HHS Public Access

Author manuscript

J Am Chem Soc. Author manuscript; available in PMC 2018 July 19.

Published in final edited form as:

J Am Chem Soc. 2017 July 19; 139(28): 9534–9543. doi:10.1021/jacs.7b01399.

Heparan Sulfate Microarray Reveals That Heparan Sulfate–Protein Binding Exhibits Different Ligand Requirements

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Abstract

Heparan sulfates (HS) are linear sulfated polysaccharides that modulate a wide range of physiological and disease-processes. Variations in HS epimerization and sulfation provide enormous structural diversity, which is believed to underpin protein binding and regulatory properties. The ligand requirements of HS-binding proteins have, however, been defined in only a few cases. We describe here a synthetic methodology that can rapidly provide a library of well-defined HS oligosaccharides. It is based on the use of modular disaccharides to assemble several selectively protected tetrasaccharides that were subjected to selective chemical modifications such as regioselective *O*- and *N*-sulfation and selective desulfation. A number of the resulting compounds were subjected to enzymatic modifications by 3-*O*-sulfotransferases-1 (3-OST1) to provide 3-*O*-sulfated derivatives. The various approaches for diversification allowed one tetrasaccharide to be converted into 12 differently sulfated derivatives. By employing tetrasaccharides with different backbone compositions, a library of 47 HS-oligosaccharides was prepared and the resulting compounds were used to construct a HS microarray. The ligand requirements of a number of HS-binding proteins including fibroblast growth factor 2 (FGF-2), and the chemokines CCL2, CCL5, CCL7, CCL13, CXCL8, and CXCL10 were examined using the array. Although all proteins recognized multiple compounds, they exhibited clear differences in structure–binding characteristics. The HS microarray data guided the selection of compounds that

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b01399.
Full experimental details and characterization of the compounds (PDF)

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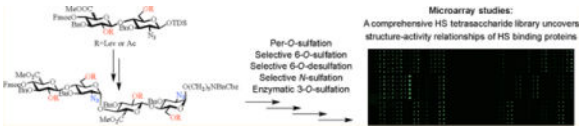
Notes

The authors declare no competing financial interest.

could interfere in biological processes such as cell proliferation. Although the library does not cover the entire chemical space of HS-tetrasaccharides, the binding data support a notion that changes in cell surface HS composition can modulate protein function.

Graphical abstract



INTRODUCTION

Heparan sulfates (HS) are linear *O*- and *N*-sulfated polysaccharides that reside on the cell surface and in the extracellular matrix (ECM) of virtually all mammalian tissue types[1] where they interact with numerous signaling proteins, growth factors and ECM components.[2] The interaction between HS and proteins is critical for many biological processes including cell–cell and cell–matrix interactions, cell migration and proliferation, growth factor sequestration, chemokine and cytokine activation,[3] and tissue morphogenesis during embryonic development.[4] Alteration in HS expression has been associated with disease,[5] and for example, significant changes in the composition of proteoglycans occur in the stroma surrounding tumors, which supports tumor growth and invasion. Many pathogens including bacteria, viruses, and parasites attack host cells by binding to HS, which is often a decisive factor for infection.[6]

HS chains are assembled in the Golgi apparatus on core proteins by initially forming a linear polymer composed of alternating *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) moieties.[7] The resulting polymer is modified by a series of processing reactions involving *N*-deacetylation, *N*-sulfation, epimerization of GlcA to L-iduronic acid (IdoA), and *O*-sulfation resulting in relatively short segments that are sulfated interspersed by regions of unmodified sugars. The considerable structural complexity of HS arises from incomplete sulfation and epimerization leading to at least 20 different disaccharide moieties. Analyses of HS isolated from different mammalian tissues indicate the existence of tissue-specific compositions. Furthermore, immunohistochemical analysis using antibodies that recognize specific HS-epitopes have uncovered unique patterns of HS-motifs within tissues.[8] These observations support a model in which HS structural diversity is not random, but regulated in a context dependent manner, which makes it possible to recruit specific binding partners leading to specific physiological or disease processes.

Although it is widely accepted that HS is an information-rich polymer, the oligosaccharide structures that mediates particular biological processes has been defined in only a few cases.[7] This stems from the fact that HS is structurally very complex arising from a complex biosynthetic pathway. Lack of structure–activity relationships for HS-binding proteins makes it difficult to understand the physiology and pathology of HS at a molecular level and greatly complicates harnessing its therapeutic potential.

Glycoarrays are emerging as a key glycomics technology because they require only very small amounts of oligosaccharides for fast and systematic evaluation of carbohydrate–protein interactions.[9] The development of HS oligosaccharide arrays has, however, been stymied by the difficulties of preparing large collections of these compounds[10] and previously reported HS arrays contained relatively small numbers of compounds that can not provide detailed structure–activity relationships.[11] To address this deficiency, we describe here a synthetic methodology that can provide large numbers of differently sulfated HS oligosaccharides by selective sulfation of hydroxyls and amines of a limited number of tetrasaccharides prepared by a modular approach. It readily provided an unprecedented library of 47 HS-oligosaccharides, which were used for the development of a microarray. The HS microarray was employed to examine ligand requirements of a number of HS-binding proteins including fibroblast growth factor 2 (FGF-2), the chemokines CCL2, CCL5, CCL7, CCL13, CXCL8, and CXCL10. Although each protein recognized multiple compounds, it was found that different HS-binding proteins exhibit different structure–binding relationships. The HS microarray data made it possible to select compounds that can interfere in biological processes such as growth factor induced cell proliferation. Although the library does not cover the entire chemical space of HS-tetrasaccharides, the array data support a notion that changes in cell surface HS composition can modulate protein function.

RESULTS AND DISCUSSION

A sufficiently large library of HS-tetrasaccharides was expected to provide an attractive means to determine structure–binding relationships for HS-binding proteins, which in turn would offer lead compounds that can interfere in cellular processes such as cell proliferation. An important motivation to prepare HS-tetrasacharides is that the potential structural diversity is of an order that was expected to be synthetically assessable. Although HS tetrasaccharides cannot mediate processes such as protein oligomerization, they can be complexed with high affinity.[11g,12] Structural studies also support that HS-tetrasaccharides can provide representative ligands for HS binding proteins, and for example a crystal structure of a ternary FGF2-FGFR1-heparin octasaccharide[13] showed that a tetrasaccharide fragment makes intimate contacts with FGF-2 whereas FGFR1 makes only direct interactions with a disaccharide component. A crystal structure of ROBO1 with a heparin octasaccharide also indicated that only a tetrasaccharide fragment makes intimate contacts with the protein.[14] Larger HS-oligosaccharides are required for the formation of a ROBO1-Slit1-HS complex. A heparin binding protein, such as antithrombin-III, requires a pentasaccharide for optimal binding and biological activity,[15] however, shorter fragments can reveal structural elements important for binding and biological activity. Thus, a HS-tetrasaccharide library was expected to be a useful resource for obtaining structure–binding relationships and begin to address whether different HS-binding proteins exhibit different preferences for HS-oligosaccharides.

We envisaged that selective sulfation of hydroxyls and amines of partially protected tetrasaccharides, prepared by a modular synthetic approach,[16] should give access to numerous compounds for array development. The attraction of such a strategy is that one complex oligosaccharide, which requires a relatively large number of chemical steps for its preparation, can be employed for the synthesis of several different sulfates derivatives.

First, tetrasaccharides **13**–**21** (Figure 1) were prepared by parallel combinatorial glycosylations of glycosyl donors **8**–**12** with glycosyl acceptors **1**–**7**[16a] to give in each case only the α-anomeric product in acceptable to good yields (**13**, 31%; **14**, 51%; **15**, 44%; **17**, 86%; **18**, 58%; **19**, 50%; **20**, 42%; and **21**, 77%). The resulting compounds differ in the composition of iduronic and glucuronic acids and the position of Lev esters. The latter functionality can selectively be cleaved with hydrazine acetate to give hydroxyls that can be sulfated using pyridinium sulfate. Reduction of the azides to amines, followed by sulfation or acetylation and removal of the remaining protecting groups, gives entry into a range of HS-tetrasaccharides. Selective sulfation of the hydroxyls and amines was expected to provide entry into additional HS-oligosaccharides. To demonstrate the power of such an approach, we describe in detail the conversion of **19** into 12 different HS-oligosaccharides (compounds **25**, **26**, **31**–**34**, **39**–**42**, **45**, and **46**; Scheme 1). Similar diversification of compounds **16**, **18**, and **21**, which have different backbone compositions, is described in the Supporting Information (Schemes S1–S3).

