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Using structurally defined oligosaccharides to understand the interactions between proteins and heparan sulfate

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Abstract

Heparan sulfate (HS) is widely present on the animal cell surface and in the extracellular matrix. HS achieves its biological functions by interacting with proteins to change proteins' conformation, oligomerization state and cellular location. The challenging question to study HS is how to dissect the relationship between the structures of HS and the biological activities. In the past several years, crucial techniques have been developed to overcome this challenge. A novel chemoenzymatic method to synthesize structurally defined HS oligosaccharides has offered a key access to this class of sulfated carbohydrate molecules. Recent rapid progress of HS microarray technology allows screening of the interaction of a target protein with a large number of HS oligosaccharides. The improved availability of HS oligosaccharides and HS microarray analysis will undoubtedly accelerate the investigation of the contribution of the specific sulfated carbohydrate structures of HS in a wide range of biological contexts.

Graphical Abstract

Heparan sulfate (HS), a linear sulfated polysaccharide, plays essential physiological roles and binds to hundreds of HS-binding proteins (HSBPs). Present on the cell surface and in the extracellular matrix space, HS interacts with proteins on the cell surface as well as the

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Conflict of Interest

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proteins around the cells, notably in the extracellular matrix. The interaction of HSBPs and HS adds a layer of regulations, *i.e.* altering oligomerization forms for unique functions, gaining binding affinity to different binding patterns, and positioning proteins at specific cellular locations. Appearing early in the metazoan evolution, HS displays a wide range of variations in sulfations and the content of iduronic acid (IdoA) in both invertebrates and higher organisms. One group of studies suggest that the HS from vertebrate cells has a higher level of sulfation and iduronic acid than the HS from *C.elegans* and *Drosophila* [1, 2]. However, other studies on HS from different invertebrates and chordates show that there is no clear structural difference in the HS between mammals and lower organisms [3, 4].

HS polysaccharides consist of the disaccharide repeating unit of glucosamine (GlcN) and glucuronic acid (GlcA) or IdoA (Fig 1). The disaccharide units are repeated 50–200 times in a HS polysaccharide chain to form a macromolecule with molecular weight of 10,000 to 80,000 Da. Each GlcN unit is capable of carrying *O*-sulfo groups at C₃ and C₆ positions or an *N*-sulfo group at C₂ position, and GlcA or IdoA unit may carry an *O*-sulfo group at the C₂ position. Epimerase converts GlcA to an IdoA residue, while sulfo groups are transferred by four different classes of HS sulfotransferases. There are around 21 commonly identified disaccharide species. In principle, a large number of saccharide sequences can be built from 21 disaccharide building blocks (Table I); however, only a limited number of oligosaccharides have been confirmed to be present in HS [5].

HSBP cover a wide range of biological functions. For example, HS binds to fibroblast growth factors (FGFs) and fibroblast growth factor receptors to control cell proliferation and differentiation [6]. It also binds to antithrombin, a protease inhibitor that regulates the blood coagulation process [7], as well as chemokines to modulate inflammation responses [8]. To understand the biological significance of individual HS-HSBP interaction, it is of paramount importance to dissect the structural detail of the interaction. In one aspect, there is a need to identify the contribution of the side chains of HSBPs amino acid residues to the HS binding event. This can be accomplished through site-directed mutagenesis studies of the protein. In another aspect, there is a need to identify the contribution of sulfation patterns and the size of the HS oligosaccharide. The latter is a much more difficult task because of the unavailability of structurally defined HS oligosaccharides. In the past, a HSBP-binding domain was isolated using a protective digestion approach, a technique used for studying the interactions between DNA and proteins. In this experiment, a mixture of HS isolated from cells was incubated with HSBP to allow complex formation, followed by heparin lyases digestion [9]. Because HSBP would protect bound HS fragment from the digestion of heparin lyases, the size and structure of the bound HS oligosaccharide could be further analyzed by gel filtration and disaccharide composition analysis, respectively. While this method can reliably determine the size of the resultant HS oligosaccharide, the compositional analysis only reveals the identity of the disaccharide components of an oligosaccharide [10], but no saccharide sequence information can be obtained. As a result, for most of the HSBPs, the structural requirement for binding at the oligosaccharide level is entirely lacking. Recently, an advanced mass spectrometry method has been reported to study the binding of HS and HSBP; however, the analysis requires the use of structurally defined HS oligosaccharides [11].

