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Recent advances in the synthesis of extensive libraries of heparan sulfate oligosaccharides for structure–activity relationship studies

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Abstract

Heparan sulfate (HS) is a linear, sulfated and highly negatively-charged polysaccharide that plays important roles in many biological events. As a member of the glycosaminoglycan (GAG) family, HS is commonly found on mammalian cell surfaces and within the extracellular matrix. The structural complexities of natural HS polysaccharides have hampered the comprehension of their biological functions and structure–activity relationships (SARs). Although the sulfation patterns and backbone structures of HS can be major determinants of their biological activities, obtaining significant amounts of pure HS from natural sources for comprehensive SAR studies is challenging. Chemical and enzyme-based synthesis can aid in the production of structurally well-defined HS oligosaccharides. In this review, we discuss recent innovations enabling the syntheses of large libraries of HS and how these libraries can provide insights into the structural preferences of various HS binding proteins.

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Introduction

Heparan sulfate (HS) is an important class of polysaccharides that plays roles in a wide range of biological events, including blood coagulation, cell differentiation, inflammatory responses, tumor metastasis and viral infections [1,2]. A thorough understanding of the biological activities of HS can lead to the development of novel therapeutics. HS consists of repeating disaccharide units of glucosamine (GlcN)-α-1→4 linked to a uronic acid, which can be either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) [2]. In addition, the 2-*O* on uronic acid, 6-*O* and 3-*O* on GlcN can be sulfated. However, these sulfate modifications are often incomplete, thus resulting in high heterogeneity of naturally-existing HS polymers [3,4]. This structural complexity and heterogeneity have hampered efforts to understand the detailed structure–activity relationships (SAR) of HS. The interactions of HS with its biological receptors can be highly structurally specific [5,6*]. Hence, it is critical to obtain pure HS containing a variety of sulfation sequences and backbone structures to advance the understanding of HS activities. As it is challenging to isolate sufficient quantities of defined HS from natural sources due to its heterogeneity, synthesis is the preferred method to access HS sequences.

Significant breakthroughs in the synthesis of HS oligosaccharides have been accomplished over the last two decades [7–10*]. HS sequences having the length approaching those of the HS polysaccharides can now be prepared [11–13]. On the other hand, to accelerate SAR studies, the availability of libraries of HS structures becomes critical. This is highlighted by recent studies where through screening of libraries of diverse HS sequences, key structural features of HS such as 3-*O* sulfation and the length of non-sulfated *N*-acetylation domain have been identified for interactions with a variety of HS binding proteins including tau, high mobility group 1 protein, the receptor binding domain of SARS-CoV-2 spike protein, and chemokines [14–18].

Construction of HS libraries is challenging owing to the numerous sulfation alternatives (*N*-, 3-*O*, and 6-*O* of GlcN and 2-*O* position of GlcA/IdoA) and variability in backbone architecture. Given that the synthesis of a single HS oligosaccharide can take more than 40 steps, innovative methods are needed to enable the preparation of large HS libraries. There have been many excellent reviews on the synthesis of HS oligosaccharides [7–10*]. Rather than providing a comprehensive summary on HS synthesis, we focus our discussion on synthetic strategies toward HS libraries with emphasis on the advances achieved during the last two years, including the synthesis of comprehensive libraries of HS tetrasaccharides.

