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Synthetic heparin and heparan sulfate oligosaccharides and their protein interactions

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Heparin and heparan sulfate bind a host of basic proteins that take advantage of the sugar's dense structural information. The significance of these interactions in various aspects of development, physiology, and disease stimulated keen interest in evaluating structure–activity relationships. The well-defined heparin and heparan sulfate oligosaccharides needed for these studies can be mainly accessed by chemical synthesis and, more recently by chemoenzymatic means. The various synthetic strategies available to chemical synthesis have recently enabled the acquisition of several regular and irregular sequences, including a number of dodecasaccharides, through improved coupling methods and judicial protecting group manipulations. Controlled chain elongation and critical application of modification enzymes allowed the generation of well-defined constructs via chemoenzymatic synthesis. Investigations of various protein interactions with the synthetic constructs delivered valuable information that could aid future drug development endeavors.

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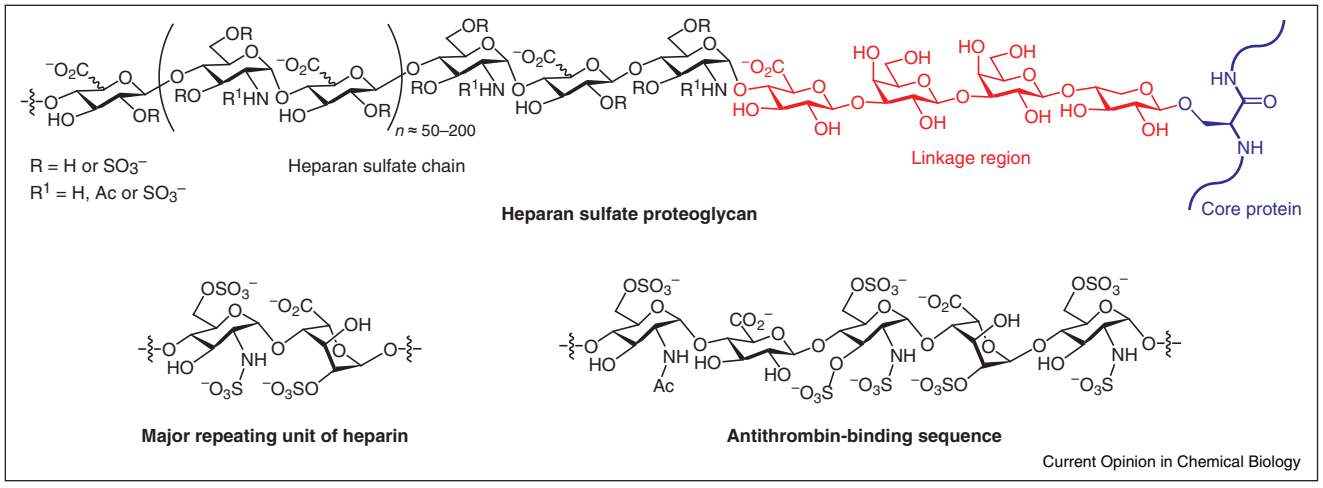
Introduction