Thus, the Fmoc of **19** was removed under mild basic conditions followed by acetylation and then Lev esters were cleaved by treatment with hydrazine acetate in a mixture of methanol and dichloromethane to give compound **22** in a yield of 77%. The resulting hydroxyls of **22** were sulfated with pyridinium sulfur trioxide to give compound **23** in a yield of 81%. Next, the esters of **23** were hydrolyzed by first treating the compound with LiOH in a mixture of hydrogen peroxide and THF, and then sodium hydroxide in methanol. The azido moiety of the resulting compound was reduced with trimethyl phosphine in THF in the presence of NaOH to give amine **24** in a yield of 70%, which was sulfated with a large excess of pyridinium sulfur trioxide in the presence of triethylamine or acetylated with acetic anhydride. The benzyl ethers and benzyloxycarbamate of the latter compounds were removed via a two-step hydrogenation over Pd/C and $Pd(OH)_2$/C to give HS oligosaccharides **25** and **26**. Alternatively, treatment of **22** with 4 equiv of pyridinium sulfur trioxide in DMF for 30 min at 0 °C resulted in selective sulfation of primary hydroxyls to give a mixture of monosulfate **27** (48%) and bis-sulfate **28** (35%), which could readily be separated by column chromatography over Iatrobeads. The same compounds were obtained when sulfur trioxide trimethylamine, which is a less reactive sulfating reagent, was employed at elevated temperature. Thus, it is evident that primary hydroxyls are much more reactive toward sulfation than secondary ones, and furthermore the hydroxyl at the distal $GlcN_3$ moiety is significantly more reactive than a similar hydroxyl at proximal $GlcN_3$ residue. Sites of sulfate esters were identified by downfield shifts of ring protons (0.4 ppm) and carbons (4 ppm). NMR spectra were fully assigned by ^1H, TOCSY (total correlation spectroscopy) and ^1H–^{13}C HSQC (heteronuclear single quantum coherence spectroscopy) experiments. The NMR data showed homogeneity of the compounds. Saponification of the esters, reduction of the azides to amines following by *N*-sulfation or *N*-acetylation and finally hydrogenation, gave the HS oligosaccharides **31**–**34**. It was found that after ester hydrolysis the intermediates could be readily purified by C18 column chromatography.

Selective removal of sulfate esters offered an opportunity to prepare additional derivatives. Thus, **23** was transformed into the pyridinium salt and treatment with *N*, *O*-bis(trimethylsilyl) acetamide (BTSA) in pyridine at 60 °C for 2 h resulting only in the

removal of primary sulfate esters to give derivatives **35** (50%) and **36** (27%).[17] The primary sulfate esters can be selectively hydrolyzed in the presence of secondary ones and furthermore the primary sulfate at the proximal $GlcN_3$ moiety is more susceptible to treatment with BTSA than the one at the distal $GlcN_3$ residue. Standard *N*-modification and deprotection procedures gave entry into derivatives **39**–**42**. It was also possible to only modify the amines, which gave entry into compounds **45** and **46**.

Three additional tetrasaccharides (**16**, **18**, and **21**) having different backbone compositions were subjected to similar manipulation to give an additional 19 sulfated oligosaccharides (Schemes S1–S3). It was found that the proximal glucosamine moiety of a tetrasaccharide having a GlcA-GlcN-IdoA-GlcN (**21**) backbone could selectively be sulfated to give additional compounds. Tetrasaccharides **13**–**15**, **17**, and **20** were subjected to conventional modification approaches to give five additional HS oligosaccharides that are low sulfated or possess differently modified amino groups (Schemes S4–S8).

Next, we explored whether the library of HS oligosaccharides could be further expanded by further sulfation using sulfotransferases.[18] In particular, there was a lack of compounds in the library modified by 3-*O*-sulfates, which is a rather rare modification important for anticoagulant activity, cellular entry of viruses, and associated with embryonic development and cancer.[19] Seven different 3-*O*-sulfotransferases (3-OST) isoforms have been identified for vertebrates, each having a unique specificity for glucosamine residues of HS.[19] The substrate specificity of 3-OST1 has been studied in detail and this enzyme has a relatively broad substrate specificity requiring an *N*-sulfonate and iduronyl residue with/without 2-O-sulfate at the reducing end of the targeted residue.[20] Tetrasaccharides **47**, **49**, **51**, and **53** were selected as putative substrates for recombinant 3-OST1 and as expected incubation in the presence of PAPS gave derivatives **48**, **50**, **52**, and **54**, respectively (Scheme 2). Any remaining starting material could easily be removed by a weak anion exchange (diethylaminoethyl cellulose) column chromatography.

The synthetic HS oligosaccharides were modified with an aminopentyl spacer, and thus a microarray could be constructed by piezoelectric non-contact printing on *N*-hydroxysuccinimide (NHS)-activated glass slides.[21] All samples were printed as replicates of 6 (1 mM in a sodium phosphate (50 mM), pH 9.0 buffer) and, after overnight incubation in a saturated NaCl chamber, the remaining activated esters were quenched with ethanolamine (100 mM) to give slides ready for screening experiments.

First, we examined the HS ligand requirements of fibroblast growth factor 2 (FGF-2 or basic FGF), which can mediate diverse functions such as morphogenesis, maintaining tissue homeostasis and regulating metabolic processes by binding and dimerizing FGF receptors (FGFRs) in a HS cofactor assisted manner.[7] Dysfunction of FGF-2 signaling contributes to many human diseases, such as metabolic disorders including chronic kidney disease and insulin resistance as well as many acquired forms of cancers, therefore, interfering in FGF-2 signaling offers many opportunities for drug discovery. Binding studies using chemically modified heparin and small numbers of synthetic compounds[11a–i,22] has provided some information about the ligand requirements of FGF-2 offering an appropriate starting point to validate the array technology.

Sub-arrays were incubated with different concentrations of FGF-2 in PBS containing BSA (1%) for 1 h followed by washing and exposure to a rabbit anti-FGF-2 antibody followed by an anti-rabbit antibody labeled with AlexaFluor 532 (Figures 2a and S4). To analyze the data, the compounds were organized according to increasing numbers of sulfates and the IdoA vs GlcA content (Figure 2e and Table S1). Figure 2a shows the lowest concentration at which good responsiveness was observed to achieve an appropriate dynamic range (see Figure S4 for additional concentrations). As expected an increase in concentration of FGF-2 resulted in a greater responsiveness. It is apparent that FGF-2 recognizes all highly sulfated compounds (Figure 2a), indicating that it seems to tolerate sulfate esters at all positions. The compounds having intermediate levels of sulfation (three and four sulfates) exhibited clear structure–activity relationships. The largest increases in binding were observed when a 2-*O*-sulfate (**3-I** vs **2-H**, **4-F** vs **3-H**, **4-D** vs **3-C**, **5-C** vs **4-E**, and **5-B** vs **4-G**) and *N*-sulfate (**4-D** vs **3-D**, **5-E** vs **3-F**, and **5-D** vs **3-K**) are added. The importance of a 2-*O*-sulfate for binding is further supported by comparing the responsiveness of compounds **3-B** and **3-C**, which are isomers having a sulfate ester at C-2 or C-6 of the proximal disaccharide moiety, and only the compound **3-B** which has a 2-*O*-sulfate exhibited binding. Similar trends were observed for other compounds such as **4-D** and **4-E**, which also differ in the position of sulfate esters. The structure–activity data also show that the 2-*O*-sulfation needs to be at IdoA residues (**3-A** vs **3-B** and **4-A** vs **4-D**) and compounds that have such a residue at GlcA do not show binding. These results agree with previous studies that have indicated that 2-*O*- and *N*-sulfates are critical for FGF-2 binding.[22a,b]

To validate the array data, binding studies were performed by surface plasmon resonance (SPR). FGF-2 was immobilized on NHS-activated groups of a CM5 sensor chip surface and titration experiments were performed with a selected set of synthetic compounds (**1-A**, **2-B**, **2-G**, **3-B**, **3-I**, **4-D**, **4-E**, **5-A**, **5-D**, and **6-A**) that showed weak, moderate and strong binding to FGF-2 in the microarray experiments. Gratifyingly, the SPR response units for the various compounds correlated well with the microarray responses (Figure 2b). The titration curves for **5-A** and **5-D** fitted well to a two-state binding mode (Figure S1a,c) and K_D values of 0.41 and 1.2 μM, respectively were determined. A contact time experiment whereby the dissociation phase of binding sensorgrams is analyzed as a function of time confirmed the two-state binding model for compounds **5-A** and **5-D** (Figure S1b,d), and as expected, longer contact times resulted in slower rates of dissociation. A model in which the HS oligosaccharide first forms a weak complex with the protein, which is followed by maturation to tight binding, can rationalize this observation.