Early efforts in HS library synthesis

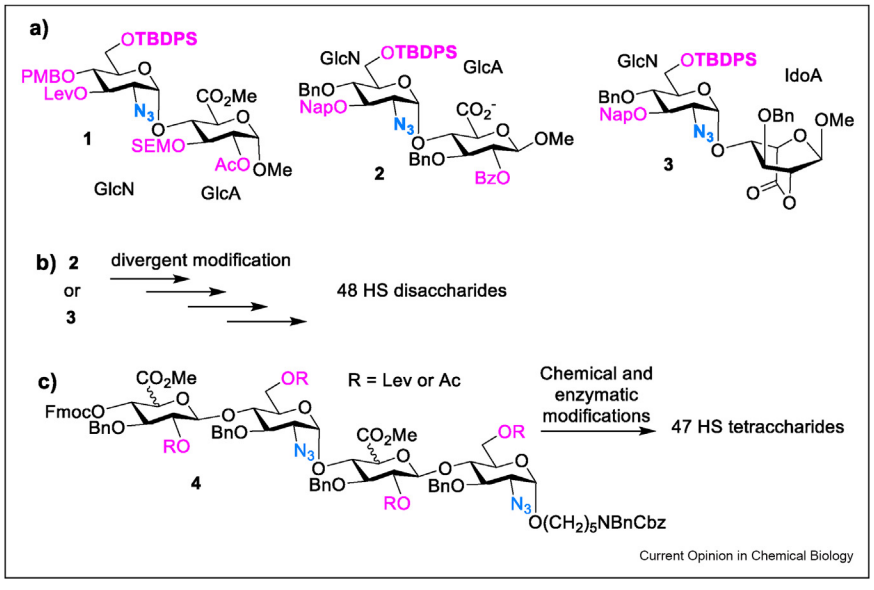
With the diverse HS structures to be prepared in a library synthesis, a common strategy employed is to design building blocks with strategically placed protective groups, which can be selectively removed for differential sulfation. An early example is the GlcN-GlcA disaccharide building block **1**, where the hydroxyl groups were protected with *tert*-butyldiphenylsilyl (TBDPS), *p*-methoxybenzyl (PMB), levulinoyl (Lev), 2-trimethylsilylethoxymethyl (SEM), and acetyl (Ac) (Scheme 1a) [19*]. These protective groups could be removed individually without affecting others and the newly liberated hydroxyl group was sulfated to generate six monosulfated and partially deprotected HS disaccharides. Shortly after, the synthesis of a comprehensive HS disaccharide library was accomplished [20]. From eight monosaccharides, 16 differentially protected disaccharides were generated, which were selectively deprotected and sulfated to produce 48 HS disaccharides, thereby covering all possible HS disaccharide sequences [20]. In the second generation approach, a divergent method was designed to obtain the comprehensive library of 48 HS-based disaccharides from just two common orthogonally protected disaccharide building blocks **2** and **3** (Scheme 1b) [21*].

Going beyond HS disaccharides, the syntheses of 12 HS oligosaccharides ranging from di-to hexa-saccharides with varying sulfation patterns through disaccharide modules were reported [22]. In another study, a divergent strategy was established combining chemical and enzymatic methods, which led to the successful preparation of over 20 hexasaccharides [23,24]. Furthermore, another HS library was obtained by starting with several selectively protected tetrasaccharides **4** [25*]. Conditions were identified to modify the structures through regioselective *O*- and *N*-sulfation, as well as selective desulfation of sulfates installed. This was followed by enzymatic modification with 3-*O*-sulfotransferase-1 to provide 3-*O*-sulfated HS derivatives (Scheme 1c). Through these diversification methods on the tetrasaccharides, a library of 47 HS oligosaccharides was prepared.

Recent efforts toward the synthesis of comprehensive HS tetrasaccharide libraries

For more comprehensive SAR studies, a broader chemical space of HS should be covered. For HS

Scheme 1



a) Structures of HS disaccharide intermediates **1**–**3**; b) Divergent chemical modification of disaccharides **2** and **3** could produce the comprehensive library of 48 HS disaccharides; c) Chemical and enzymatic modification of tetrasaccharide intermediates **4** led to a library of 47 HS tetrasaccharides.

tetrasaccharides, considering all variations of *N*-, 6-*O* sulfations of the GlcN and 2-*O* sulfation of the uronic acid with the possibilities of having both GlcA and IdoA in the backbone, there are 256 possible structures. To produce a library of such a size, multiple hurdles need to be overcome.