Proteoglycans are vital components of cell surfaces and extracellular matrices of animal tissues [1,2]. They are complex macromolecules that comprise a core protein and one or more conjugated glycosaminoglycans (GAGs) — linear polymers with repeating disaccharide backbones. While the protein segments displayed notable activities [3,4], the vast majority of proteoglycan functions are associated with GAGs of which heparan sulfate (HS) is the most heterogeneous and most widespread [5]. Alternating 1 → 4-linked α-D-glucosamine (GlcN) and either β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) make up the extended HS backbone (Figure 1). Potential sulfations may occur at C3 and C6 of GlcN and at C2 of the uronic acid (UA), and the GlcN amine function may be sulfonated, acetylated or unsubstituted. These variations account to 48 disaccharide possibilities within the chain. However, only about half of those were observed in *Nature*, likely due to biological restrictions that also granted tissue-specific sulfonation patterns and intermittent swatches of unsulfated regions [6]. Hundreds of basic proteins, implicated in fertilization, growth and development, bacterial and viral infections, wound healing, immune response, and cancer progression among others, take advantage of the rich structural diversity of HS [7]. HS grant proteins localized availability near the cell surface and facilitate various means of delivering intended functions. The biomedical significance of these interactions prompted intense investigations aiming to determine the structural features optimally required for function. The antithrombin activation by the HS analog heparin leading to the development of the anticoagulant fondaparinux has long inspired the study of HS–protein associations [8]. Sequestered *in vivo* by mastocytes, heparin is generated similar to HS and carries the same disaccharide variations. It is, however, more homogeneous with N-sulfonated and 6-O-sulfonated GlcN (GlcNS6S) and 2-O-sulfonated IdoA (IdoA2S) occupying most of the chain [9]. The binding of antithrombin with a distinct 3-O-sulfonated pentasaccharide sequence in heparin triggers the exposure of the protease reactive center loop capable of deactivating factors IIa and Xa of the coagulation cascade [10].

The heparin and HS fragments isolated from natural sources are typically unsuitable for structure–activity relationship evaluations because of their polydispersity and structural ambiguity. Chemoenzymatic alterations of such fragments and that of heparosan, the N-acetyl-D-glucosamine (GlcNAc)–GlcA copolymer harvested from *Escherichia coli* strain K5 [11,12], to afford certain defined features only supplied partial information on the structural requirements for binding [13]. Here, the non-uniform starting materials, the incomplete enzymatic transformations, and the difficulties in reaction monitoring and product purification are persistent concerns. Despite the considerable effort and resources involved, chemical synthesis remains the most common and reliable source of well-defined heparin and HS oligosaccharides [14–16,17•]. The chemical approach grew in

Figure 1



Structure of a proteoglycan with attached heparan sulfate chain and some notable structures found in heparin.

sophistication by adopting advances in mainstream carbohydrate chemistry and discovering novel ways in dealing with the challenges associated with the complex structure of heparin and HS. On the other hand, chemoenzymatic approach progressed by dealing with the problem of selectivity during enzymatic modifications [18]. The recent methodologies in generating defined heparin and HS oligosaccharides and the information obtained from their biological evaluations are the subject of the present review.

