown that it has a strict ligand requirement, and removal of any of the sulfates results in considerable loss of binding and biological activity. We anticipated that microarray binding studies with AT-III would validate its robustness and provide an opportunity to investigate the influence of backbone modifications on binding. The HS microarray was incubated with biotinylated AT-III (Enzyme Research Laboratories), and binding was visualized using streptavidin-AlexaFluor635

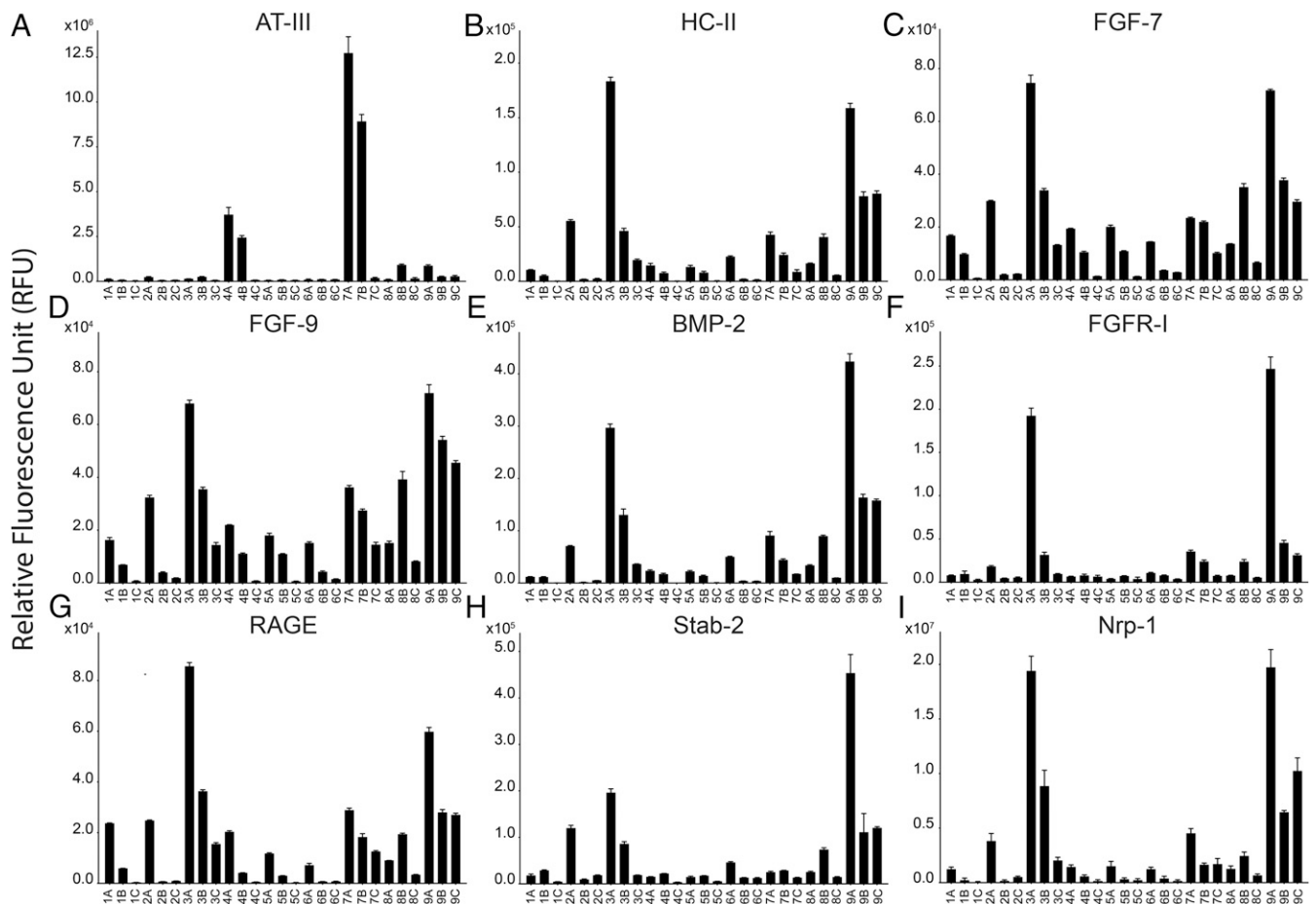
(BioLegend) (Fig. 4A and dose-response in *SI Appendix, Fig. S24*). As anticipated, compound **7A** that embeds the Fondaparinux sequence gave the strongest binding. A compound lacking a 6-OS on the central glucosamine (**7B**) gave a slightly lower responsiveness whereas a compound lacking the 3-OS did not exhibit binding, which is in agreement with previous report SAR data.

The array results also confirmed that the central GlcNS3S moiety needs to be flanked at the reducing and nonreducing end by an IdoA and GlcA moiety, respectively. Lack of 2-OS at the IdoA moiety (**4A** and **4B**) flanking the central GlcNS3S resulted only in a slight decrease in binding. In contrast to previous report that indicated that the nonreducing GlcA can be changed to IdoA2S without significant loss of activity, our findings indicates this modification (**9A**) greatly impairs binding (24). To validate the array studies, the anti-Factor Xa activity of the hexasaccharides was measured using a commercially available two-stage chromogenic assay (Biophen Anti-Xa kit). Initially, the screening was performed at 3 nM, 10 nM, 30 nM, and 5  $\mu\text{M}$  final concentration, and Fondaparinux and buffer were included as a positive and negative control, respectively (Fig. 5A and *SI Appendix, Fig. S3*). Only the identified hits of the microarray (**4A**, **4B**, **7A**, and **7B**) showed anti-FXa activity similar to that of Fondaparinux. Compounds having an IdoA2S-GlcNS3S  $\pm$  6S-IdoA2S motif (**9A** and **9B**) had low inhibitory activity ( $\sim 10$  to 20% at 30 nM concentration; *SI Appendix, Fig. S3A*), which is in agreement with the array data. Intriguingly, a non 3-OS containing hexasaccharide (compound **8C**; *SI Appendix, Fig. S3B*) reduced Factor Xa activity by  $>80\%$  at 5  $\mu\text{M}$ . We determined half-maximal inhibitory concentrations ( $\text{IC}_{50}$ ) for compounds **4A**, **4B**, **7A**, and **7B** (Fig. 5B and C), which were 7.4, 10.5, 32.2, and 9.4 nM, respectively, and comparable to Fondaparinux ( $\text{IC}_{50} = 4.9$  nM; *SI Appendix, Fig. S4*).