The first significant obstacle is the access to building blocks. Traditional approaches for building block preparation start from commercially-available monosaccharides. These approaches typically require 6–15 synthetic steps to generate monosaccharide building blocks with protective group patterns suitable for stereoselective glycosylation reactions and post-glycosylation synthetic manipulations and sulfation. One innovative strategy to expedite building block access is to utilize naturally-existing polysaccharides [26**]. Inspired by a method developed for chondroitin sulfate synthesis [27], heparin polysaccharide was hydrolyzed using aqueous triflic acid followed by esterification, NH_2 to N_3 conversion, and acetylation to give disaccharide **5** in 20% overall yield (Scheme 2a). Subsequently, **5** was converted to disaccharide **6** in six steps, from which disaccharide donor **7** and acceptor **8** bearing a fluorous tag were prepared to produce tetrasaccharide **9** (Schemes 2a and 2b) [28**]. The preparation of the disaccharides **7** and **8** starting from the heparin polysaccharide not only removed the need to perform one challenging glycosylation, i.e., the 1,2-*cis* glycosylation between GlcN and IdoA, but also reduced the number of synthetic steps needed by approximately 50% as compared to the traditional strategy of starting from the monosaccharide building blocks [26**,28**].

A critical consideration in preparing a comprehensive tetrasaccharide library is reducing the total number of synthetic steps required to access the large number of compounds. One strategy is to design key tetrasaccharides bearing strategically-positioned, orthogonally-deprotectable groups at potential sulfation sites. As a step toward the comprehensive HS tetrasaccharide library, tetrasaccharide **10** (Scheme 2c) was synthesized with a GlcN-GlcA-GlcN-IdoA backbone [29**]. Tetrasaccharide **10** bears TBDPS, fluorenylmethoxycarbonyl (Fmoc), 2-naphthylmethyl (Nap), and Lev groups at the four potential *O*-sulfation sites, with the two nitrogen moieties of the GlcNs differentiated as azide and trifluoroacetamide respectively. Suitable reaction conditions were established to orthogonally remove any of these four *O*-protective groups in good yields without affecting any other (Scheme 2c). The resulting partially deprotected tetrasaccharides were subsequently sulfated and further modified. Through this divergent approach, a library of 64 HS tetrasaccharides (**15**–**78**) bearing systematically varied *N*-sulfation, 2-*O*, and 6-*O* sulfations on the GlcN-GlcA-GlcN-IdoA backbone (Figure 1) was successfully produced at 2–5 mg scale for each member of the library [29**].