The activity of tetrasaccharides **1-A**, **5-D**, and **6-A** was further evaluated in cellular assays. First, we examined whether the synthetic compounds could inhibit FGF-2 binding to mouse lung endothelial cells with heparin as a positive control. As can be seen in Figure 2c, compounds **5-D** and **6-A** inhibited FGF-2 binding in a dose dependent manner, whereas compound **1-A** did not exhibit any activity, which is in agreement with the microarray and SPR data. Heparin inhibited FGF-2 binding at high concentrations, whereas at low concentrations additional recruitment of FGF-2 was observed, which is in agreement with literature data.[23] At low concentration, heparin is believed to function as a co-receptor to bridge FGF-FGFR complex formation by interacting with both FGF and FGFR, thereby

recruiting additional FGF to the cell surface. Importantly, in cell function studies, the high affinity synthetic compounds **5-D** and **6-A** and heparin, but not compound **1-A**, could inhibit FGF induced proliferation of endothelial cells (Figure 2d), which is in agreement with inhibition of FGF-2 binding on the endothelial cell surface. Therefore, the synthetic compounds **5-D** and **6-A** exhibited an inhibitory activity on FGF-2 similar to heparin.

Next, we examined whether other HS-binding proteins may exhibit different preferences for the HS-oligosaccharides. For this purpose, recombinant human CCL2 (MCP-1), CCL7 (MCP-3), CCL13 (MCP-4), CXCL10 (IP-10), CCL5 (RANTES), and CXCL8 (IL-8) with a C-terminal HA tag were incubated with the microarray and detected by a rabbit anti-HA antibody followed by anti-rabbit conjugated with AlexaFluor 532. Although in general a higher level of sulfation led to increases in binding (Figures 3, S3, and S5–S10), distinct differences in binding patterns were observed for the different proteins.

Although CCL2, CCL7, and CCL13 belong to the same chemokine subfamily and share high sequence homology (62–71%), they exhibit clear differences in structure–binding relationships (Figures 3a,c and S2c). For CCL2, substantial binding was observed for highly sulfated compounds only and it appears that this protein does not exhibit a preference for IdoA or GlcA, *N*-sulfate, 6-*O*-sulfate or 2-*O*-sulfate. However, a compound having a 3-*O*-sulfate (**5-A**) showed the strongest responsiveness and this modification may be important for optimal binding properties. CCL7 and CCL13 exhibited more promiscuous binding behavior and most compounds having three or more sulfates showed rather strong responsiveness. A previously identified glycosaminoglycan-binding deficient mutant of CCL2 in which Ala replaced the basic amino acids Arg-18 and Lys-19[24] showed no binding to any of the HS oligosaccharides tested (Figure 3b). The CCL7 mutant K18AK19A[25] also exhibited very substantial decreases in oligosaccharide binding, validating the microarray results and prior studies of this chemokine (Figure S2b).

CXCL10 (Figure 3d) and CCL5 (Figure S2d) showed a preference for 6-*O*-sulfates over 2-*O*-sulfates, and for example binding of compound **4-E**, bearing a 6-*O*-sulfate at the non-reducing glucosamine, was substantially stronger than for **4-D** which is an isomeric compound having a sulfate ester at the C-2 position of the distal iduronic acid moiety. This trend was, however, different for tetrasaccharides having a different backbone, and for example compounds **4-I** and **4-H** showed different activities. Adding 2-*O*-sulfate(s) on compound **4-I** to form compounds **5-D** and **6-A** did not significantly affect binding (same is true for compounds **4-G** and **5-C**). Both proteins do not tolerate a glucuronic acid at the reducing glucosamine moiety (compounds **4-A**, **4-B**, **4-F**, and **4-G** are poor binders). Replacing a nonreducing GlcA with IdoA moiety and including a 2-*O*-sulfate at this residue ensured strong responsiveness (**4-E**, **4-J**, **4-K**, **6-A**). For a number of compounds the array data for CXCL10 was validated by SPR binding experiments (Figure S11). CXCL8 (Figure S2e) has a broader substrate preference and also binds to compounds such **4-I** and **5-C**. These results highlight that structure activity relationships for the chemokines are complex.

We explored whether HS-oligosaccharides that bind to a chemokine can interfere in cellular processes. CXCL10, which is an interferon-inducible chemokine, has potent chemotactic activity. It can inhibit proliferation of endothelial cells *in vitro*, and possesses angiostatic and

antitumor effects *in vivo*.[26] These properties are associated with CXCL10's ability to bind to HS.[27]

Mouse lung endothelial cells were treated with CXCL10 or BSA as negative control and cell proliferation was measured. As expected, CXCL10 significantly reduced cell proliferation, which was enhanced by heparin. Interestingly, compounds **4-J** and **4-K**, which both showed strong responsiveness in the array screen for CXCL10, also enhanced the CXCL10-mediated inhibition of cell proliferation, whereas tetrasaccharide **4-I**, which did not exhibit binding to CXCL10, showed no responsiveness (Figure 3e). These results demonstrate that array binding results can be translated to biologically relevant responses.

The interaction of HS with chemokines causes the formation of protein gradients that regulate cellular processes such as the migration of cells into inflamed tissues, tissue repair and development.[3,28] It has been thought that there is functional redundancy among chemokines, however, recent studies indicate a substantial degree of specificity and plasticity in the homo- and hetero-oligomerization of chemokines and their binding to receptors. The latter processes often depend on glycosylaminoglycans (GAG) such as heparan sulfate as co-receptor. The data presented here support a notion that chemokines exhibit different selectivities for HS epitopes, and thus it is likely that these differences influences chemokine oligomerization and receptor binding and activation. It is known that endothelial cells of different tissue types express different chemokine profiles.[29] Furthermore, it has been observed that different endothelial tissues can express unique patterns of HS-motifs.[30] Thus, it is likely that HS and chemokine expressions operate synergistically to regulate the above-mentioned biological processes.

CONCLUSIONS

It has been suggested that HS encodes information because of its potential structural diversity.[12b] Cell-type specific HS expression may result in the selective recruitment of HS binding proteins leading to the activation of specific signal transduction pathways.[31] Defining ligand requirements of a large number of HS-binding proteins is a critical step for validating such a model and the HS-oligosaccharide array reported here provides such opportunities. It was found that all proteins recognized multiple HS-oligosaccharides, however, clear differences were observed in the structure–binding data supporting a notion that different HS-binding proteins recognize different patterns of sulfation. A number of chemokines, such as CCL7, exhibited stronger responsiveness when the number of sulfates increased and appear to have no preference for IdoA vs GlcA or positions of sulfate moieties. Other HS binding proteins recognized potently all highly sulfated HS-tetrasaccharides (five to six sulfates) but exhibited clear preferences for derivatives having intermediate levels of sulfation (three to four sulfates). For example, FGF-2 preferentially bound tri- and tetra-sulfates HS-oligosaccharides having iduronic acid moieties modified by sulfate esters at C-2. Thus, for these proteins it appears that sulfates at specific positions are important for binding whereas others are tolerated but do not substantially improve binding. Heparan sulfate is rich in domains that have low and intermediate levels of sulfation,[7b] and thus selective recognition of such structural elements is probably biologically relevant. Other chemokines such as CXCL10 showed clear preferences even for the highly sulfated

tetrasaccharides and for these cases sulfates at specific positions are important for binding whereas others are not tolerated. The structure–binding relationship for these chemokines is, however, complex and backbone composition influenced which sulfate groups are important or tolerated for binding.

Binding experiments using SPR validated array data. Furthermore, it was found that compounds that exhibited strong responsiveness in the array screening for FGF-2 and CXCL10 could interfere in cell proliferation, whereas weak binders did not possess this property. These experiments indicate that array data can be translated to biologically relevant properties.

Although a relatively large collection of HS tetrasaccharides has been prepared, it is important to note that it contains relatively few sequences having a GlcA2S or GlcN3S moiety, which have been associated with a number of important biological processes.[19,32] Thus, the interpretation of the array data requires care because the most favorable ligand for a given HS-binding protein may not be present in the library. Furthermore, the premise of the reported approach is that tetrasaccharides can reveal informative structure–binding relationship.[11g,12] Tetrasaccharides may, however, not represent full binding epitopes, and larger structures may bind with higher affinity and selectivity. The technology described here makes it possible to prepare larger collections of HS oligosaccharides and combined with progress in sequencing of HS and cellular activity measurements will provide important means to uncovering the possible existence of a HS code.[12b] Tetrasaccharide ligands uncovered by array screening can be employed to design larger HS oligosaccharides to probe in a systematic manner the importance of the length of HS oligosaccharides for binding selectivities.