We also examined the HS binding specificity of heparin cofactor-II (HC-II), which is another serine protease inhibitor implicated in blood coagulation (35). Its primary, secondary, and tertiary structures bear considerable homology to AT-III (36). A computational screening approach, using a virtual library of HS hexasaccharides, identified an unusual sequence composed of consecutive GlcA 2-OS as a potent activator, which was validated by activity measurements of several synthetic compounds (37). Our array data indicate that the binding selectivities of AT-III



**Fig. 3.** Structural analysis of hexasaccharides by NMR spectroscopy. (A) Structure of hexasaccharides with core trisaccharide GlcA-GlcNS-GlcA with variable 3- and/or 6-O-sulfation on central glucosamine. (B) Stacked plots showing  $^1\text{H}$  NMR of compounds **1A**, **1B**, and **1C**; blue highlighted area, presence of 3-OS results in distinct downfield shift of  $\text{GlcN}_{\text{H-3}}$ ; green highlighted area, presence of 3-OS results in characteristic split and downfield shift of  $\text{GlcN}_{\text{H-2}}$ . (C) The  $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR of compound **1A**; blue highlighted area, characteristic C-3 of 3-O-sulfated GlcN; green highlighted area, characteristic C-2 of 3-O-sulfated GlcN. (D) The  $^1\text{H}$ - $^1\text{H}$ -TOCSY NMR spectrum of compound **1A**; characteristic correlation between ring protons (anomeric H-1, H-2, and H-3 protons) of 3-OS-bearing central GlcN.



**Fig. 4.** Screening various HS recognizing proteins for their binding against an array of synthetic hexasaccharides printed at 100  $\mu\text{M}$  concentration in a replicate of six. (A) AT-III/Human serpinC1 (10  $\mu\text{g}/\text{mL}$ ). (B) HC-II/Human serpinD1 (3  $\mu\text{g}/\text{mL}$ ). (C) Human FGF-7 (0.3  $\mu\text{g}/\text{mL}$ ). (D) Human FGF-9 (0.1  $\mu\text{g}/\text{mL}$ ). (E) Human BMP-2 (1  $\mu\text{g}/\text{mL}$ ). (F) Human FGFR-1/CD331 (10  $\mu\text{g}/\text{mL}$ ). (G) RAGEWRAGE RAGE/advanced glycosylation end product AGER (1  $\mu\text{g}/\text{mL}$ ). (H) Human stabilin-2/Stab-2 (30  $\mu\text{g}/\text{mL}$ ). (I) Mouse Nrp-1 (30  $\mu\text{g}/\text{mL}$ ). List of protein source (type of fusion tag, if present), primary antibodies, and secondary antibodies (type of fluorophore, if present) screened against HS microarray are presented in *SI Appendix, Table S1*. For dose–response of various HS recognizing protein screened for their binding to HS microarray, see *SI Appendix Fig. S2*. Data are presented as mean  $\pm$  SD ( $n = 4$ ). Representative data are shown for each protein, which was repeated at least three times.

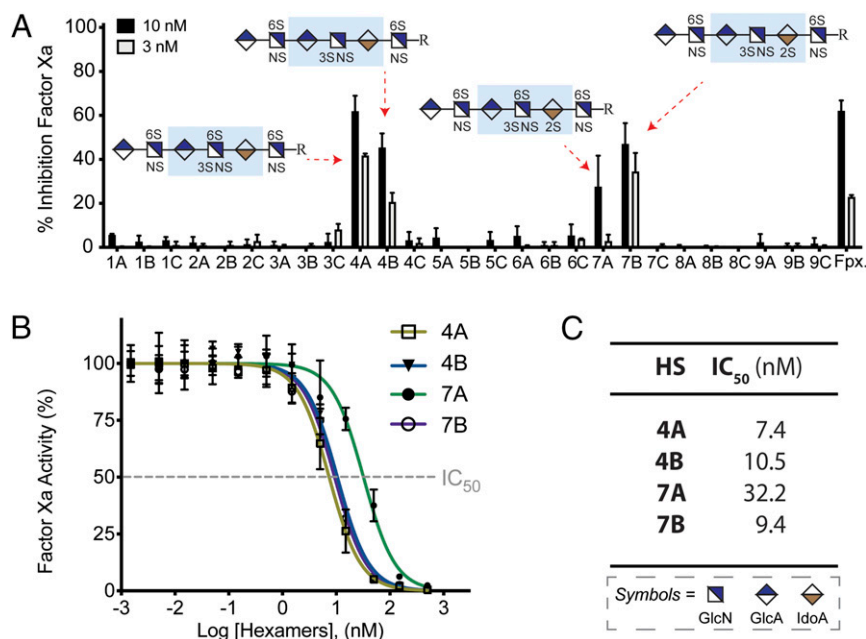
and HC-II are very different, but both have a dependence for a 3-OS moiety (Fig. 4B). Specifically, HC-II strongly bound to compounds **3A** and **9A**, indicating that an IdoA2S-GlcNS3S6S sequon is important for binding. It tolerates GlcA and IdoA2S neighboring the GlcNS3S6S moiety but not any other residues. The dependence of 3-*O*-sulfation appears to be sequence dependent, and removal of this moiety at **3A** ( $\rightarrow$ **3C**) resulted in a greater reduction in binding compared to a similar perturbation of **9A** ( $\rightarrow$ **9C**). The data also show that 6-OS of the GlcNS3S6S moiety contributes substantially to binding, because **3B** and **9B** gave a much lower responsiveness than corresponding **3A** and **9A**, respectively.