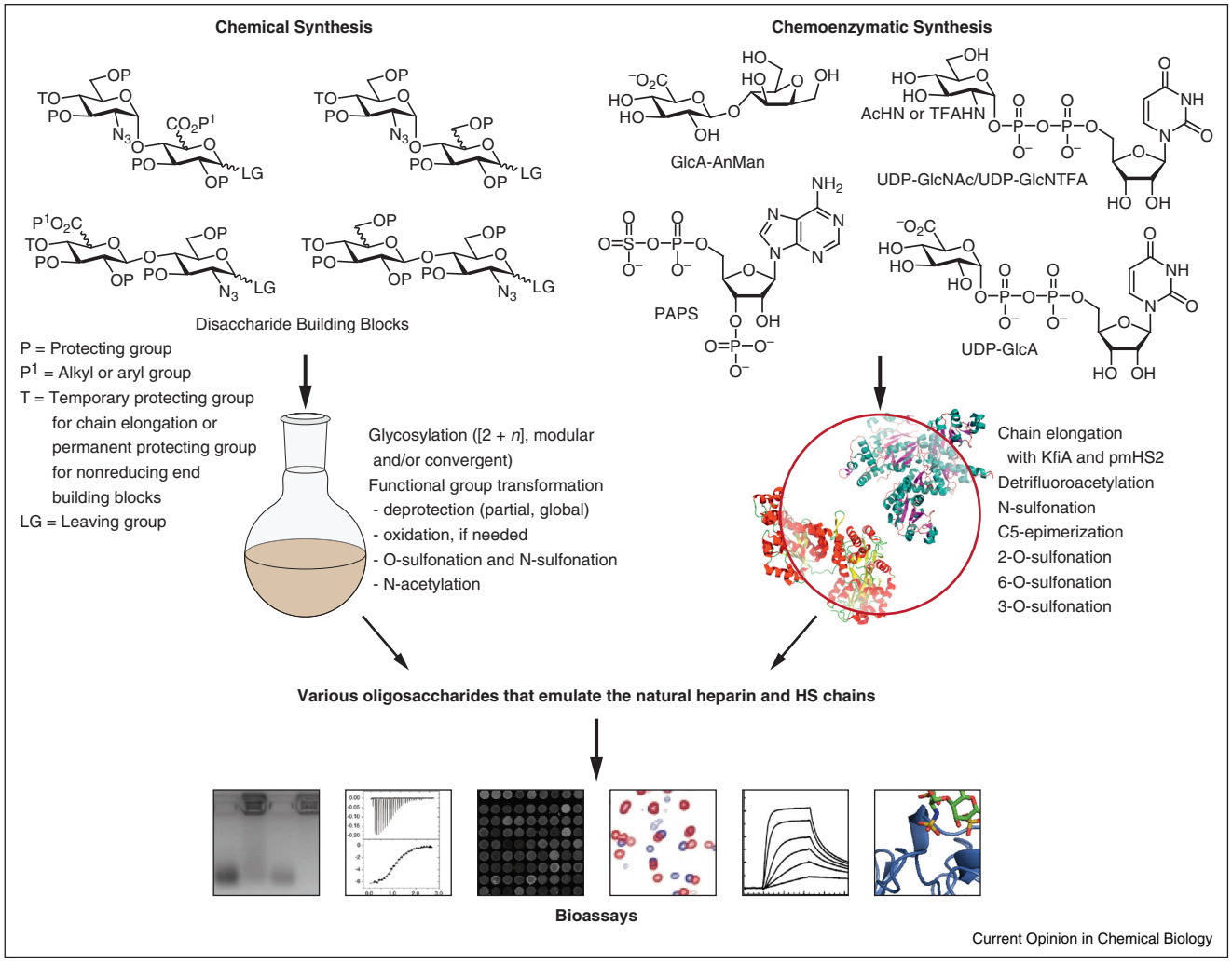
Chemical synthesis

As a synthetic target for many years, numerous strategies were disclosed addressing the challenges in the chemical synthesis of heparin and HS oligosaccharides [17•]. Orthogonal protecting groups at key positions played central roles in the transformations, ensuring the regioselectivity and stereoselectivity in glycosylation as well as the functional group pattern of the target constructs. Additionally, unnatural functional groups, such as linkers that are tailor-made for assay purposes, can be conveniently installed on the sugar chain. Novel and improved methodologies in recent reports contributed in increasing the variety and complexity of the synthesized structures.

The repeating disaccharide nature of heparin and HS motivated the generation of disaccharide building blocks to form longer skeletons (Figure 2). As enzymatic degradation of the natural compound delivers oligosaccharides with repeating UA–GlcN backbone, past syntheses often leaned toward disaccharide building blocks corresponding to this sequence. Notably, a higher number of recent efforts were developed using the GlcN–UA disaccharide precursor. The latter approach acknowledges the greater difficulty in α-glucosaminylation relative to 1,2-*trans* glycosylation involving the UA precursor. With azide masking the 2-amine function, α-glucosaminylation particularly relies on anomeric effect. α-Stereoselectivity is further enhanced by an acceptor with axially oriented hydroxyl nucleophile [19], the remote participation by acyl groups, and the steric influence of bulky groups. In particular, Hung showed that *tert*-butyldiphenylsilyl and *p*-bromobenzyl groups at the respective 6-O and 3-O positions of a glucosaminyl donor confer full α-stereoselectivity regardless of leaving group, activator, and acceptor [20•]. *tert*-Butyldimethylsilyl group at 4-O also provided similar effect on stereoselectivity [21•]. The UA precursor can be made with the carboxyl function already present before chain assembly. Conversely, oxidation may be carried out, typically using 2,2,6,6-tetramethyl-1-piperidinyloxyl free radical, until the relevant glycosidic bonds have formed to avoid reactivity and epimerization issues. The acquisition of the L-idose or IdoA derivative is another main concern. Fueled by the high price of the unprotected monosaccharide, several synthetic strategies were developed using cheaper starting materials [22]. There are recent updates concerning the formation of 1,6-anhydro-L-idose by Hung [23] and introduction of various protecting groups in the D-xylose-derived IdoA derivative by Seeberger [24]. Alternatively, Gardiner's group disclosed a new method via D-xylodialdose involving the stereoselective cyanohydrin formation at C5 in the L-*ido* configuration [25].