EXPERIMENTAL SECTION

General Procedure for Selective 6-*O*-Sulfation

SO_3·Py (2 equiv per OH) was added to a solution of starting material in DMF (2 mM). The mixture was stirred first at 0 °C for 30 min and then at room temperature (RT) for 30 min until TLC ($CHCl_3$/CH_3OH, 90/10, v/v) indicated completion of the reaction. Next, triethylamine and CH_3OH (1/1, v/v, 1 mL) were added, and stirring was continued for 30 min, after which the mixture was concentrated *in vacuo*. The residue dissolved in CH_3OH was applied to a column of Iatrobeads (1.5 g), which was eluted with a gradient of CH_3OH in $CHCl_3$ (from 96/4 to 88/12, v/v). Fractions containing product were concentrated *in vacuo* and the residue was dissolved in water passed through a column of Biorad 50 × $8Na^+$ resin (0.6 × 5 cm) using CH_3OH or H_2O as the eluent to provide after lyophilization the product as the sodium salt.

General Procedure for Selective De-6-*O*-sulfation

The starting material in methanol was passed through a column of ion-exchange resin (Amberlite IR-120, H+ form, 0.5 mL) with pyridine (0.2 mL) in the receiving flask to give the pyridinium salt. The solution was concentrated under reduced pressure to give a residue that was dissolved in pyridine. *N,O*-bis(trimethylsilyl)acetamide (40 equiv per sulfate) was

added to the solution and the resulting reaction mixture was stirred at 60 °C for 2 h until TLC ($CHCl_3/CH_3OH$, 90/10, v/v) indicated completion of the reaction. Then the solvents were evaporated under reduced pressure and the residue was co-evaporation with methanol and water three times (1/1, v/v, 2 mL). The resulting residue was purified by C18 column chromatography using a gradient of water and methanol (from 90/10 to 5/95, v/v). Fractions containing product were converted into the sodium salt form as described above.

General Procedure for 3-OST1-Mediated 3-*O*-Sulfation

First, 0.1 mg 3-OST1 (0.85 mg/mL) was added to a solution of 0.1 mg tetrasaccharide, PAPS (40 μM final concentration) in 0.5 mL pH 7 buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM $MnCl_2$, 5 mM $MgCl_2$, and 1% Triton X-100. The reaction mixture was incubated at 37 °C for 7 h after which 0.1 M NaOH was added until pH = 9. The mixture was directly loaded onto a DEAE column (0.6 × 2.5 cm), which was eluted with a gradient of aqueous ammonium bicarbonate (from 0.1 to 1.5 M). Fractions containing product were converted into sodium salt form as described above.

General Procedure for Selective *N*-Sulfation

The starting material was dissolved in CH_3OH (1 mL for 0.006 mmol) and triethylamine (0.3 mL) and 0.1 M NaOH (2 equiv per NH_2) were added. The mixture was cooled (0 °C) and SO_3·Py (5 equiv per NH_2) was added. The progress of the reaction was monitored by TLC (silica gel TLC, EtOAc/pyridine/water/CH_3CO_2H, 8/5/3/1, v/v/v/v). Two additional portions of SO_3·Py (5 equiv per NH_2) were added after 1 and 2 h. After stirring for additional 4 h at 0 °C, the reaction mixture was diluted with water and concentrated *in vacuo*. The residue was passed through a short column of Biorad 50 × 8Na+ resin (0.6 × 5 cm) using CH_3OH and H_2O (90/10, v/v) as eluent. Appropriate fractions were concentrated under reduced pressure. The resulting residue was dissolved in water and then applied to a column of C18 silica gel, which was eluted with a gradient of H_2O and CH_3OH (from 90/10 to 40/60, v/v). Fractions containing pure product were concentrated under reduced pressure to provide *N*-sulfated product. The resulting product was *N*-acetylated according to general procedure for global *N*-acetylation (see SI for details). The product was converted into sodium salt form as described above.

Other General Synthetic Procedures

The procedures for the preparation of tetrasaccharides, cleavage of Lev esters, *O*-sulfation, saponification of methyl esters and de-*O*-acetylation, reduction of azide groups, global *N*-acetylation, *N*-sulfation, and global debenzylation have been reported previously[16a] and are described in the SI.

Glycan Array Screening and Analyses

Microarrays were constructed by piezoelectric non-contact printing of the synthetic HS oligosaccharides modified with an aminopentyl spacer on *N*-hydroxysuccinimide (NHS)-activated glass slides (Nexterion Slide H). All samples (100 μM and 1 mM) were printed (drop volume ~450 pL, 1 drop per spot) at 50% relative humidity as replicates of 6 in sodium phosphate (50 mM), pH 9.0 buffer. After overnight incubation in a saturated NaCl

chamber (providing a 75% relative humidity environment), the remaining activated esters were quenched with ethanolamine (100 mM) in sodium phosphate (50 mM), pH 9.0. Sub-arrays (18 × 18 spots) were incubated with 50 μL recombinant human FGF-2 (100-18B, PeproTech) at different concentrations (0.5, 2, and 5 μg/mL) in PBS containing BSA (1%; A7030, Sigma-Aldrich) for 1 h followed by washing and exposure to 50 μL rabbit anti-human FGF-2 antibody (500-P18, PeproTech) for 1 h, then followed by 50 μL anti-rabbit antibody labeled with AlexaFluor 532 (A-11009, Life Technologies) for 1 h. Wash steps involved three successive washes of the whole slides with PBS-Tween 1%-BSA 0.1% (1×) and deionized H_2O (2×) with 5 min soak times. Similarly, 50 μL recombinant proteins CCL2 (0.5 and 5 μg/mL), CCL2 mutant R18AK19A (0.5, 2, and 5 μg/mL), CCL5 (0.5, 2, and 5 μg/mL), CCL7 (1, 2, and 5 μg/mL), CCL7 mutant K18AK19A (1 and 5 μg/mL), CCL13 (2 and 5 μg/mL), CXCL8 (0.5, 2, and 5 μg/mL), and CXCL10 (0.5, 2, and 5 μg/mL) with a C-terminal HA tag were exposed to the microarray for 1 h and detection was accomplished by 50 μL rabbit anti-HA antibody (71–5500, Invitrogen) for 1 h followed by 50 μL anti-rabbit antibody labeled with Alexa Fluor 532 for 1 h. All incubation and wash steps were performed at RT. Washed arrays were dried by centrifugation and immediately scanned for AlexaFluor 532 on a GenePix 4000 B microarray scanner (Molecular Devices). The detection gain was adjusted to cancel out saturation. See Figure S2 for a representative array image. Mean fluorescent intensities minus mean background were calculated and data were fitted using Prism software (GraphPad Software, Inc.). Bar graphs represent the mean ± SD for each compound. Each experiment has been repeated three times. The lowest possible protein concentration was employed at which good responsiveness was observed to achieve an appropriate dynamic range.

Surface Plasmon Resonance (SPR)

The surface of a CM5 sensor chip (Biacore Inc., GE Healthcare, USA) was activated using freshly mixed *N*-hydroxysuccimide (NHS; 100 mM) and 1-(3-(dimethylamino)propyl)-ethylcarbodiimide (EDC; 391 mM) (1/1, v/v) in water. Next, FGF-2 (45 μg/mL) or CXCL10 (50 μg/mL) in aqueous NaOAc (10 mM, pH 5.5) was passed over the surface until a ligand density of ~4500 RU or ~1500 RU, respectively, was achieved. Quenching of the remaining active esters was accomplished by passing aqueous ethanolamine (1.0 M, pH 8.5) over the surface of the chip. The control flow cell was activated with NHS and EDC, which was then treated with ethanolamine. HBS-EP (0.01 M HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20; pH 7.4) was employed as the running buffer for immobilization, binding and kinetic analysis. Serial dilutions of each compound in HBS-EP buffer at a flow rate of 30 μL/min was employed for association and dissociation at a temperature of 25 °C. Two 30 s injections of aqueous NaCl (2.0 M) at flow rates of 50 and 30 μL/min was employed for regeneration to achieve baseline status. Data were fitted to a two-state binding model using BIAcore T100 evaluation software (Biacore Inc., GE Healthcare).

Endothelial Cell Surface FGF-2 Binding Assay

An immortalized mouse lung endothelial cell line[33] was used in the cell surface FGF-2 binding study. Briefly, endothelial cells (80% confluent) were harvested with trypsin (0.25%) and plated (15 000/100 μL/well) onto a 96 well tissue culture plate (SIAL0596, Sigma). After 24 h, cells were fixed with 100 μL 2% paraformaldehyde (PFA) for 15 min at

RT and then gently washed three times (3 min each) with 400 μL PBS. The plate was blocked with 100 μL BSA (1%) in PBS (BSA-PBS) for 1 h at RT and then incubated with 100 μL biotinlyated-FGF-2 (1 μg/mL; BFF-H8117, ACROBiosystems) in the absence or presence of test compound or heparin at 0.5, 5, or 50 μg/mL for 1 h at RT. After three washes (3 min each) with 400 μL BSA-PBS, 100 μL streptavidin-HRP (500 ng/mL; 21130, Thermo scientific) in BSA-PBS was added for 1 h at RT. After five washes (3 min each) with 100 μL BSA-PBS, the endothelial cell surface bound HRP was measured using an Ultra TMB-Elisa kit (34028, Thermo Scientific) according the manufacturer's protocol.