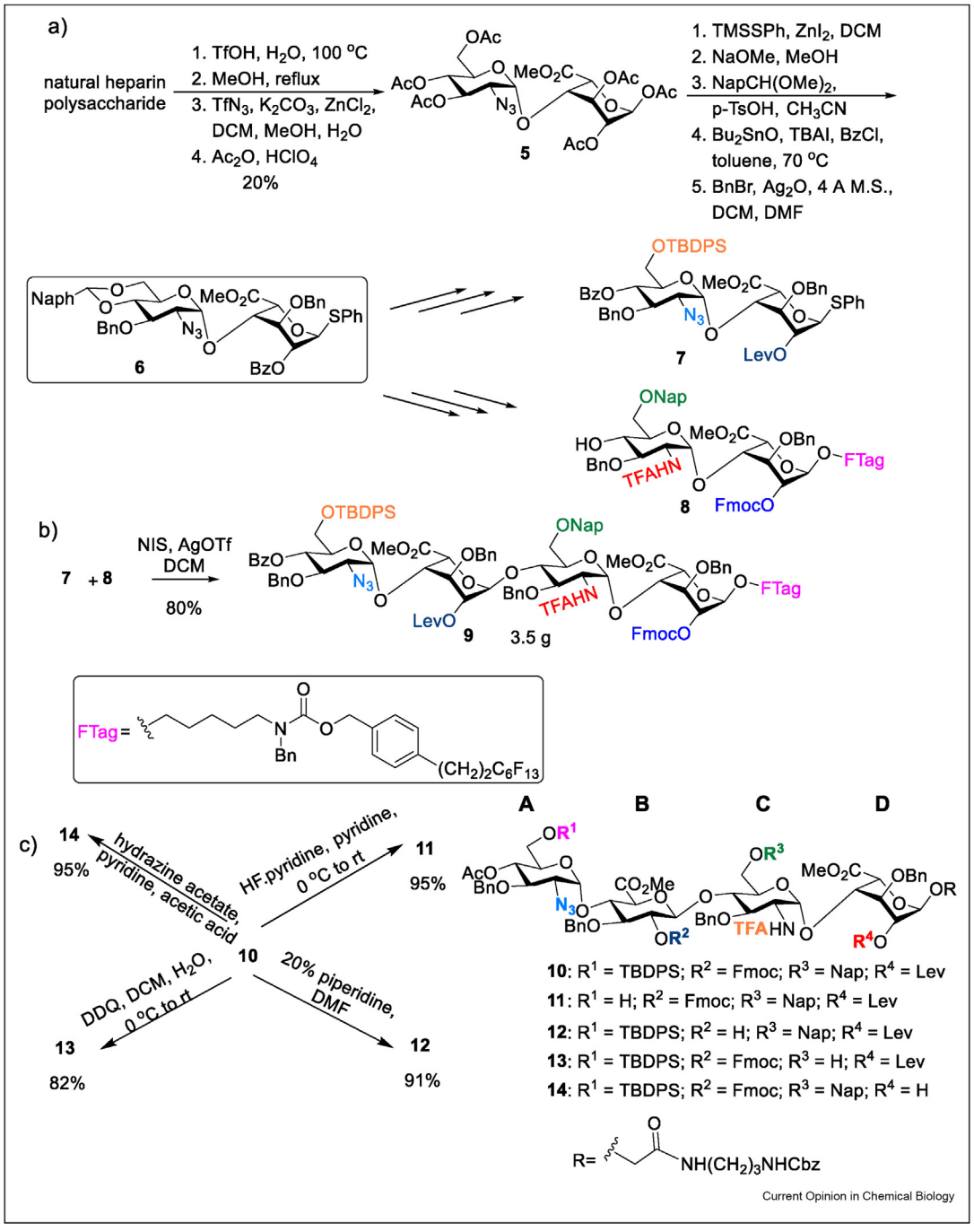
A second HS tetrasaccharide library with 64 members bearing the GlcN-IdoA-GlcN-IdoA backbone was prepared from the key tetrasaccharide intermediate **9** [28**]. Tetrasaccharide **9** was synthesized efficiently from the disaccharide building blocks derived from heparin polysaccharide in 3.5 g scale (Schemes 2a and b). In a similar manner as the synthesis of tetrasaccharides **15**–**78**, **9** was orthogonally deprotected and sulfated [28**]. From this single universal building block **9**, 64 HS tetrasaccharides **79**–**142** (Figure 1) encompassing all combinations of 2-*O*-, 6-*O*-, and *N*-sulfations on the tetrasaccharide GlcN-IdoA-GlcN-IdoA backbone were generated at 5–15 mg scales in 86–95% purities.

Expediting HS library synthesis through solid-phase and fluorous tag-supported strategies

Solid-phase-supported glycan synthesis can significantly streamline the overall process, which is particularly attractive for library preparation. During the previous two decades, substantial breakthroughs in solid-phase-based glycosylation have been achieved [30–36]. While several sulfated carbohydrates have been prepared on solid phase [36], solid-phase-supported glycosylation toward HS synthesis is underdeveloped. In 2019, with the automated glycan assembly (AGA) platform Glyconeer, a hexasaccharide GlcN-glucose (Glc)-GlcN-Glc-GlcN-Glc backbone **145** was synthesized on solid phase (Scheme 3a) [37]. Disaccharide glycosyl trichloroacetimidate donors **143** and **144** with the pre-formed 1,2-*cis* linkage between GlcN-Glc was utilized for solid-phase glycosylation. To enhance the overall glycosylation yields, 5–10 eq of the disaccharide donor was used and each glycosylation reaction was performed twice. The excess donor hydrolyzed during the synthesis was collected on the synthesizer, purified, and converted back to the trichloroacetimidate donor to recover some of the high-value building blocks.