Interesting, a number of other proteins exhibited similar structure-binding profiles compared to HC-II. For example, Nrp-1, which has been implicated in angiogenesis and axon guidance (11), also preferentially binds hexasaccharides **3A** and **9A** (Fig. 4I). Previous studies have shown that binding of this protein to HS is dependent on the presence of a 3-OS group, and, in particular, it was selectively retained on an HS affinity matrix modified by 3-OST-1 or 3-OST-2. The effect of 3-*O*-sulfation was cell autonomous, and, based on collapse assay using neurons derived from 3-OST-1– and 3-OST-2–deficient mice, activity appears to depend on the expression of 3-OST-2 (11). This

observation indicates that the latter sulfotransferase can install the 3-OS motifs of compounds **3C** and **9C**. It is the expectation that the identified ligands are attractive for incorporation into biomaterial scaffolds to provide bioactive cues to modulate Nrp-1 activity for neuronal patterning and functional vasculature (38).

A number of growth factors and growth factor receptors also demonstrated a binding preference for 3-*O*-sulfated structures. For example, basic fibroblast growth factor receptor 1 (FGFR1) is highly selective for HS oligosaccharides **3A** and **9A**, and derivatives lacking a 3-OS, 6-OS, or 2-OS at the central Ido2S-GlcNS3S6S bound poorly (Fig. 4F). Other growth factors such as FGF-7 (Fig. 4C) and FGF-9 (Fig. 4D) also showed strong responsiveness to compounds **3A** and **9A** but exhibited some promiscuity and also bound other compounds. The array data also verified the dependence of HS modified by 3-OS for binding of RAGE (Fig. 4G) and stabilin-2 (Fig. 4H). A previous report asserted that a decasaccharide represents a minimal oligosaccharide for stabilin-1 receptor binding (13); however, our data indicate that a hexasaccharide having a distinct sulfation pattern and backbone structure is a proper ligand for this protein.

We have also discovered an HS-binding protein that has a dependence for 3-OS–modified HS. Bone morphogenetic protein-2 (BMP-2) is approved by the Food and Drug Administration for



**Fig. 5.** Anticoagulant activity of hexasaccharides. (A) Factor Xa inhibition assays, HS hexamers were screened at concentrations 10 and 3 nM, respectively, with Fondaparinux (Fpx.) as positive control; higher concentration screening data are presented in *SI Appendix, Fig. S3*, symbol structures of identified hits are presented, and the variable core region is highlighted in blue. (B) Determination of IC<sub>50</sub> of **4A**, **4B**, **7A**, and **7B**; an overlay of inhibition curve is presented, and individual inhibition curves are presented in *SI Appendix, Fig. S4*. (C) Table for IC<sub>50</sub> value of identified hits. Symbol nomenclature for HS backbone monosaccharides is presented in the dashed box. Data are presented as mean  $\pm$  SD (for A and B,  $n = 3$ ). All experiments were performed three times at the minimum. R = O(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>.

bone regeneration; however, its therapeutic range is small, and there are many reported cases of adverse clinical effects due to overdosing (39). In an elegant study, this problem was addressed by affinity purification of HS using immobilized BMP-2 (40). Co-administration of this HS preparation with BMP-2 greatly enhances bone formation in vitro and in vivo and was much more efficacious than mucosal HS or heparin. Our array studies indicate that BMP-2 preferentially binds to HS oligosaccharides modified by a 3-OS moiety such as the hexasaccharides **3A** and **9A** (Fig. 4E). Sequences such as **9A** are devoid of anticoagulation activity and are attractive to be developed as scaffolds (38) for modulation of BMP-2 activity for bone regeneration.

Collectively, the array data show that many HS-binding proteins require an IdoA2S-GlcNS3S6S sequon for binding. They exhibit variable dependence for a 2-OS and 6-OS moiety and a GlcA or IdoA2S neighboring the GlcNS moiety.

#### Inhibition of Herpes Simplex Virus 1 Infection by HS Oligosaccharides.

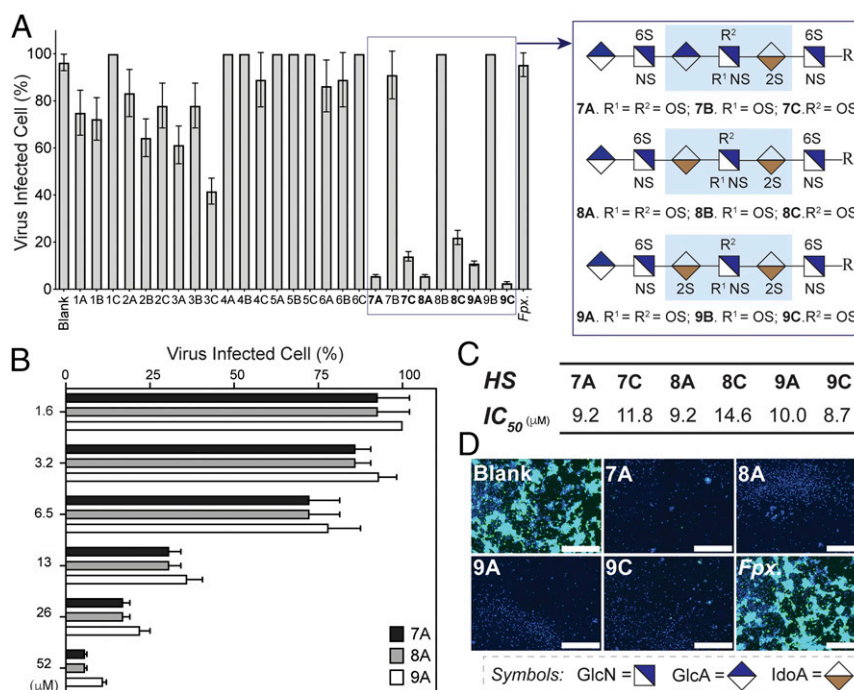
Herpes simplex virus type 1 (HSV-1), which belongs to neurotropic alphaherpesvirus subfamily, is a human pathogen that can cause chronic mucocutaneous and sometimes genital lesions (41, 42). HSV-1 infection is initiated by binding of the viral envelope glycoproteins gB and gC to HS proteoglycans. Next, the HS-virion complex shuttles to the cell surface where a third glycoprotein, gD, engages with one of the entry receptors: herpes virus entry mediator, nectin-1, or 3-*O*-sulfated HS. This interaction then triggers a series of events involving two additional HSV-1 glycoproteins, gH and gL, which ultimately cause viral capsid penetration into the cell (43, 44).