Endothelial Cell Proliferation Assay

Endothelial cell proliferation in the presence of FGF-2 (233-FB, R&D Systems) or CXCL10 (4277-1000, BioVision) with bovine serum albumin (BSA) as control were performed using the xCELLigence RTCA DP system (ACEA Biosciences Inc., San Diego, CA, USA). Electrical impedance was monitored continuously for 24 h and reported as cell index.[34] The background impedance was set according the manufacturer's instructions with 150 μL DMEM containing fetal bovine serum (0.5%; FBS, Atlanta Biologicals). Immortalized mouse lung endothelial cells were seeded (5,000/well) in replicates of 4 in DMEM medium containing FBS (0.5%), BSA (50 ng/mL), FGF-2 (50 ng/mL) or CXCL10 (1 μg/mL) without or with the test HS compounds or heparin at 50 μg/mL (final volume 150 μL/well). The seeded cells were left to equilibrate at RT for 30 min and then grown at 37 °C in a humidified atmosphere with 5% CO_2. Data were analyzed using the xCELLigence software (Version 1.2.1) and the cell index expressed as mean ± SD.

Statistical Analysis

Statistical significance was determined using one-way ANOVA with Bonferroni's multiple comparison test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Institute of General Medical Sciences (Research Resource for Integrated Glycotechnology P41GM103390 to G.-J.B. and grants R01AI-118985 and R21AI121918 to T.M.H.) from the National Institutes of Health. We thank Drs. Xuesong Chen and Rob Burgess (RayBiotech) for guidance with microarray studies and Lina Mullen for cloning the HA-tagged chemokines.

References

1. (a) Bishop JR, Schuksz M, Esko JD. Nature. 2007; 446:1030–1037. [PubMed: 17460664] (b) Li L, Ly M, Linhardt RJ. Mol BioSyst. 2012; 8:1613–1625. [PubMed: 22513887]
2. Kreuger J, Kjellen L. J Histochem Cytochem. 2012; 60:898–907. [PubMed: 23042481]
3. Allen SJ, Crown SE, Handel TM. Annu Rev Immunol. 2007; 25:787–820. [PubMed: 17291188]
4. Kraushaar DC, Dalton S, Wang L. Biol Chem. 2013; 394:741–751. [PubMed: 23370908]
5. (a) Lindahl U, Kjellen L. J Intern Med. 2013; 273:555–571. [PubMed: 23432337] (b) Vlodavsky I, Iozzo RV, Sanderson RD. Matrix Biol. 2013; 32:220–222. [PubMed: 23499526]

6. Kamhi E, Joo EJ, Dordick JS, Linhardt RJ. Biol Rev Camb Philos Soc. 2013; 88:928–943. [PubMed: 23551941]
7. (a) Turnbull J, Powell A, Guimond S. Trends Cell Biol. 2001; 11:75–82. [PubMed: 11166215] (b) Xu D, Esko JD. Annu Rev Biochem. 2014; 83:129–157. [PubMed: 24606135]
8. (a) van Kuppevelt TH, Dennissen M, van Venrooij WJ, Hoet RMA, Veerkamp JH. J Biol Chem. 1998; 273:12960–12966. [PubMed: 9582329] (b) Lawrence R, Olson SK, Steele RE, Wang L, Warrior R, Cummings RD, Esko JD. J Biol Chem. 2008; 283:33674–33684. [PubMed: 18818196]
9. (a) de Paz JL, Horlacher T, Seeberger PH. Methods Enzymol. 2006; 415:269–292. [PubMed: 17116480] (b) Oyelaran O, Gildersleeve JC. Curr Opin Chem Biol. 2009; 13:406–413. [PubMed: 19625207] (c) Park S, Gildersleeve JC, Blixt O, Shin I. Chem Soc Rev. 2013; 42:4310–4326. [PubMed: 23192235]
10. (a) Dulaney SB, Huang XF. Adv Carbohydr Chem Biochem. 2012; 67:95–136. [PubMed: 22794183] (b) Mende M, Bednarek C, Wawryszyn M, Sauter P, Biskup MB, Schepers U, Brase S. Chem Rev. 2016; 116:8193–8255. [PubMed: 27410264]
11. (a) Noti C, de Paz JL, Polito L, Seeberger PH. Chem -Eur J. 2006; 12:8664–8686. [PubMed: 17066397] (b) de Paz JL, Spillmann D, Seeberger PH. Chem Commun. 2006:3116–3118.(c) de Paz JL, Noti C, Seeberger PH. J Am Chem Soc. 2006; 128:2766–2767. [PubMed: 16506732] (d) de Paz JL, Moseman EA, Noti C, Polito L, von Andrian UH, Seeberger PH. ACS Chem Biol. 2007; 2:735–744. [PubMed: 18030990] (e) Shipp EL, Hsieh-Wilson LC. Chem Biol. 2007; 14:195–208. [PubMed: 17317573] (f) Park TJ, Lee MY, Dordick JS, Linhardt RJ. Anal Biochem. 2008; 383:116–121. [PubMed: 18722996] (g) Maza S, Macchione G, Ojeda R, Lopez-Prados J, Angulo J, de Paz JL, Nieto PM. Org Biomol Chem. 2012; 10:2146–2163. [PubMed: 22294265] (h) Roy S, El Hadri A, Richard S, Denis F, Holte K, Duffner J, Yu F, Galcheva-Gargova Z, Capila I, Schultes B, Petitou M, Kaundinya GV. J Med Chem. 2014; 57:4511–4520. [PubMed: 24786387] (i) Dulaney SB, Xu YM, Wang P, Tiruchinapally G, Wang Z, Kathawa J, El-Dakdouki MH, Yang B, Liu J, Huang XF. J Org Chem. 2015; 80:12265–12279. [PubMed: 26574650] (j) Yang J, Hsieh PH, Liu X, Zhou W, Zhang X, Zhao J, Xu Y, Zhang F, Linhardt RJ, Liu J. Chem Commun. 2017; 53:1743–1746.
12. (a) Hu YP, Zhong YQ, Chen ZG, Chen CY, Shi Z, Zulueta MM, Ku CC, Lee PY, Wang CC, Hung SC. J Am Chem Soc. 2012; 134:20722–20727. [PubMed: 23240683] (b) Zong C, Huang R, Condac E, Chiu Y, Xiao W, Li X, Lu W, Ishihara M, Wang S, Ramiah A, Stickney M, Azadi P, Amster IJ, Moremen KW, Wang L, Sharp JS, Boons GJ. J Am Chem Soc. 2016; 138:13059–13067. [PubMed: 27611601]
13. Schlessinger J, Plotnikov AN, Ibrahimi OA, Eliseenkova AV, Yeh BK, Yayon A, Linhardt RJ, Mohammadi M. Mol Cell. 2000; 6:743–750. [PubMed: 11030354]
14. Fukuhara N, Howitt JA, Hussain SA, Hohenester E. J Biol Chem. 2008; 283:16226–16234. [PubMed: 18359766]
15. van Boeckel CAA, Petitou M. Angew Chem, Int Ed Engl. 1993; 32:1671–1690.
16. (a) Arungundram S, Al-Mafraji K, Asong J, Leach FE III, Amster IJ, Venot A, Turnbull JE, Boons GJ. J Am Chem Soc. 2009; 131:17394–17405. [PubMed: 19904943] (b) Zong C, Venot A, Dhamale O, Boons GJ. Org Lett. 2013; 15:342–345. [PubMed: 23293947] (c) Dhamale OP, Zong C, Al-Mafraji K, Boons GJ. Org Biomol Chem. 2014; 12:2087–2098. [PubMed: 24549353]
17. (a) Takano R, Matsuo M, Kameihayashi K, Hara S, Hirase S. Biosci Biotechnol Biochem. 1992; 56:1577–1580.(b) Roy S, Lai H, Zouaoui R, Duffner J, Zhou H, P Jayaraman L, Zhao G, Ganguly T, Kishimoto TK, Venkataraman G. Glycobiology. 2011; 21:1194–1205. [PubMed: 21515908]
18. Xu Y, Masuko S, Takieddin M, Xu H, Liu R, Jing J, Mousa SA, Linhardt RJ, Liu J. Science. 2011; 334:498–501. [PubMed: 22034431]
19. Thacker BE, Xu D, Lawrence R, Esko JD. Matrix Biol. 2014; 35:60–72. [PubMed: 24361527]
20. Nguyen TK, Arungundram S, Tran VM, Raman K, Al-Mafraji K, Venot A, Boons GJ, Kuberan B. Mol BioSyst. 2012; 8:609–614. [PubMed: 22116385]
21. Heimburg-Molinaro J, Song X, Smith DF, Cummings RD. Curr Protoc Protein Sci. 2011; 64:12.10.1–12.10.29.
22. (a) Guimond S, Maccarana M, Olwin BB, Lindahl U, Rapraeger AC. J Biol Chem. 1993; 268:23906–23914. [PubMed: 7693696] (b) Ishihara M, Kariya Y, Kikuchi H, Minamisawa T,

Yoshida K. J Biochem. 1997; 121:345–349. [PubMed: 9089410] (c) Miller GJ, Hansen SU, Avizienyte E, Rushton G, Cole C, Jayson GC, Gardiner JM. Chem Sci. 2013; 4:3218–3222.