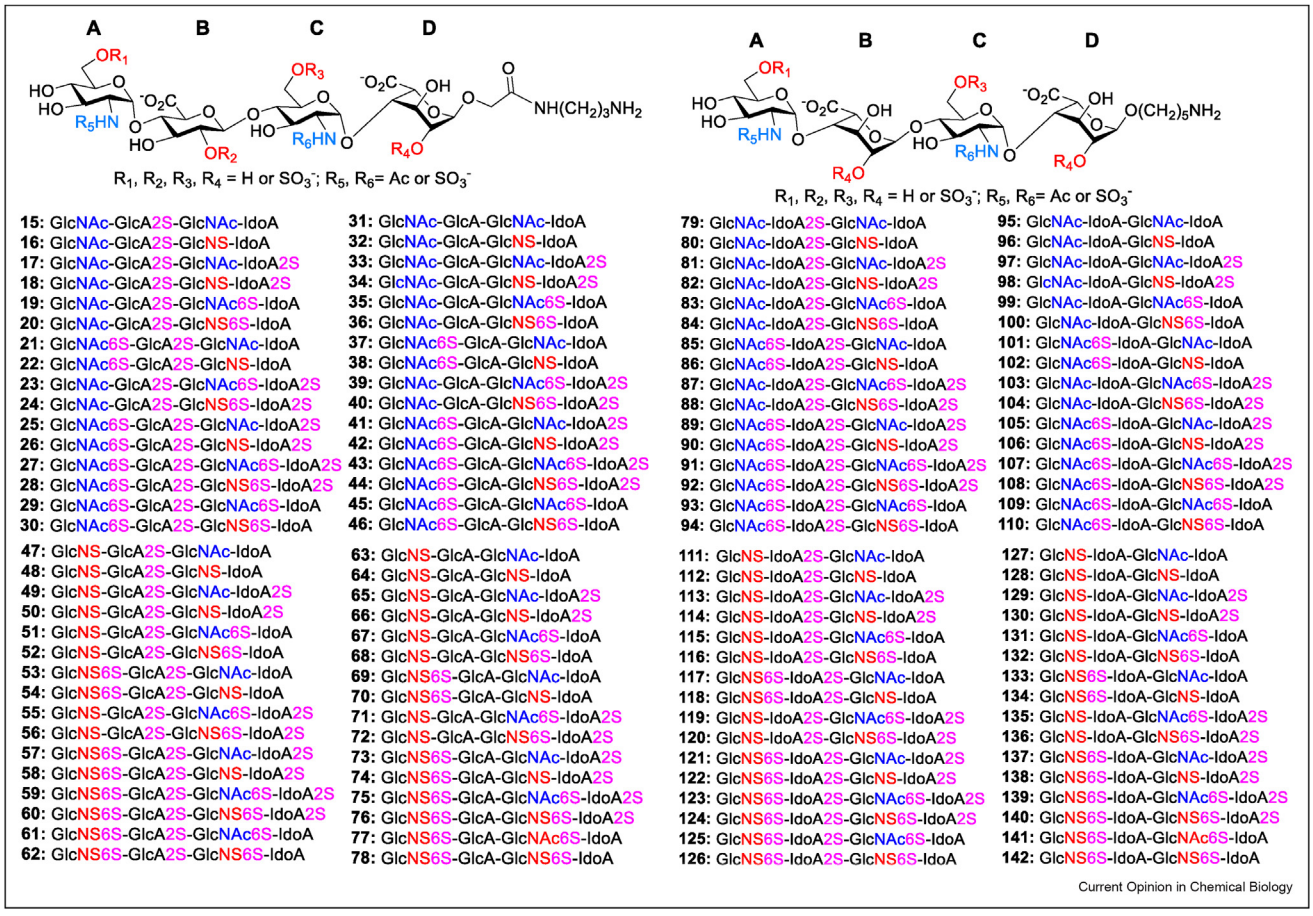
In order to overcome some of the limitations of solid-phase-supported glycosylation—including the need for large excess of the donor to enhance the reaction yield and the difficulty in monitoring the reaction progress—a significant innovation of the synthesis of the library of **79**–**142** was the introduction of a fluorous tag (FTag) to the reducing end of tetrasaccharide **9** (Scheme 2b) [28**]. The lightly fluorous tag C_6F_{13} in **9** did not significantly impact the reactivity or solubility of the building blocks in common organic solvents. As a result, the solution-phase-based glycosylation reactions could be performed with the FTag building blocks using near stoichiometric amounts of the building block, which is a significant advantage [28**]. Furthermore, the desired FTag bearing HS glycans could be readily separated from the non-fluorous impurities through fluorous solid-phase extraction (FSPE). This greatly reduced the time