It has been shown that 3-*O*-sulfation of HS has a profound effect on HSV-1 infection (10), and it appears all 3-OST isotypes, except 3-OST-1, can generate HSV-1 entry receptors (45). Previous studies have also indicated that the minimal sequence that can bind to gD protein is an octasaccharide ( $\Delta$ UA-GlcNS-IdoA2S-GlcNAc-IdoA2S-GlcNS-IdoA2S-GlcNH<sub>2</sub>6S3S, binding

constant  $K_d = 18.0 \mu\text{M}$ ) (46). This conclusion is, however, based on studies employing redundant structures that are either derived from HS oligosaccharides generated by heparin lyase digestion or a small number of chemically synthesized structures (29, 46, 47). Thus, there is an urgent need to systematically analyze the influence of HS structure on HSV infectivity. We anticipated that our hexasaccharide library would be attractive to examine the importance of 6-*O*-, 3-*O*-, and, 2-*O*-sulfation and C-5 epimerization of HS to inhibit viral infectivity.

Thus, the library of hexasaccharides was screened for inhibitory activity for HSV-1 infection of human corneal epithelial cells (HCEs). A fluorescence assay using GFP reporter HSV-1 virions mixed with the hexasaccharides (**1A** to **1C** through **9A** to **9C**, at 100  $\mu\text{g}/\text{mL}$  concentration) was performed to determine the ratio of infected cells vs. total cells (DAPI nuclear stain) (Fig. 6A) (48, 49). Next, IC<sub>50</sub> was determined for compounds exhibiting potent inhibition at 100  $\mu\text{g}/\text{mL}$  concentration (**7A**, **7C**, **8A**, **8C**, **9A**, and **9C**; Fig. 6B). All the compounds exhibited low micromolar inhibitory activity (Fig. 6C), with compound **9C** being the most potent (IC<sub>50</sub> = 8.7  $\mu\text{M}$ ), having a structure similar to a previously identified octasaccharide inhibitor (47). A number of the identified hexasaccharide inhibitors are modified by a 3-OS moiety (**7A**, **8A**, and **9A**). However, the corresponding compounds lacking 3-OS (**7C**, **8C**, and **9C**) are also potent inhibitors, whereas derivatives in which the 6-OS is omitted failed to invoke inhibition (**7B**, **8B**, and **9B**). These findings indicate that the backbone structure and sulfation pattern are important for inhibitory activity, and it appears that a GlcNS6S-IdoA2S sequon is critical for activity. There is no dependence on the nature of the nonreducing uronic acid moiety, and a compound having GlcA (**7A**, **7C**), IdoA (**8A**, **8C**), or IdoA2S (**9A**, **9C**) at this position exhibited similar activities. On the other hand, perturbations at the reducing end uronic acid resulted in a loss of activity (**3A**, **3C**, **6A**, and **6C**). Strikingly, the pentasaccharide Fondaparinux, which bears structural similarity to hexasaccharide **7A**, showed no





**Fig. 6.** Inhibition of HSV-1 infection of HCEs (RCB1834 HCE-T) by HS hexasaccharides. (A) Screening of hexasaccharides at 100  $\mu$ g/mL, untreated and infected blank as positive control, and Fpx. was used as a 3-OS standard; box highlighted area shows HS oligosaccharides that elicited more than 80% reduction in virus infection; symbol structure of the hexamers and influence of sulfation site on viral infection; data presented are an average of three independent experiments. (B) Determination of IC<sub>50</sub> values of 3,6-OS hits (7A, 8A, and 9A); 6-OS hits (7C, 8C, and 9C) are in *SI Appendix, Fig. S5A*. (C) Table of IC<sub>50</sub> value of identified hits, 7A, 7C, 8A, 8C, 9A, and 9C. (D) GFP reporter HSV-1 infection assays; images for compound 7B, 8B, 8C, and 9B are in *SI Appendix, Fig. S5B*. (Scale bar, 100  $\mu$ m.) Symbol nomenclature for HS backbone monosaccharides is presented in dashed box. Data are presented as mean  $\pm$  SD (for A and B,  $n = 3$ ). All experiments were performed at least as three independent experiments. R = O(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>.

inhibitory activity, suggesting that a hexasaccharide constitutes to minimal chain length for inhibitory activity. Although the composition of HS expressed by HCE is not known yet, the inhibition assay provides information about the types of HS epitopes recognized by HSV-1.