A better way to identify the structures of HS oligosaccharides that are responsible for binding to HSBPs would be to prepare a panel of structurally defined oligosaccharides and test their binding affinity to HSBPs [12]. This method was successfully used to prove the structure of antithrombin-binding pentasaccharide in heparin, and the pentasaccharide was subsequently developed into an anticoagulant drug known as fondaparinux [13]. The design of structurally defined pentasaccharides was built on a large amount of work from the isolation and characterization of naturally occurring polysaccharides as reviewed by Ricard-Blum and Lisacek [14]. This approach however has not been possible as a general method to prove relationship between the binding affinity of a HSBP and the structure of HS due to the lack of structurally defined HS oligosaccharides. Despite extensive efforts [15–19], it is technically challenging to synthesize sufficient amount of HS oligosaccharides using a purely chemical synthetic approach for biological studies, especially those larger than octasaccharides with complex sulfation patterns. In recent years, an alternative chemoenzymatic approach has emerged to offer a shorter synthetic route to synthesize HS oligosaccharides [20–23]. The newly developed chemoenzymatic approach demonstrates the ability to make diverse oligosaccharides with sufficient quantities for a wide range of biological analysis [24]. The chemoenzymatic method for the synthesis of HS oligosaccharides has been reviewed extensively [24], including a recent article published by Tsai and colleagues in this Journal [25]. Furthermore, a number of HS oligosaccharides synthesized by the chemoenzymatic approach are commercially available (www.glycantherapeutics.com). In this review, we will cover the latest structural insights of HS-HSBP interactions gained through using structurally defined oligosaccharides.

3-O sulfated HS oligosaccharides in protein binding

Sulfation at the hydroxyl group of C₃ position of glucosamine is a rare modification of HS [5]. The 3-*O*-sulfation is carried out by a family of enzyme known as 3-*O*-sulfotransferase (Hs3st). It remains largely a mystery as to why the Hs3st family has greatly expanded in vertebrates to contain 7–8 different isoforms [26]. Presumably, different Hs3st isoforms have somewhat different oligosaccharide substrate preferences and the resulting sequence would bind to distinct HSBPs. However, until now only a few HSBPs have been identified to bind selectively to 3-*O*-sulfated HS [27]. The difficulty of identifying HSBPs that bind selectively/preferentially to 3-*O*-sulfated HS is partly due to a lack of sufficient quantity of structurally defined HS oligosaccharides containing the 3-*O*-sulfated glucosamine. Without such oligosaccharides, specific contribution of the 3-*O*-sulfate group to binding cannot be determined with high degree of certainty. Although two 3-*O*-sulfated octasaccharides were synthesized using a chemical approach, the compounds are not readily available due to the lengthy synthesis [18]. By employing a *de novo* chemoenzymatic synthesis scheme, the structurally defined 3-*O*-sulfated HS oligosaccharides are now available. These oligosaccharides helped identify a couple of novel HSBPs that recognize 3-*O*-sulfated HS [22, 28].

In one recent study, neuropilin-1 (Nrp-1), a cell surface receptor which plays essential roles in both axon guidance and angiogenesis, was found to bind preferentially to HS modified by Hs3st [29]. In this study, HS dodecasaccharides (GlcNAc6S-GlcA-GlcNS6S±3S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S±3S-IdoA2S-GlcNS6S-GlcA-pNP, where

pNP represents *p*-nitrophenyl) that differs only on the number of 3-*O*-sulfate groups they carry (0, 1 or 2) were compared in their ability to inhibit Nrp-1-dependent neuron growth cone collapse. Interestingly, the IC₅₀ values of the dodecasaccharides that bear two and one 3-*O*-sulfate groups were reduced to 1/12 and 1/3 of the dodecasaccharide that bears no 3-*O*-sulfate, suggesting a strong binding preference of Nrp-1 to 3-*O*-sulfated HS. Combined with the data obtained from Hs3st-2 and Hs3st-1 deficient mice, this study provided strong evidence that HS modified by Hs3st-2 likely contains the most ideal, and physiologically relevant 3-*O*-sulfate containing structure for interacting with Nrp-1 on neuron. It has been reported that 3-*O*-sulfation shifts the conformation equilibrium of neighboring IdoA2S residues between skew boat (²S_O) conformation and chair conformation (¹C₄) [30] [28]. However, there is no sufficient evidence to conclude that the increased binding affinity between 3-*O*-sulfated dodecasaccharides and Nrp-1 is attributed to the ²S_O conformation of IdoA2S residues in the dodecasaccharides.