Scheme 2



a) Disaccharide donor **7** and acceptor **8** could be derived from heparin polysaccharides; b) Synthesis of FTag-bearing protected HS tetrasaccharide **9**; c) The key HS tetrasaccharide intermediate **10** could be orthogonally deprotected to expose specific hydroxyl groups to be sulfated for divergent modifications.

Figure 1



Structures of the HS tetrasaccharides **15**–**142** encompassing all combinations of 2-*O*-, 6-*O*-, and *N*-sulfations on the GlcN-GlcA-GlcN-IdoA and GlcN-IdoA-GlcN-IdoA tetrasaccharide backbones [28**,29**].

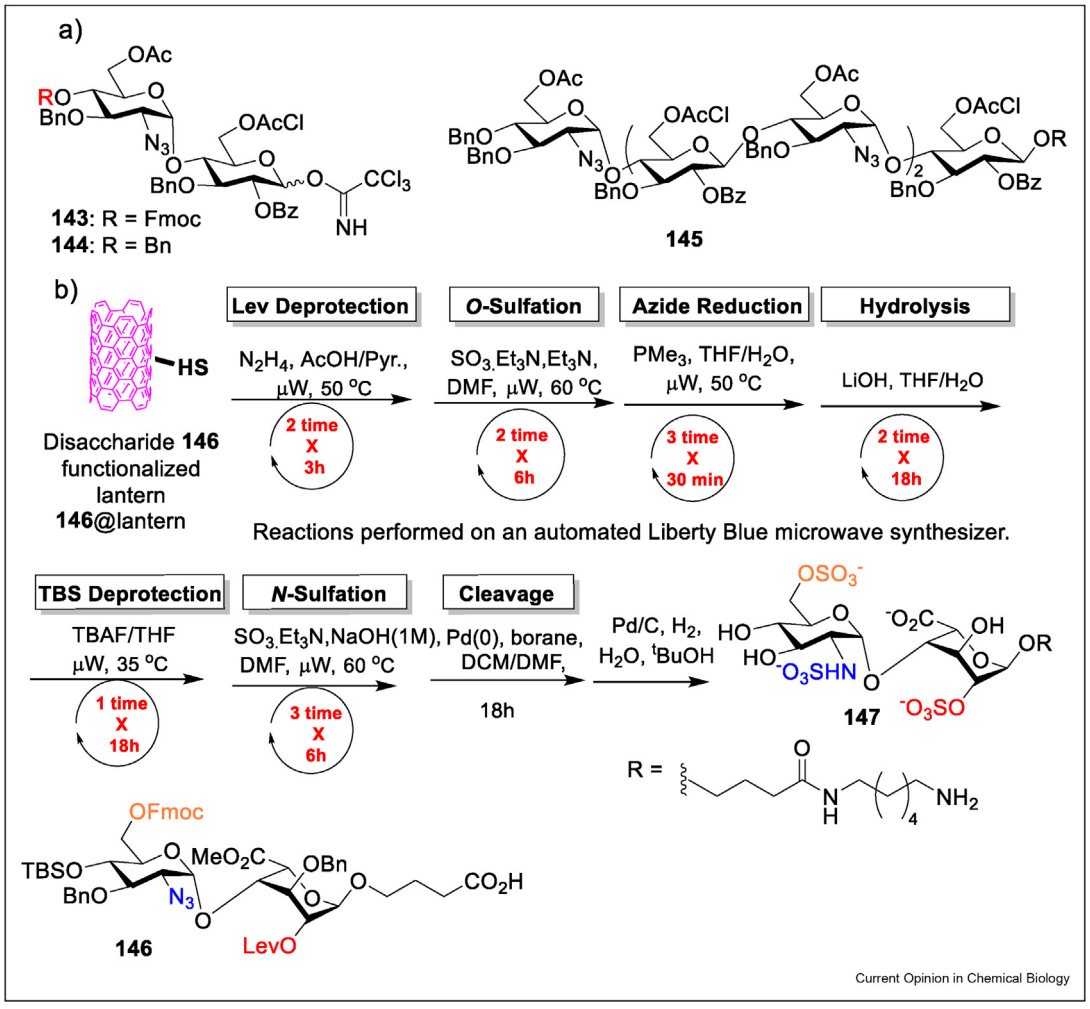
needed to purify the highly charged intermediates in HS synthesis, thus expediting the overall synthetic process.