Intrigued by the observation that several compounds devoid of 3-OS can inhibit viral infectivity, we investigated the HS ligand requirements of gD. For this purpose, the HS-microarray was incubated with gD (Abcam) followed by incubation with biotinylated anti-gD antibody (Abcam) and streptavidin-AlexaFluor635 (ThermoFisher). The results showed that the protein has a preference for hexasaccharides modified by 3-OS (*SI Appendix, Fig. S2C*).

Previous studies have shown that 3-*O*-sulfation of HS can generate a fusion receptor for HSV-1 gD for mediating cell entry and spread (50). However, binding of glycoproteins gB and gC to HS precedes gD binding. Thus, it is conceivable that the hexasaccharides examined herein inhibit viral infection by competitively binding to gB or gC, thereby blocking initial cell attachment. Previously reported inhibition studies using synthetic HS oligosaccharides attributed inhibition of infection to gD blockage. These studies have been performed either without the non-3-OS analog or at very low MOI (multiplicity of infection) and short incubation time, and did not address the involvement of other viral glycoproteins (29, 47).

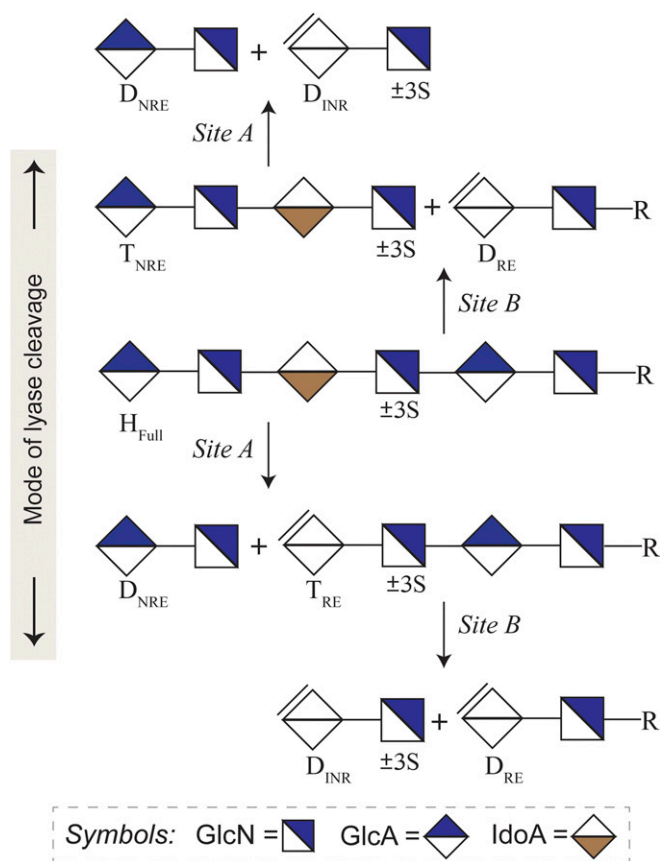
**The Use of the Hexasaccharide Library to Probe Substrate Requirements of Heparin Lyases.** Heparin lyases (I, II, and III) isolated from *Flavobacterium heparinum* or *Bacteroides eggertii* are important enzymes for the depolymerization of Hep/HS (18). These enzymes can cleave glycosidic linkages between GlcN and uronic acid by  $\beta$ -elimination and generate product saccharides containing an unsaturated uronic acid residue at the nonreducing end (51, 52). Understanding the substrate requirement of

lyases is important for the production of LMWHs with higher anticoagulant activity and improved pharmacokinetic profiles (19, 53, 54), for the preparation of HS standards for MS (17), and for the development of bioanalytical methods for Hep/HS (16).

It is known that lyase I cleaves the glycosidic linkage between GlcNS and sulfated IdoA residues, and lyase II cleaves the glycosidic linkage between *N*-sulfated or acetylated GlcN and GlcA or IdoA residues, whereas lyase III action is limited to GlcN and GlcA residues (*SI Appendix, Fig. S6A*) (51). Furthermore, there are data to support that lyases cannot cleave the glycosidic linkage at the nonreducing end of 3-*O*-sulfated glucosamine (site A; Fig. 7) (15, 16). In the case of lyase II, this resistance is attributed to Asn405, and an engineered enzyme with a less sterically demanding Ala405/Gly405 mutation may overcome this resistance and broaden lyase II substrate specificity (55). Despite this knowledge, fine substrate specificities of lyases are not known, hampering the use of these enzymes for the depolymerization of heparin and HS into oligosaccharides for structure and activity relationship studies.

We anticipated that our collection of synthetic HS hexasaccharides having diverse backbones (GlcA-GlcA, IdoA-GlcA, GlcA-IdoA, and IdoA-IdoA; *SI Appendix, Fig. S6B*) and key modifications such as 6-*O*-sulfation and 3-*O*-sulfation (6-OS and 3-OS) on glucosamine (GlcN), and 2-*O*-sulfation (2-OS) on uronic acid residues (HexA) would allow establishment of substrate requirements of heparin lyases. Cleavage of the hexasaccharides at site A (Fig. 7) will release an unsaturated tetrasaccharide (T<sub>RE</sub>), and a saturated disaccharide (D<sub>NRE</sub>). Alternatively, cleavage at site B will release an unsaturated disaccharide (D<sub>RE</sub>) and a saturated tetrasaccharide (T<sub>NRE</sub>). The tetrasaccharides can then be cleaved to give a saturated D<sub>NRE</sub>, unsaturated D<sub>NR</sub>, and unsaturated D<sub>RE</sub>. Thus, resistance to a





**Fig. 7.** Heparin lyases substrate requirements for the digestion of 3-OS-bearing HS. Mode of lyase cleavage: Lyase can cleave intact hexasaccharide ( $H_{Full}$ ) at site A—nonreducing end (NRE) uronic acid—and/or site B—reducing end (RE) uronic acid—to release different tetrasaccharides ( $T_{RE}$  and  $T_{NRE}$ ) and disaccharides ( $D_{NRE}$ ,  $D_{INR}$ , and  $D_{RE}$ , where INR stands for inner disaccharide). Symbol nomenclature for HS backbone monosaccharides is presented in the dashed box. For illustration purposes, HS modifications other than 3-OS were not shown in the model substrate ( $H_{Full}$ ).

cleavage at site A or B will generate a saturated or unsaturated tetrasaccharide, respectively, whereas full resistance will give the hexasaccharide starting product.