23. Goodger SJ, Robinson CJ, Murphy KJ, Gasiunas N, Harmer NJ, Blundell TL, Pye DA, Gallagher JT. J Biol Chem. 2008; 283:13001–13008. [PubMed: 18281281]
24. Lau EK, Paavola CD, Johnson Z, Gaudry JP, Geretti E, Borlat F, Kungl AJ, Proudfoot AE, Handel TM. J Biol Chem. 2004; 279:22294–22305. [PubMed: 15033992]
25. Salanga CL, Dyer DP, Kiselar JG, Gupta S, Chance MR, Handel TM. J Biol Chem. 2014; 289:14896–14912. [PubMed: 24727473]
26. Luster AD, Greenberg SM, Leder P. J Exp Med. 1995; 182:219–231. [PubMed: 7790818]
27. Campanella GS, Colvin RA, Luster AD. PLoS One. 2010; 5:e12700. [PubMed: 20856926]
28. Salanga CL, Handel TM. Exp Cell Res. 2011; 317:590–601. [PubMed: 21223963]
29. Hillyer P, Male D. Immunol Cell Biol. 2005; 83:375–382. [PubMed: 16033532]
30. Allen BL, Filla MS, Rapraeger AC. J Cell Biol. 2001; 155:845–858. [PubMed: 11724824]
31. Yu TW, Bargmann CI. Nat Neurosci. 2001; 4(Suppl):1169–1176.
32. (a) Lindahl B, Eriksson L, Lindahl U. Biochem J. 1995; 306(Pt 1):177–184. [PubMed: 7864807] (b) Yamada S, Murakami T, Tsuda H, Yoshida K, Sugahara K. J Biol Chem. 1995; 270:8696–8705. [PubMed: 7721774] (c) Dejima K, Takemura M, Nakato E, Peterson J, Hayashi Y, Kinoshita-Toyoda A, Toyoda H, Nakato H. J Biol Chem. 2013; 288:34384–34393. [PubMed: 24133213] (d) Hsieh PH, Xu Y, Keire DA, Liu J. Glycobiology. 2014; 24:681–692. [PubMed: 24770491] (e) Boothello RS, Sarkar A, Tran VM, Nguyen TK, Sankaranarayanan NV, Mehta AY, Alabbas A, Brown S, Rossi A, Joice AC, Mencio CP, Quintero MV, Kuberan B, Desai UR. ACS Chem Biol. 2015; 10:1485–1494. [PubMed: 25742429] (f) Sankaranarayanan NV, Strebel TR, Boothello RS, Sheerin K, Raghuraman A, Sallas F, Mosier PD, Watermeyer ND, Oscarson S, Desai UR. Angew Chem, Int Ed. 2017; 56:2312–2317.
33. Qiu H, Jiang JL, Liu M, Huang X, Ding SJ, Wang L. Mol Cell Proteomics. 2013; 12:2160–2173. [PubMed: 23649490]
34. (a) Martinez-Serra J, Gutierrez A, Munoz-Capo S, Navarro-Palou M, Ros T, Amat JC, Lopez B, Marcus TF, Fueyo L, Suquia AG, Gines J, Rubio F, Ramos R, Besalduch J. OncoTargets Ther. 2014; 7:985–994.(b) Ke N, Wang X, Xu X, Abassi YA. Methods Mol Biol. 2011; 740:33–43. [PubMed: 21468966]

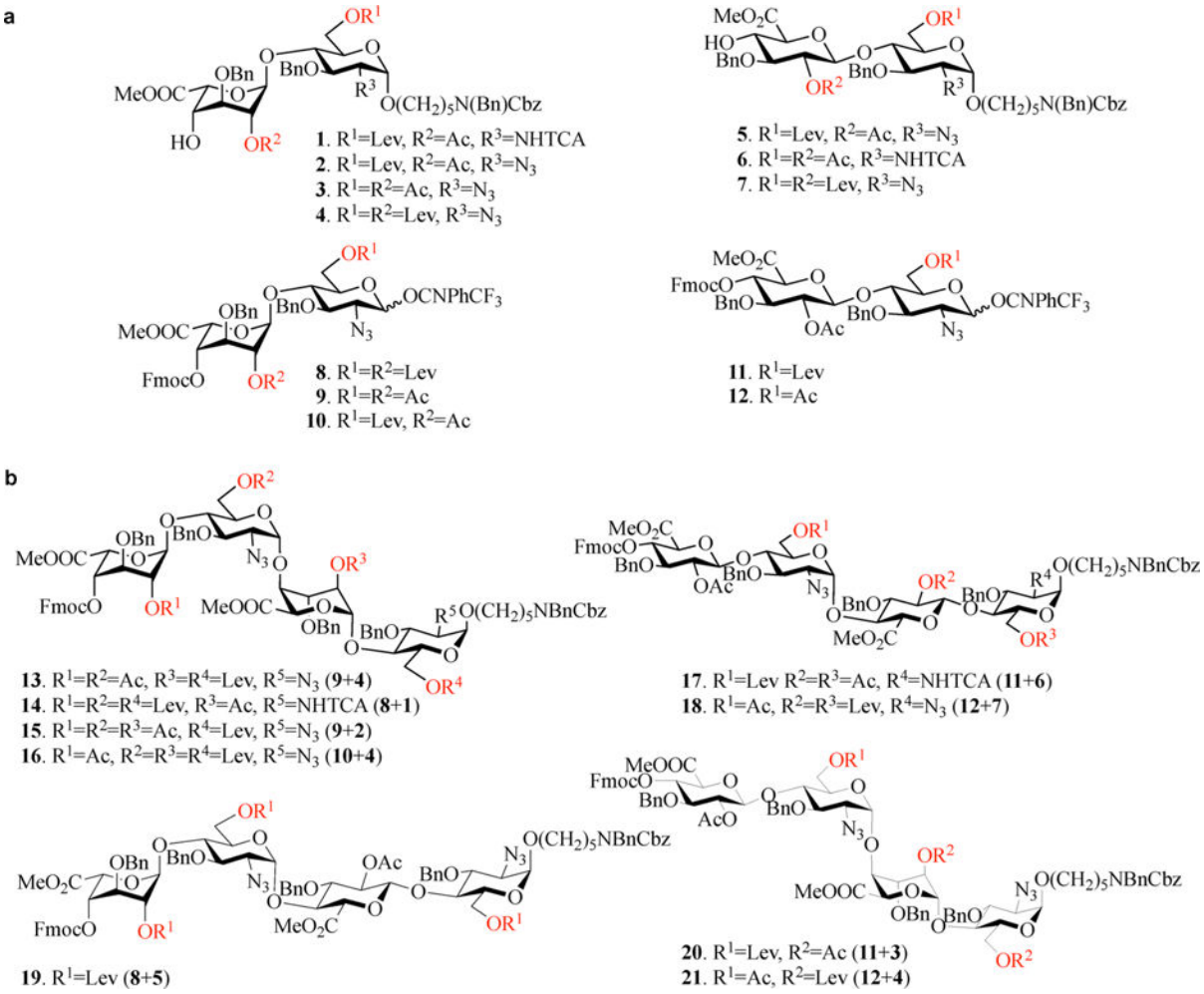
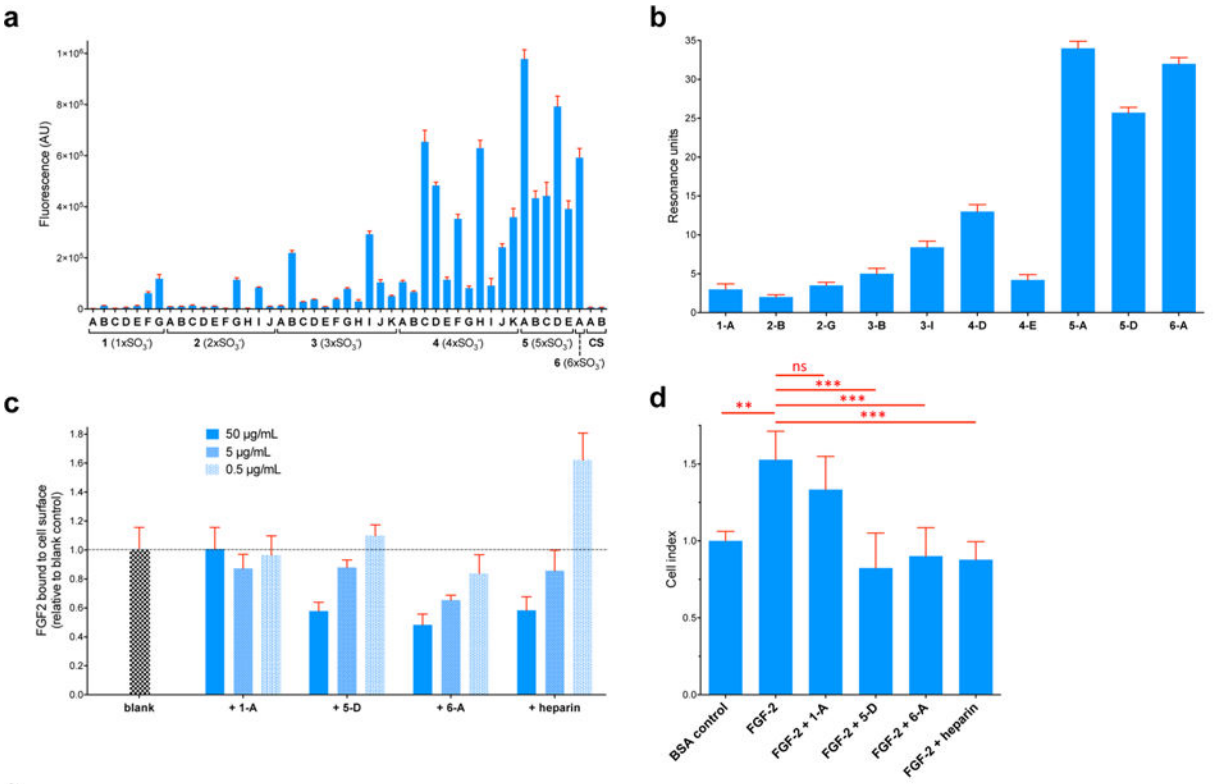