Another approach to expedite the library preparation is to immobilize the key synthetic intermediate on solid-phase support such as Synphase Lanterns for post-glycosylation transformations [38**]. Performing post-glycosylation deprotection and sulfations on solid phase avoids the need to purify the highly polar products. A CEM Liberty Blue Microwave Automated Synthesizer was used to automate the entire process of *O*- and *N*-sulfations, deprotection and cleavage on the Lantern. For example, the HS disaccharide **146** functionalized lantern (**146@lantern**) was subjected to Fmoc cleavage, Lev deprotection, *O*-sulfation, azide reduction, hydrolysis, *tert*-butyldimethylsilyl (TBS) deprotection, and *N*-sulfation (Scheme 3b). The synthesizer was programmed to carry out the 7 synthetic steps from **38** in an automated fashion in 96 h, leading to **147** in 60% overall yield, which represents an average of 92% yield for each synthetic transformation (Scheme 3b). A library of 16 HS disaccharides with diverse sulfation patterns was prepared via this method [38**]. Compared to the traditional solution phase-based synthesis, this new strategy significantly improved the overall synthetic efficiency, as it led to a reduction of over 80% of the number of column purification steps needed from the disaccharide intermediates.

The comprehensive HS library could yield critical insights into SAR of HS oligosaccharides

The availability of two extensive libraries of HS oligosaccharides enabled systematic investigations into the structural determinants important for molecular recognition by HS-binding proteins [28**,29**]. The tetrasaccharides were attached onto glycan microarrays, and their binding interactions were analyzed with FGF2 and fibroblast growth factor 4 (FGF4), which play important roles in a wide range of developmental processes and human diseases such as asthma, cancer, and cardiovascular disease [39–41]. The results

Scheme 3



a) Disaccharide building blocks **143** and **144** were used to synthesize hexasaccharide backbone **145** on solid phase. b) HS backbone disaccharide **146** was immobilized on Lanterns, and microwave-assisted automated synthesis was performed to expedite the post-glycosylation modifications, including sulfation, to form HS disaccharide **147**. The reactions indicated in black rectangles were performed by the automated synthesizer.

revealed that 2-*O*- sulfation at both IdoA residues is important for FGF2 recognition: loss of a single 2-*O*-sulfate group resulted in a significant reduction in FGF2 binding {**124** (100% of the relative signal intensity) vs **140** (45%) and **126** (39%)}, whereas removal of both 2-*O*-sulfate groups eliminated binding {**142** (3%)}. Although the *N*-sulfate groups were both essential {**124** (100%) vs **91** (2%)}, *N*-sulfation was more important at the GlcN residue at the non-reducing end than that closer to the reducing end {**123** (70%) vs **92** (28%)} [28**]. In contrast, the absence of any or both 6-*O*-sulfate groups resulted in a relatively minor decrease in binding {**124** (100%) vs **120** (77%), **122** (63%) and **114** (68%)}, demonstrating that 6-*O*-sulfation plays a minor role for FGF2 recognition. In addition, a new data analysis method utilizing the sulfation logos has been established [28**], which is prompted by the need to analyze and visualize the extensive data available from the large libraries.