Another study investigated the structural requirements of HS and Wnt3a interaction using a panel of structurally defined oligosaccharides ranging from tetrasaccharide to dodecasaccharide [31]. This small library of oligosaccharides allowed authors not only to confirm previous finding that the 6-*O*-sulfate group is essential for Wnt3a–HS interactions, but also to provide novel insight on the role of 3-*O*-sulfate groups in Wnt3a–HS interaction. In a cell-based Wnt signaling assay where binding of Wnt to cell surface HS is required for signaling, adding exogenous HS oligosaccharides inhibits signaling. It was observed that while a highly sulfated dodecasaccharide with the structure of GlcNS6S-GlcA-(GlcNS6S-IdoA2S)₄-GlcNS6S-GlcA-pNP inhibited only 20% of Wnt signaling, another dodecasaccharide with only one additional 3-*O*-sulfate group inhibited Wnt signaling by 75%. This study also determined that the minimal binding size of the oligosaccharide for Wnt3a is hexasaccharide provided that a 3-*O*-sulfate group is included.

Availability of oligosaccharides also enabled researchers to reexamine and challenge the status quo in the field. A very recent study challenges the long standing view that -GlcA-GlcNS3S6S- disaccharide unit is required for HS-antithrombin interaction (Fig 2). In this study, a total of six 3-*O*-sulfated hexasaccharides and octasaccharides were synthesized using the chemoenzymatic method [28]. Among them, one octasaccharide has the structure of GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP, where the GlcNS3S6S is flanked by two IdoA2S residues (Fig 2). This octasaccharide does not have the disaccharide unit of -GlcA-GlcNS3S6S-, but displays strong binding affinity toward antithrombin and potent anticoagulant activity as demonstrated by *in vitro* and *ex vivo* experiments [28]. Interestingly, unlike fondaparinux, the octasaccharide has a faster clearance rate in a rat model, offering a possible candidate for short-acting anticoagulant drug. This observation suggests that the structure of HS oligosaccharide not only determines its biological function, but also contributes to the clearance *in vivo*.

HS oligosaccharide microarray

Carbohydrate microarray analysis technique offers high throughput capability to screen the interactions of carbohydrates and proteins with extremely small amounts of glycan and protein samples [32–34]. A number of defined neutral saccharides, including *N*-linked and

O-linked glycans, bacterial oligosaccharides and oligosaccharides from human milk have been made for glycan microarray development through an NIH sponsored consortium (www.functionalglycomics.org). Preparation of a comprehensive HS oligosaccharide microarray to probe HS-HSBP interactions is now possible after crucial improvement on synthetic methods. The effect of having such an HS microarray is expected to be revolutionary because it could reveal the structural selectivity of many HSBPs at unprecedented resolution. The Seeberger group pioneered the development of the HS microarray for the analysis of HS interaction's with growth factors and chemokines [35, 36.] The reports demonstrated the feasibility of the HS microarray, however, only a small number of tetrasaccharides and hexasaccharides were present on their array. Since then, the number of oligosaccharides for HS microarray analysis has been steadily improved. The Huang group [37] demonstrated an array analysis of eight hexasaccharides, but unfortunately, the biologically relevant 3-*O*-sulfated hexasaccharides were not included in their study. Subsequently, a HS microarray analysis consisting of 14 different HS heptasaccharides has been reported, which covers 3-*O*-sulfated heptasaccharides [38]. The procedures for making microarray chips and the representative data from microarray analysis are shown in Fig 3. First, a synthetic oligosaccharide is immobilized on the functionalized array surface (Fig 3A). The functionalized array surface reacts only with the amino group (-NH₂) at the reducing end, therefore the oligosaccharide is affixed in a specific orientation as shown in Fig 3A. A total of 13 heptasaccharides and one pentasaccharide were used in this study [38], covering different sulfation patterns (Fig 3B). Fluorescently labeled antithrombin only bound to two heptasaccharides, comp **5** and **12**, demonstrating the structural selectivity of the binding event between antithrombin and HS (Fig 3C and 3D). It should be noted that HS array analysis can be constructed in different formats. For example, HS polysaccharides and oligosaccharides were attached to a gold surface to investigate the interaction with proteins [39, 40].

The most comprehensive HS microarray that has been prepared so far is a tetrasaccharide library containing 47 unique structures. The tetrasaccharide library was prepared using a modular synthetic approach by linking disaccharides with diverse structures [41]. Tetrasaccharides prepared in this way contain 1 to 6 sulfate groups at various positions and include both glucuronic and iduronic acids. Several HSBPs including fibroblast growth factor 2 (FGF2) and chemokines CCL2, CCL7, CCL5, CCL13, CXCL8 and CXCL10 were tested on the tetrasaccharide array. For FGF2 binding, the strict dependence on 2-*O*-sulfated IdoA is immediately clear, which validated the utility of the microarray, even though it only is comprised of relatively short tetrasaccharides. The result of the chemokine binding is especially revealing in several respects. First, each chemokine shows distinct binding preferences and seems to be able to distinguish among tetrasaccharides with the same number of sulfate groups. Second, CCL5, CCL7 and CCL13 were capable of binding mono-sulfated tetrasaccharide containing IdoA and GlcNAc6S quite well, and it is surprising that the binding to such tetrasaccharide is even better than many tetra- and penta-sulfated tetrasaccharides. At last, for all proteins tested, the most highly sulfated tetrasaccharide, IdoA2S-GlcNS6S-IdoA2S-GlcNS6S, was not the best binder. For CXCL8, CXCL10 and CXCL13, binding of tri- and tetra-sulfated tetrasaccharides were found to be 2–3 fold better than the hexa-sulfated tetrasacchrude. These discoveries were significant because it showed