Glycosylations with the same disaccharide building block is a typical route in generating heparin and HS oligosaccharide. By this approach, different lengths can be readily prepared leading to compounds with regular repeating patterns. Elongations were achieved using

Figure 2



General overview of the chemical and chemoenzymatic synthesis of heparin and HS oligosaccharides and their biological evaluation.

iterative [2 + *n*]-coupling toward the nonreducing end [23,26,27•,28,29•,30] or by the initial generation and further convergent use of long chain donors [20•,31,32]. Thus far, chemical synthesis enabled the assembly of several dodecasaccharides [26,27•,29•,31,32] on top of many shorter homologues. The ensuing functional group transformations typically involve partial deblocking of key hydroxyls, oxidation, if needed, and sulfonation using an SO_3-based reagent followed by complete removal of other O-protecting groups usually through hydrogenolysis. The azide can be converted to the amine, acetamide, or sulfonamide at a synthetically convenient stage. Because multi-O-sulfonation after chain assembly is often tricky, Huang demonstrated the feasibility of preinstalled 2,2,2-trichloroethyl sulfate esters [33]. Fluorous tags were also incorporated at either the reducing [30] or nonreducing end [32] to allow purification via fluorous solid-phase extraction. Boons showed [30] that repeated reagent treatment to afford higher yields is possible in fluorous-supported heparin and HS synthesis.

Aside from length variations, heparin and HS diversity can be emulated by changing the manner of functional group transformations from assembled skeletons — the divergent approach — and by using differentially functionalized building blocks in a modular fashion. Understandably, representing the heparin and HS diversity by synthesis becomes less feasible as length increases because of concurrent exponential increase in the number of prospective structures. Complete synthesis was only accomplished, so far, on the disaccharide level by Hung and coworkers [34••]. Using two orthogonally protected disaccharides precursors, all 48 disaccharide possibilities in the heparin and HS chains were synthesized using divergent functional group transformation. Hung [35••] and Huang [33] also applied divergent transformations of

disaccharide precursors to prepare multiple disaccharide building blocks for further use in making oligosaccharides with irregular sequences. Proper selection of protecting groups, leaving groups, and glycosylation conditions led to the successful synthesis of exotic HS structures such as the 3-O-sulfonated octasaccharide generated by Hung [35••] notable for carrying amine, acetamide, and sulfonamide groups. In several instances, common fully protected oligosaccharides (tetrasaccharide and longer) were variably transformed to create multiple final products that mainly differ in overall amine substitution [21•,26,33,36] or O-sulfonation patterns [29•,32,37]. These strategies enhance the utility of the assembled skeletons. Modular strategies coupled with divergent transformations were recently applied to make small sugar libraries [21•,29•,36,37]. In Huang's case [21•], several hexasaccharide skeletons were assembled under preactivation-based one-pot sequential glycosylation from the nonreducing to the reducing end.

The full-fledged synthesis of proteoglycan is still beyond reach. Conjugation between a heparin-like dodecasaccharide through a non-carbohydrate linker and the lysine residue of a CD4 mimetic peptide was, nonetheless, achieved [31]. More importantly, a glycopeptide derived from syndecan-1, a widespread HS proteoglycan, containing a serine-attached tetrasaccharide linkage region and includes an HS-based tetrasaccharide has been successfully synthesized [38]. The synthesis encountered glycosylation and functional group transformation issues, but the know-how gained here could lay the groundwork for the preparation of more complex targets.