The binding results with FGF4 emphasize the distinct and opposing contributions of each *N*-sulfate group in the tetrasaccharide sequence, as well as critical distinctions in the HS binding specificities of FGF4 and FGF2 [28**]. The FGF4 sulfation preferences differ markedly from those of FGF2, as certain tetrasaccharides were preferentially recognized by FGF4 but not FGF2 [e.g., **139** (67% vs. 3%) and **142** (52% vs. 3%)]. These results highlight the exciting possibility of selective regulation of FGFs *in vivo* by fine tuning the structures of HS.

Administration of the anti-coagulant drug heparin (a highly sulfated form of HS) *in vivo* can trigger heparin-

induced thrombocytopenia (HIT), a life-threatening side effect [42,43]. HIT is caused by immune responses against the complex formed by heparin with Platelet Factor-4 (PF4). To better understand the SAR of HS binding with PF4, PF4 was screened on the microarray bearing HS tetrasaccharides [29**]. The results suggested that the number as well as the location of sulfates can significantly influence PF4 binding. For example, while **55** is one of the strongest binders of PF4 identified in the library, compound **50** interacts little (6.6%) with PF4, despite the fact that both compounds contain four sulfates. Comparison of the binding of PF4 vs those of FGF2 demonstrated that several compounds (**18**, **24**, **40**, **42**, **44**, **50**, **72**, **74** and **76**) are strong binders with FGF2 with low binding to PF4, suggesting they can be promising leads for FGF2-based therapeutics with reduced risks of HIT [29**]. These results highlight the power of a comprehensive library, which can decipher the fine characteristics of the SAR of HS and provide exciting leads to target important biomedical events.

Conclusion and future prospectives

With the appreciation of the multifaceted biological functions of HS, it is important to better understand the SAR of HS with their biological targets. Building on the synthesis of comprehensive HS disaccharide libraries, two 64-membered libraries of HS tetrasaccharides have been successfully prepared, which represents the largest, most comprehensive HS library to date. The key innovation in these syntheses includes the design of a single tetrasaccharide building block that can be divergently modified to produce 64 different HS tetrasaccharides. Furthermore, the installation of a fluorous tag on the building blocks significantly aids in the purification of the highly polar, charged intermediates. Derivation of the building blocks from naturally existing heparin polysaccharide and automation, for example using a solid-phase support such as Synphase Lanterns, can further expedite the syntheses.

The availability of comprehensive libraries of HS can provide tremendous opportunities to establish detailed SARs of HS. The HS tetrasaccharide libraries have already helped decipher the differential structural preferences of two members of the FGF protein family. In addition, lead compounds with high FGF2 but low PF4 binding have been identified from the library, a result that suggests their potential as therapeutics with reduced possibilities for HIT. Nonetheless, the current library still represents a fraction of the enormous chemical space of HS. Future efforts will expand on the facile preparation of the building blocks on large scales and the design of automated synthetic routes to increase the number of compounds in the library. Innovative strategies need to be further developed to enable the generation of even larger HS libraries to probe their fascinating biological functions.

CRediT statement

Sherif Ramadan: Conceptualization (supporting); Writing – original draft (leading); Writing – review and editing (supporting); **Morgan Mayieka**: Conceptualization (supporting); Writing – original draft (supporting); Writing – review and editing (supporting); **Nicola L. B. Pohl**: Conceptualization (supporting); Writing – review and editing (supporting); **Jian Liu**: Conceptualization (supporting); Writing – review and editing (supporting); **Linda C. Hsieh-Wilson**: Conceptualization (supporting); Writing – review and editing (supporting); **Xuefei Huang**: Conceptualization (leading); Writing – original draft (supporting); Writing – review and editing (leading).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xuefei Huang reports financial support was provided by National Institutes of Health. Jian Liu reports financial support was provided by National Institutes of Health. Nicola Pohl reports financial support was provided by National Institutes of Health. Linda Hsieh-wilson reports financial support was provided by National Institutes of Health. Jian Liu reports a relationship with Glycan Therapeutics, LLC that includes: equity or stocks. Xuefei Huang reports a relationship with Glycan Therapeutics, LLC that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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