The hexasaccharide **1A** to **1C** through **9A** to **9C** were exposed to either lyase I, II, or III from *B. eggerthii*, and the resulting products were analyzed by SEC-MS. The reducing end aminopentyl linker allowed unambiguous identification of site A and site B cleavage products. The results are summarized in Fig. 8, representative chromatograms and mass spectra are shown in *SI Appendix*, Figs. S7–S10, and MS data are provided in *SI Appendix*, Table S2. The rectangles of Fig. 8 indicate different modes of cleavage, and green represents cleavage at both sites A and B to give disaccharides  $D_{NRE}$ ,  $D_{INR}$ , and  $D_{RE}$ ; blue represents cleavage only at site A to give  $D_{NRE}$  and  $T_{RE}$ ; orange indicates cleavage only at site B to give  $D_{RE}$  and  $T_{NRE}$ ; red represents full resistance, and, in this case, only the starting hexasaccharide ( $H_{Full}$ ) was detected.

In the case of lyase I, compounds having an IdoA2S residue toward the reducing end (**7A** to **9C**) were cleaved at both sites. Under the experimental conditions, the compounds were not fully digested, and similar abundances of products were observed (*SI Appendix*, Fig. S7;  $T_{RE}$  vs.  $T_{NRE}$ ;  $D_{RE}$  vs.  $D_{NRE}$ ), indicating similar activities at each cleavage site. Interestingly, derivatives having an unmodified GlcA or IdoA at the same position were only cleaved at site A, highlighting that, at the nonreducing end of a hexasaccharide, 2-*O*-sulfation is not required for cleavage

(**1A** to **2C** and **4A** to **5C**). Furthermore, the results demonstrate that sulfation of the central GlcN residue (**1A** vs. **1B** vs. **1C**) does not influence the cleavage. In contrast to previous reports, which indicated resistance of 3-OS-bearing structures at site A, we found this site to be highly susceptible to lyase I cleavage and not affected by 3-OS (**1A** and **1B**, **2A** and **2B**, **3A** and **3B**, etc.). The previous studies employed heparinase I from *F. heparinum* and heparin as model substrate, which primarily consist of GlcNS  $\pm$  6S  $\pm$  3S-IdoA2S disaccharide unit (75 to 95%) thus, lacking sufficient structural diversity for enzyme specificity studies (16, 19). In the current study, we have employed lyases from *B. eggerthii*, and the observed differences in cleavage patterns may be caused by either differences in substrate specificity between flavobacterial and *Bacteroides* heparinases or the use of a structurally wider collection of HS motifs.

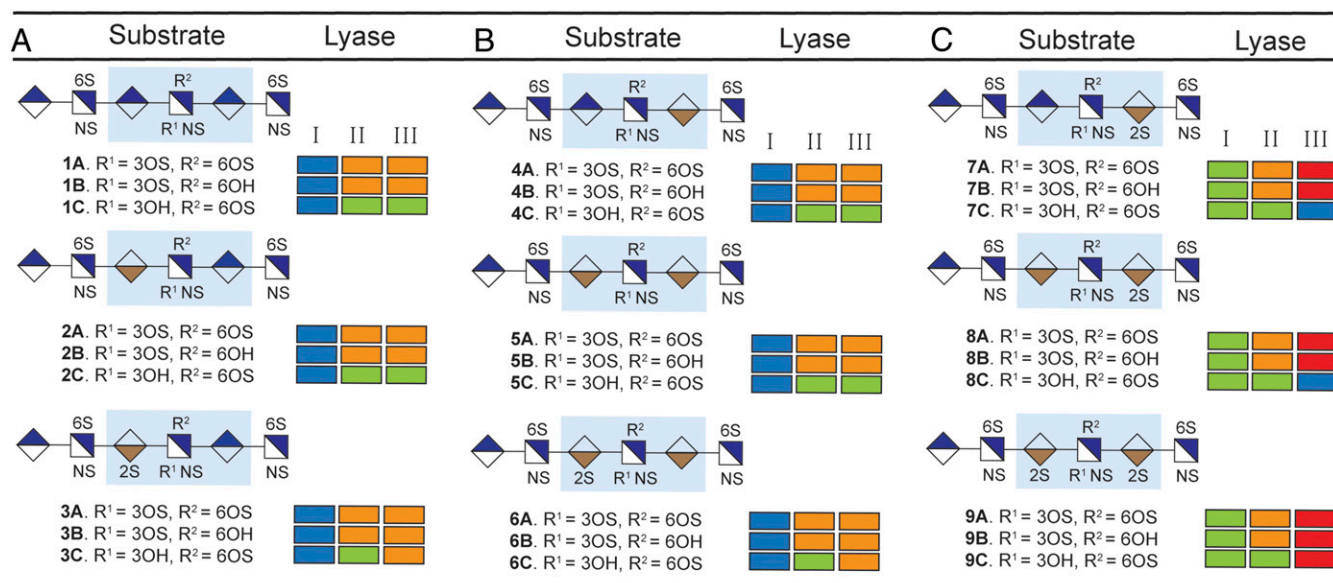
Lyase II is known to be the most promiscuous of the three enzymes cleaving both high- and low-sulfated domains (51, 52). As anticipated, exhaustive digestion with lyase II of compounds lacking a 3-OS motif (**1C**, **2C**, **3C**, etc.; green rectangles in Fig. 8) afforded primarily disaccharides ( $D_{NRE}$ ,  $D_{INR}$ , and  $D_{RE}$ ), demonstrating that the sulfation pattern (GlcA/IdoA vs. IdoA2S) and C-5 epimerization of the HexA residue (GlcA vs. IdoA) does not impact digestion by lyase II. Hexasaccharides bearing either 3-OS or 3,6-OS at the central GlcN (**1A** and **1B**, **2A** and **2B**, **3A** and **3B** etc.; orange rectangles in Fig. 8) released only  $T_{NRE}$  and  $D_{RE}$  products, demonstrating resistance at site A. This observation confirms the intricate role of 3-OS on GlcN to protect the downstream glycosidic bond from cleavage (15, 56). Our results highlight that an additional 6-OS at this residue does not affect the resistance.