Figure 1.

Modular synthesis of HS oligosaccharides. (a) Modular disaccharide building blocks. (b) Assembled tetrasaccharides.



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1-A	GlcA-GlcNAc-GlcA2S-GlcNAc	2-F	GlcA-GlcNAc6S-IdoA-GlcNAc6S	3-H	IdoA-GlcNS6S-GlcA-GlcNS	4-I	IdoA-GlcNS6S-IdoA-GlcNS6S
1-B	GlcA-GlcNAc-IdoA2S-GlcNAc	2-G	IdoA2S-GlcNAc6S-GlcA-GlcNAc	3-I	IdoA2S-GlcNAc6S-GlcA-GlcNAc6S	4-J	IdoA2S-GlcNS6S-IdoA-GlcNAc6S
1-C	GlcA-GlcNAc-IdoA-GlcNAc6S	2-H	IdoA-GlcNAc6S-GlcA-GlcNAc6S	3-J	IdoA-GlcNS-IdoA-GlcNS6S	4-K	IdoA2S-GlcNAc6S-IdoA2S-GlcNAc6S
1-D	IdoA2S-GlcNAc-GlcA-GlcNAc	2-I	IdoA-GlcNS-IdoA-GlcNS	3-K	IdoA-GlcNAc6S-IdoA2S-GlcNAc6S	5-A	GlcA-GlcNS3S-IdoA2S-GlcNS6S
1-E	IdoA-GlcNAc6S-GlcA-GlcNAc	2-J	IdoA-GlcNAc6S-IdoA-GlcNAc6S	4-A	GlcA-GlcNS-GlcA2S-GlcNS6S	5-B	GlcA-GlcNS6S-IdoA2S-GlcNS6S
1-F	IdoA-GlcNAc-IdoA-GlcNAc6S	3-A	GlcA-GlcNS-GlcA2S-GlcNS	4-B	GlcA-GlcNS6S-GlcA-GlcNS6S	5-C	IdoA2S-GlcNS6S-GlcA-GlcNS6S
1-G	IdoA-GlcNAc6S-IdoA-GlcNAc	3-B	GlcA-GlcNS-IdoA2S-GlcNS	4-C	GlcA-GlcNS3S-IdoA2S-GlcNS	5-D	IdoA-GlcNS6S-IdoA2S-GlcNS6S
2-A	GlcA-GlcNS6S-GlcA-GlcNAc	3-C	GlcA-GlcNS-IdoA-GlcNS6S	4-D	GlcA-GlcNS-IdoA2S-GlcNS6S	5-E	IdoA-GlcNS3S6S-IdoA-GlcNS6S
2-B	GlcA-GlcNAc-GlcA2S-GlcNAc6S	3-D	GlcA-GlcNAc-IdoA2S-GlcNS6S	4-E	GlcA-GlcNS6S-IdoA-GlcNS6S	6-A	IdoA2S-GlcNS6S-IdoA2S-GlcNS6S
2-C	GlcA-GlcNAc6S-GlcA-GlcNAc6S	3-E	GlcA-GlcNS6S-IdoA-GlcNS	4-F	IdoA2S-GlcNS6S-GlcA-GlcNS	CS-A	GlcA-GalNAc-GlcA-GalNAc4S
2-D	GlcA-GlcNAc-IdoA-GlcNS6S	3-F	GlcA-GlcNAc6S-IdoA2S-GlcNAc6S	4-G	IdoA-GlcNS6S-GlcA-GlcNS6S	CS-B	GlcA-GlcNS6S-GlcA-GlcNAc
2-E	GlcA-GlcNAc-IdoA2S-GlcNAc6S	3-G	IdoA2S-GlcNS-GlcA-GlcNS	4-H	IdoA-GlcNS-IdoA2S-GlcNS6S		

Abbreviations: D-glucuronic acid (GlcA); *N*-acetyl-D-glucosamine (GlcNAc);glucosamine (GlcN); L-iduronic acid (IdoA); *N*-acetylgalactosamine (GalNAc)

Figure 2.

Binding of synthetic heparan sulfate oligosaccharides to FGF-2. (a) Binding of FGF-2 (0.5 μg/mL) to the HS oligosaccharide microarray at 1 mM ($n = 6$). (b) SPR binding of immobilized FGF-2 to various tetrasaccharides (100 μM) used as analytes. (c) Inhibition of FGF-2 binding to mouse endothelium cell surface by various tetrasacharides and heparin. (d) Inhibition of FGF-2 induced cell proliferation by various tetrasacharides and heparin measured in real time by an xCELLigence RTCA DP system. Asterisks indicate statistically significant difference (** $P < 0.01$, *** $P < 0.001$) and ns indicates no significant difference. Bar graphs in panels a-d represent the mean ± SD. (e) Compound numbering and structures of the tetrasaccharide library.