Chemoenzymatic synthesis

With some exemptions, the enzymes that give heparin and HS their remarkable structure can be expressed in *E. coli* in adequate quantities [13,39••]. Together with heparosan's availability as starting material [12], the chemoenzymatic approach attracted interests as a method for generating heparin-like and HS-like compounds with certain desired characteristics. The synthetic cost was brought down significantly by regeneration [40] or enzymatic synthesis [41] of the sulfotransferase coenzyme 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which can also be radiolabeled with ^{35}S to aid detection during purification and analysis. Chemical synthesis also offered access to starting materials of defined lengths to address the polydispersity of heparosan chains [20•,42]. While particular modifications can be introduced with high regioselectivity, however, controlling the extent and location of the modification amid numerous residues in the substrate to afford a well-defined structure is difficult. Despite many concerns, chemoenzymatic treatment of heparosan sufficiently provided long oligosaccharides for bioassays that are impractical by current chemical means. It even effectively delivered a $^{13}C/^{15}N$-labeled HS-based octasaccharide from a purposely labeled heparosan polymer [43].

A recent shift in chemoenzymatic synthesis allowed the preparation of defined heparin-based and HS-based compounds. Alternating application of two bacterial enzymes, KfiA from *E. coli* strain K5 and pmHS2 from *Pasteurella multocida*, permitted controlled polymer elongation using uridine diphosphate (UDP) derivatives of GlcNAc and GlcA, respectively (Figure 2) [39••]. A convenient starting unit in this case is GlcA–AnMan (AnMan: 2,5-anhydromannitol) obtained by chemical degradation of heparosan [44]. GlcA–AnMan is also amenable to functionalization, such as adding a fluorous tag [45,46]. KfiA cannot add GlcN and selective N-deacetylation is not feasible by enzymatic or chemical means. Fortunately, N-trifluoroacetyl glucosamine (GlcNTFA) can also be added by KfiA, providing selective access to the free amine and its sulfonation with N-sulfotransferase (NST) and PAPS [45]. NST is the truncated version of the natural bifunctional enzyme N-deacetylase/N-sulfotransferase. Conversely, pmHS2 adds GlcA to substrates with nonreducing GlcNAc, GlcNTFA, and the N-sulfonated GlcN (GlcNS) residues, but not GlcN [42]. C5-epimerase (C5-epi) acts on GlcA residues flanked by GlcNS at its nonreducing side and GlcNS or GlcNAc at its reducing side [45]. The GlcA residue transformation into IdoA is known to be reversible, but a GlcNAc three residues away at the nonreducing side was found to influence the irreversible IdoA generation [47]. Concurrent treatment with C5-epi and 2-O-sulfotransferase causes the selective formation of IdoA2S. While the 6-O-sulfotransferase isoforms 1 and 3 appear to indiscriminately act on different GlcN residues [46], the 3-O-sulfotransferase (3-OST) isoform-1 only sulfonate GlcNS6S with an unsulfonated UA at its nonreducing side [39••]. Other 3-OST isoforms provide differing substrate specificities [39••,48,49]. By proper order and selection of enzymatic treatment, Liu generated various well-defined compounds, including a heptasaccharide carrying the antithrombin-binding sequence at a good yield and scale [50••]. His laboratory also achieved a 21-mer oligosaccharide that has anti-IIa and anti-Xa activities [51•]. Because contiguous GlcNS residues are present in that construct, however, reversible epimerization occurred on C5-epi treatment resulting to a mixture of compounds.

Protein interactions with synthetic heparin and HS oligosaccharides

Crucial structural features and parameters involved in protein interactions are revealed by various techniques, including surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), microarray analysis, affinity electrophoresis, fluorescence resonance energy transfer, various competition assays, NMR perturbation, X-ray crystallography, and many others (Figure 2). The kinetic and thermodynamic aspects of the interaction and the

participating residues and functional group on both binding entities are beneficial in generating a good picture of the encounter [7].