Previous studies have shown that lyase III can cleave HS at sites in which the GlcN residue is GlcNAc, GlcNAc6S, GlcNS, or GlcNS6S, while the HexA residue is limited to GlcA or IdoA lacking a sulfate (18). Studies using a heparin-derived tetrasaccharide (57) and hexasaccharide (19) indicated that a GlcNS3S residue also causes lyase III resistance. As shown in Fig. 8, all compounds having a GlcNS3S or GlcNS3S6S were not cleaved at site A. As anticipated, an IdoA2S moiety also caused resistance at its reducing (site B for **7C** and **8C**) and nonreducing end (site A for **3C** and **6C**), whereas all hexasaccharides lacking 3-OS and 2-OS substitutions (**1C**, **2C**, **4C**, and **5C**) were cleaved at both sides to give disaccharides. Interestingly, compounds having a GlcNS3S or GlcNS3S6S moiety and IdoA2S residue at the reducing position (**7A**, **7B**, **8A**, **8B**, **9A**, and **9B**; red rectangles in Fig. 8) were fully resistant to lyase III treatment. A compound having two IdoA2S (**9C**) residues and lacking a 3-OS was also not digested by this enzyme.

We have uncovered that the action of lyase I at site A is not influenced by 6-OS, 3-OS, 2-OS, and C-5 epimerization, whereas, for site B, it requires an IdoA2S moiety and tolerates any GlcN variation (Fig. 9). In the case of lyase II, the presence of 3-OS makes site A resistant to cleavage, yet it tolerates other modifications on HexA, and, for site B, it shows promiscuity and can endure any modifications on both GlcN and HexA. For lyase III, the hexasaccharides bearing 3-OS are resistant to cleavage at site A and show sensitivity for 3-OS at site B; however, both sites A and B exhibit complete resistance when 2-OS is present at HexA.

## Discussion

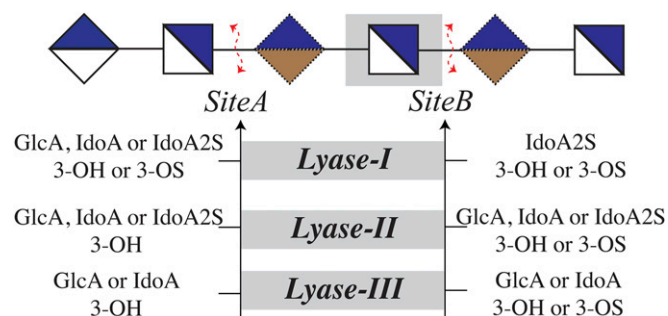
A modular synthetic approach has been developed that can readily provide HS oligosaccharides with or without a 3-OS ester. The approach was employed to prepare a library of 27 hexasaccharides that differ in backbone composition and modification of the central GlcN moiety by either a 3-OS, 6-OS, or 3,6-OS. The compounds were printed as a glycan microarray to examine ligand requirement of HS-binding proteins. The established binding selectivities of AT-III compared very well with anti-Factor Xa activity,



**Fig. 8.** Summary figure of heparin lyase (I, II, and III) activities. Cleavage site color code: Blue rectangle represents cleavage at site A, orange rectangle represents cleavage at site B, green rectangle represents cleavage at both sites A and B, and red rectangle represents resistant substrates with no cleavage. (A) Hexasaccharides having GlcA toward the reducing end of central GlcN. (B) Hexasaccharides with IdoA toward reducing end of central GlcN. (C) Hexasaccharides with IdoA2S toward reducing end of central GlcN. Variable trisaccharide core UA ± 2S-GlcNS ± 6S ± 3S-UA ± 2S is highlighted in light blue.

demonstrating robustness of the array platform. In addition to a hexasaccharide that embeds the Fondaparinux sequence, a compound lacking a 6-OS on the central glucosamine also gave strong responsiveness. Removal of the 2-OS of the neighboring IdoA moiety also had a relatively small impact on binding, but all other perturbations abolished activity, confirming the importance of the backbone composition for binding and activity. Many of the other examined HS-binding proteins also bound a subset of the compounds, demonstrating that the backbone and sulfation pattern is critical for binding selectivity. The microarray data showed that a lack of a 3-OS or 6-OS impacts the binding differently. Furthermore, the backbone structure (IdoA vs. GlcA) also impacts the binding in a substantial way. The backbone structure influences the 3D presentation of sulfates, and thus the examined binding events are not simply governed by electrostatic interactions. Future structural studies are required to understand binding selectivities at a structural level, and the availability of synthetic ligands will facilitate such endeavors. Surprisingly, many of the examined proteins have a requirement for an IdoA2S-GlcNS3S6S sequon with variable dependence for the 2-OS and 6-OS moiety and a GlcA or IdoA2S neighboring the GlcNS3S moiety. These results indicate that cells can create HS sequences for the selective recruitment of HS-