clearly that chemokine-HS interactions can be highly specific, and that exhaustive sulfation may have a negative impact on binding. It is hard to imagine that such in-depth structure insight can be obtained using traditional methods.

The next goal is to expand the structural coverage on the HS microarray analysis. The availability of structurally defined HS oligosaccharides is critical in order to achieve this goal. Compared to chemical synthesis, the main advantage of the chemoenzymatic method is that it is relatively easier to prepare longer oligosaccharides. The synthesis is readily repeatable at the milligram scale for further studies, *i.e.* co-crystallization complexes of oligosaccharides and HSBPs and the intervention of biological systems using soluble oligosaccharides. A recent paper reports the synthesis of a library of HS oligosaccharides covering 65 different compounds using the chemoenzymatic method [42], which will be printed on the microarray chips for the microarray analysis. It should be noted that HS-functionalized cell culture plates have been reported for screening functional roles of HS in live cells, offering a new dimension for high throughput methods beyond the currently described HS microarray analysis [43]. The advancement in the screening techniques and availability of homogeneous HS oligosaccharides will undoubtedly accelerate HS-related research toward the understanding the structural and selectivity of HS in various biological systems.

In summary, HS is an essential glycan and plays a wide range of physiological and pathophysiological functions. In the past, the availability of structurally defined oligosaccharides and a high throughput screening technique have been the major road blocks in HS-related research. The success of new HS synthetic techniques and the introduction of HS microarray analysis will play significant roles in accelerating the studies in understanding the functions of HS in different biological contexts.

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Fig. 1.
Structures of disaccharide repeating units of HS.

Fig. 2.
Structures of the 3-*O*-sulfated octasaccharide and fondaparinux. The key disaccharide motifs that are responsible for binding to antithrombin are boxed.

Fig. 3.

Images of HS-array analysis. Panel A shows schematic reactions for immobilization of an oligosaccharide on a functionalized array slide. Panel B shows the symbolic structures of 14 HS oligosaccharides that are immobilized on the array slide for microarray analysis. Panel C shows the schematic interaction of a fluorescently labeled antithrombin interacts with the oligosaccharides (1–7) on the array slide. Panel D shows the histogram of fluorescence intensity analysis when the array slide was hybridized with fluorescently labeled antithrombin. Here, the antithrombin was labeled with Alexa Fluo[®] 488. The histogram data have been published in our previous publication [38].

Table I

Commonly observed HS disaccharides

	Abbreviated structure	Disaccharide Code [*]
1	GlcA-GlcNAc	G0A0
2	GlcA-GlcNH ₂	G0H0
3	GlcA-GlcNS	G0S0
4	GlcA-GlcNAc6S	G0A6
5	GlcA-GlcNH ₂ 6S	G0H6
6	GlcA-GlcNS6S	G0S6
7	GlcA-GlcNS3S6S	G0S9
8	GlcA2S-GlcNAc6S	G2A6
9	GlcA2S-GlcNS	G2S0
10	GlcA2S-GlcNS6S	G2S6
11	IdoA-GlcNAc	I0A0
12	IdoA-GlcNAc6S	I0A6
13	IdoA-GlcNS	I0S0
14	IdoA-GlcNS6S	I0S6
15	IdoA2S-GlcNAc	I2A0
16	IdoA2S-GlcNS	I2S0
17	IdoA2S-GlcNH ₂ 6S	I2H6
18	IdoA2S-GlcNH ₂ 3S6S	I2H9
19	IdoA2S-GlcNS3S	I2S3
20	IdoA2S-GlcNS6S	I2S6
21	IdoA2S-GlcNS3S6S	I2S9

^{*} Structure codes are used during the disaccharide analysis of HS.

The definitions of these codes are described in a paper by Lawrence and colleagues [10]: G = Glucuronic acid, I = iduronic acid, A = glucosamine, H = glucosamine with free amine, S = *N*-sulfated glucosamine, 0 = zero *O*-sulfation, 2 = 2-*O*-sulfation, 6 = 6-*O*-sulfation, 3 = 3-*O*-sulfation and 9 = 3-*O*-sulfation and 6-*O*-sulfation.