Growth factors are among the most important group of proteins that bind HS. With 48 disaccharides, Hung identified GlcNS–IdoA2S by ITC as the minimum requirement for fibroblast growth factor-1 (FGF-1) interaction that occurs at a shallow binding site in the protein revealed by X-ray analysis [34••]. Aside from the N-sulfonate and 2-O-sulfonate functionalities, the 3-O-sulfonate, if present, and the 3-hydroxyl of IdoA contribute to the encounter. FGF-2 binds the disaccharide GlcNS6S–IdoA2S, *albeit* weak [28]. Using longer sugars, Huang and Liu determined the importance of IdoA2S and GlcNS in binding to FGF-2 and an additional 6-O-sulfonate group gives a twofold affinity enhancement [42]. Jayson and coworkers showed that the interaction of a GlcNS–IdoA2S repeating sequence with vascular endothelial growth factor$_{165}$ is considerably weaker than that with FGF-2 and a long sugar, such as a dodecasaccharide, is needed for effective protein inhibition [26].

HS often assists bacterial and viral entry into host cells. SPR competitive assays indicated that the HS dodecasaccharide–CD4 mimic conjugate inhibits the binding of immobilized CD4 with gp120, a human immunodeficiency virus (HIV) envelope glycoprotein needed for cell entry [31]. Binding of the CD4 mimic exposed the adjacent coreceptor domain of gp120, which has affinity to the HS dodecasaccharide. This cooperative behavior of the conjugated components enabled the inhibition of HIV entry onto peripheral blood mononuclear cells. The dodecasaccharide carries the IdoA2S–GlcNS6S repeating unit and is at an appropriate length to cover the coreceptor domain of gp120. Conversely, two different 3-O-sulfonated octasaccharides inhibited the herpes simplex virus-1 (HSV-1) infection of Vero cells in a dosage dependent manner [35••]. A 3-O-sulfonated HS is known to interact with HSV-1 envelope glycoprotein gD enabling viral entry. The similar inhibition profiles of the two sugars suggest minor contribution of other structural features and the location of the 3-O-sulfonate group on the extent of gD binding. Heparin-binding hemagglutinin (HBHA) is the virulence factor crucial for extrapulmonary dissemination of *Mycobacterium tuberculosis*. ITC experiments identified a hexasaccharide with GlcNS6S–IdoA2S repeating sequence as the shortest sugar that interacts with HBHA in an entropically driven manner [20•].

Assays with other proteins also revealed interesting results. Competitive inhibition indicated that a heptasaccharide with GlcNS6S–IdoA2S repeats can inhibit the binding of eosinophil-derived neurotoxin (EDN) to Beas-2B cells [23]. Fluorescence-assisted carbohydrate electrophoresis showed that the shorter pentasaccharide of the same sequence possess the ability to bind eosinophil cationic protein (ECP), a close relative of EDN [52]. Using NMR perturbation, the dissociation constant of the trisaccharide GlcNS6S–IdoA2S–GlcNS6S and ECP was measured at around 15–30 μM with the nonreducing end positioned at the protein interior [53]. Concerning the Alzheimer's disease-related protease BACE-1, interaction with both sequences containing IdoA/GlcNS and GlcA/GlcNAc suggests the probability of dual binding regions [36]. Octasaccharides and longer sugars with GlcNAc6S–UA2S units showed potent binding with BACE-1 [29•]. SPR and microarray experiments with the natural cytotoxicity receptors NKp30, NKp44, and NKp46 using a compound library denote binding to the highly charged regions of the sugar, but with differing individual specificities and length dependencies [54].

Conclusion

In a molecule as complex as HS, only a small sampling of a huge structural potential have been evaluated. This reflects on the many difficulties associated with the acquisition of well-defined oligosaccharides by chemical and chemoenzymatic means. Nevertheless, current advances keep pushing the boundaries of structural complexity and the effectiveness and efficiency of the synthetic process. Recent evaluations identify the important structural features in the sugar that could form the foundations of future studies. With the antithrombin-binding sequence that started a rethinking of HS–protein interactions, the motivations for identifying new candidate drugs derived from heparin and HS are stronger than ever.

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