binding proteins requiring 3-OS. To support this notion, further studies are required to define the fine substrate specificities of 3-OST enzymes, and we anticipate that the collection of HS oligosaccharides is ideally suited for this purpose. In addition, technologies will need to be developed that can quantify, on cells and tissues, GlcNS3S-containing HS epitopes. It is anticipated that the synthetic compounds described here may provide standards to develop such methodologies. The current HS hexasaccharide library has a variable trisaccharide core and constantly flanking moieties. It is the expectation that the lead compounds can be further optimized by varying the constant regions in a systematic manner. The HS oligosaccharides were also examined as inhibitors of HSV-1 infection, which, surprisingly, showed nonessentiality of 3-OS. Currently, it is not known which HS epitopes are expressed by HCEs, and thus it is not clear yet whether the receptors used by HSV-1 have structures similar to the inhibitors identified. The synthetic HS hexasaccharides made it also possible to examine substrate specificities of lyases I, II, and III. Previously, it was found that 3-OS can cause enzymatic resistance to degradation. Our results show a complex interplay between sulfation pattern and lyase activity. The uncovered substrate specificities of these enzymes will make it possible to selectively generate HS oligosaccharide domains for liquid chromatography (LC)-MS/MS sequencing and will broaden the understanding of relationships between structures of oligosaccharide domains and their biological functions. Collectively, the results presented here demonstrate that the synthetic methodology provides an important tool to examine the biology of 3-OS-modified HS.



**Fig. 9.** Heparin lyases (I, II, and III) mode of action: Influence of 3-O-sulfation and uronic acid at potential cleavage site on HS hexamers digestion.

## Materials and Methods

See *SI Appendix* for synthetic schemes, reaction procedures, NMR/MS characterization, and NMR spectra of compounds. Also, detailed assay procedures, supplementary figures and supplementary tables for glycan microarray, GFP reporter HSV-1 infection assays, and lyases digestions are provided in *SI Appendix*.

**Chemical Glycosylation.** Glycosyl acceptor (1.0 equiv) and trifluoro-*N*-phenylacetimidate donor (1.2 equiv) were azeotroped with toluene, and then dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$  (0.2 M). Freshly activated molecular sieves (4 Å) were added under argon, and the mixture was stirred for 30 min at room

temperature (rt) and then cooled to  $-20^{\circ}\text{C}$ . Triflic acid (1.5 equiv) was added and stirred for 1 h and then quenched by the addition of pyridine. The mixture was filtered, concentrated, and purified by flash chromatography (hexanes/EtOAc, from 60/40 to 40/60, vol/vol) to give pure hexasaccharide.

**Glycan Array Screening and Analysis.** HS hexasaccharides were printed on NHS ester-activated glass slides (NEXTERION Slide H, Schott Inc.) using a Scienion sciFLEXARRAYER S3. Printed glass slide was incubated with protein of interest. After 1 h, the slide was sequentially washed by dipping in TSM wash buffer (2 min), TSM buffer (2 min), and water ( $2 \times 2$  min), and spun dry. The slide was further incubated with detection reagent for 1 h in the dark, and the same washing sequence was repeated. The slide was scanned using a GenePix 4000B microarray scanner (Molecular Devices) at the 635-nm excitation wavelength.

**Anti-Factor Xa Assays.** Anti-Factor Xa activity of hexasaccharides was determined by BIOPHEN ANTI-Xa kit (Anaira Diagnostica). The solution of AT-III (0.04 IU, 40  $\mu\text{L}$ ) and sample or control (various concentrations, 40  $\mu\text{L}$ ) was incubated at  $37^{\circ}\text{C}$  for 2 min. Factor Xa (0.32  $\mu\text{g}$ , 40  $\mu\text{L}$ ) was then introduced, mixed, and incubated at  $37^{\circ}\text{C}$  for 2 min, followed by addition of chromogenic substrate (0.032  $\mu\text{g}$ , 40  $\mu\text{L}$ ). After exactly 2 min, the reaction was stopped by citric acid (2 wt.%, 80  $\mu\text{L}$ ) and mixed, and absorbance at 405 nm was measured.

**GFP Reporter HSV-1 Infection Assays.** HCEs (RCB1834 HCE-T) were grown until 90% confluent. HSV-1 17-GFP reporter virus (48, 49) solution (0.01 MOI) was mixed with the hexasaccharides (1A to 1C through 9A to 9C, at 100  $\mu\text{g}/\text{mL}$  concentration) and incubated for 30 min at rt to initiate neutralization of the virus. The mixture was then added to HCE monolayer for 2 h to allow viral infection before the cells were washed and media replaced. At 72 h postinfection, samples were imaged using a Biotec Lionheart LX imaging system. For IC<sub>50</sub> values determination, viruses (0.01 MOI) were mixed with the identified hexasaccharides (7A, 7C, 8A, 8C, 9A, and 9C) at various concentrations (from 52  $\mu\text{M}$  to 1.6  $\mu\text{M}$ ), and infection assays were performed as described above.

**Heparan Lyases Digestion.** Heparin lyase I, II, and III was from *B. eggertii* (New England Biolabs). Each compound (1 nmol) was incubated with 1 mU of each heparin lyase in a 20- $\mu\text{L}$  reaction buffered by 50 mM ammonium formate in the presence of 2 mM calcium chloride, at  $37^{\circ}\text{C}$  overnight. The digestion products by heparin lyases were analyzed using SEC-MS.

**Data Availability.** All study data are included in the article and/or *SI Appendix*.

**ACKNOWLEDGMENTS.** This research was supported by the NIH (Grants P41GM103390 and HLBI R01HL151617 to G.-J.B.; Grants U01CA221234 and R01GM133963 to J.Z.; and Grant R01EY029426 to D.S.). We thank Dr. Jeff Esko (University of California, San Diego) for providing neuropilin-1.

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