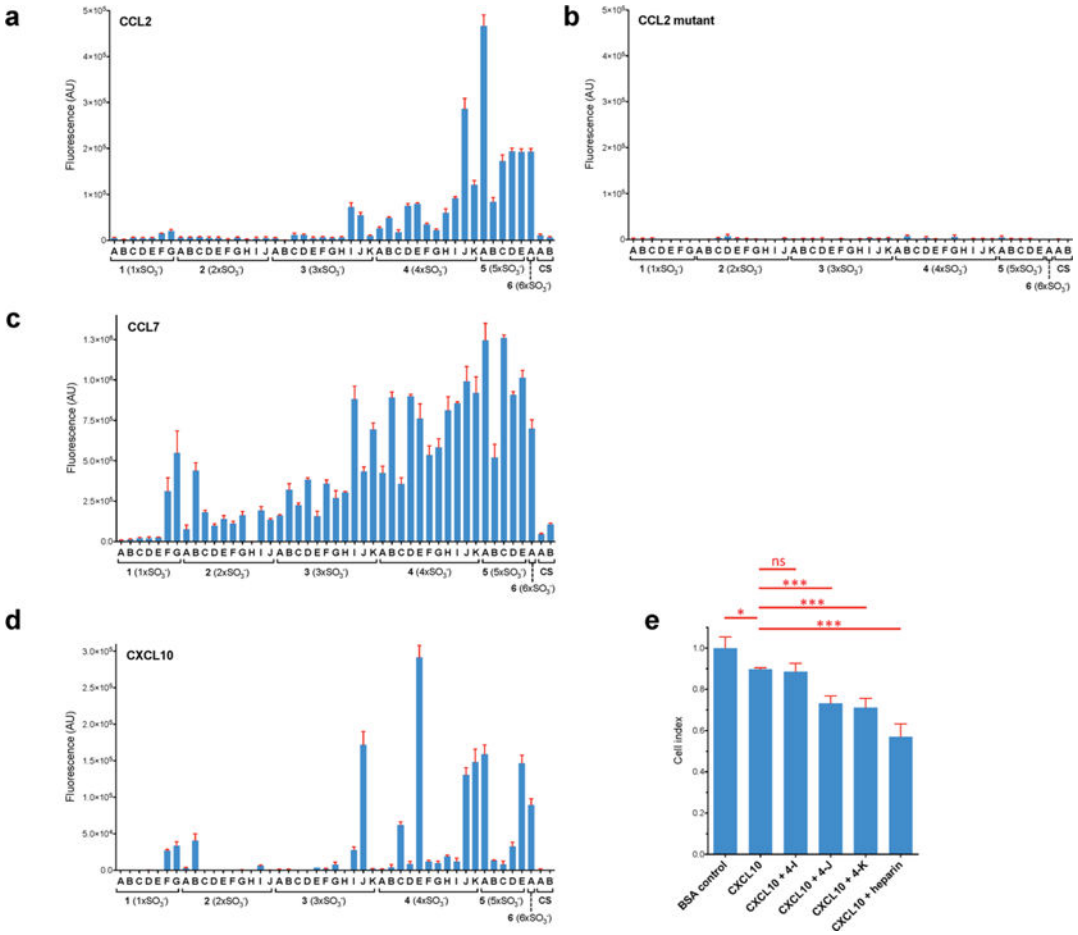
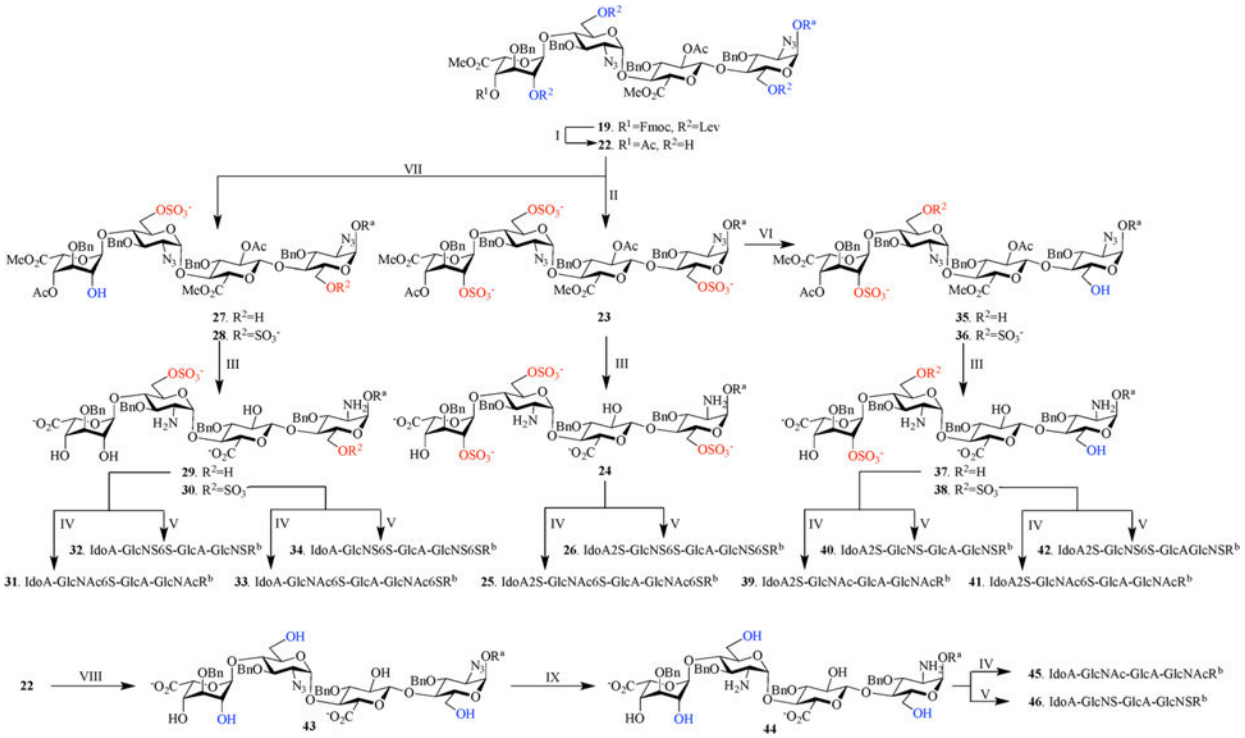


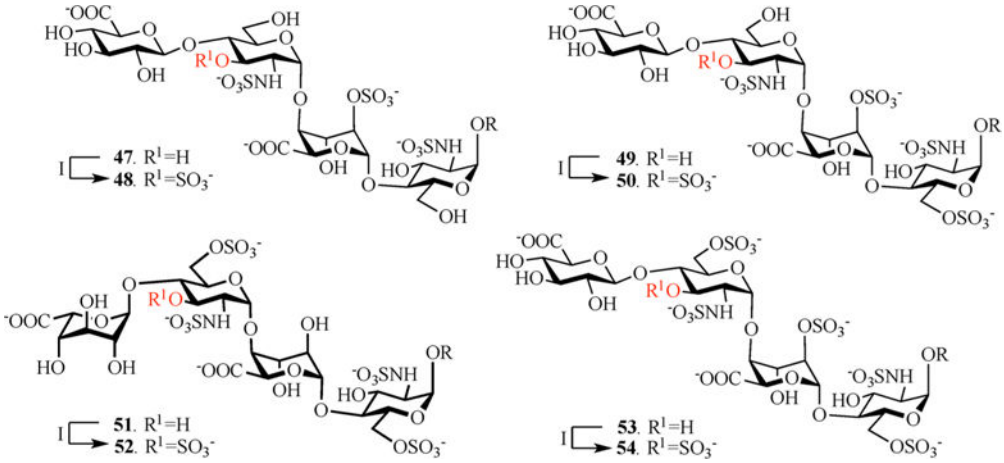
Figure 3.

Binding of different HS-binding proteins to the HS oligosaccharide microarray and subsequent biological examination. Microarray results of synthetic heparin sulfate tetrasaccharide library at 1 mM with (a) CCL2 (0.5 μg/mL); (b) CCL2 mutant R18AK19A (0.5 μg/mL; shown at same scale as CCL2); (c) CCL7 (1 μg/mL); and (d) CXCL10 (0.5 μg/mL). (e) Effect of various tetrasaccharides on the inhibition of CXCL10 on cell proliferation. Asterisks indicate statistically significant difference (* $P < 0.05$, *** $P < 0.001$) and ns indicates no significant difference. Bar graphs in panels a–e represent the mean ± SD.



Scheme 1. Diversification of Tetrasaccharide 18[a]

[a]Reagents and conditions: (I) (i) Et_3N, MeOH/DCM; (ii) Ac_2O, Pyr., DMAP; (iii) NH_2NH_2AcOH, DCM/MeOH, RT, 2 h, 77%; (II) $Py.SO_3$ excess, DMF, 2 h, 81%; (III) (i) LiOH, H_2O_2, THF, 4 h, then 4 M NaOH, MeOH, 12 h; (ii) PMe_3, THF, MeOH, 0.1 M NaOH, 1 h (**24**, 70%; **29**, 78%; **30**, 54%; **37**, 67%; **38**, 54%); (IV) (i) Ac_2O, MeOH, Et_3N, 30 min; (ii) Pd/C, H_2, MeOH, H_2O, 4 h, (iii) $Pd(OH)_2$, H_2, H_2O, 14 h (**25**, 85%; **31**, 65%; **33**, 72%; **39**, 77%; **41**, 72%, **45**, 82%); (V) (i) $Py.SO_3$, MeOH, Et_3N, 0.1 M NaOH, 12 h; (ii) Pd/C, H_2, MeOH, H_2O, 4 h; (iii) $Pd(OH)_2$, H_2, H_2O, 14 h (**26**, 67%; **32**, 68%; **34**, 77%; **40**, 69%; **42**, 77%, **46**, 71%); (VI) BTSA, Pyr., 60 °C, 2 h (**35**, 50%; **36**, 27%); (VII) $Py.SO_3$, controlled addition, DMF (**27**, 48%; **28**, 35%); (VIII) LiOH, H_2O_2, THF, 4 h, then 4 M NaOH, MeOH, 12 h, 84%; (IX) PMe_3, THF, MeOH, 0.1 M NaOH, 1 h, 87%. $R^a=(CH_2)_5NBnCbz$; $R^b=(CH_2)_5NH_2$.



Scheme 2. Tetrasaccharide Substrates for 3-OST1 and Their Modified Products[a]

[a]Reagents and conditions: (I) 3-*O*-sulfotransferase 1, PAPS, MES buffer, $MnCl_2$, $MgCl_2$, (**48**, 19%; **50**, 75%; **52**, 25%; **54**, 20%). R = $(CH_2)_5NH_2$.