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Synthetic probes of glycosaminoglycan function

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Glycosaminoglycans (GAGs) participate in many critical biological processes by modulating the activities of a wide range of proteins, including growth factors, chemokines, and viral receptors. Recent studies using synthetic oligosaccharides and glycomimetic polymers have established the importance of specific structural determinants in controlling GAG function. These findings illustrate the power of synthetic molecules to elucidate glycan-mediated signaling events, as well as the prospect of further advancements to understand the roles of GAGs *in vivo* and explore their therapeutic potential.

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Introduction

Glycosaminoglycans (GAGs) are a class of highly charged, extracellular polysaccharides that display rich structural diversity [1,2]. The two most prevalent members of the family, heparin/heparan sulfate (HS) and chondroitin sulfate (CS), are composed of repeating disaccharide units of uronic acid (L-iduronic acid or D-glucuronic acid) and hexosamine (D-glucosamine or D-galactosamine) sugars (Figure 1a). Each sugar is differentially sulfated at the hydroxyl and/or amino positions by various sulfotransferase enzymes, giving rise to hundreds of potential sulfation sequences. This structural diversity leads to a wide range of protein-binding motifs and enables modulation of cellular signaling pathways. Indeed, GAGs participate in many important signaling events such as neuronal growth [2–5], tumor progression [6], inflammation [7], and development [8]. Their involvement in both normal and disease-related processes has sparked intense interest in understanding the mechanisms and structural determinants that control GAG activity. However, the chemical complexity and diversity of these polysaccharides present a formidable challenge to elucidating their structure–function relationships.

A major barrier to understanding GAGs has been the difficulty of accessing defined structures. Well-characterized, homogeneous GAG fragments are challenging to purify from natural sources due to their strong anionic character and assorted sulfation patterns. Purified structures are usually limited to the most abundant sulfation sequences and may lack the rare, highly sulfated epitopes that are often physiologically important. Chemically modified heparin/HS polysaccharides and CS motif-enriched polysaccharides typically contain a small percentage of other sulfated epitopes that can also exert biological activity. As such, caution must be used when interpreting results obtained with natural GAGs. From a purity and selectivity standpoint, these limitations make the use of defined synthetic structures crucial for studying the structure–function relationships of GAGs.

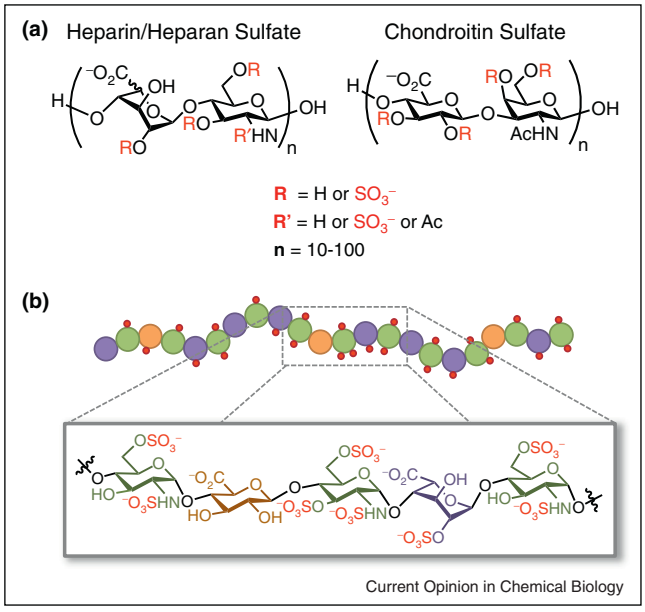
In this article, we describe some of the recent advances in the synthesis of GAG oligosaccharides and glycomimetic polymers. These advances include both synthetic organic and chemoenzymatic approaches to access defined oligosaccharides and polymerization techniques to develop simplified glycomimetics that emulate the natural multivalency of GAGs. We also highlight how these molecules have been used as probes to identify the roles of specific sulfation motifs in mediating key signaling events and cellular processes. Together, the studies suggest opportunities for the continued development of new chemical approaches to interrogate the functions of this ubiquitous, important class of polysaccharides.

Chemical synthesis of GAG oligosaccharides

The chemical synthesis of GAGs is notoriously challenging because it requires iterative, stereoselective formation of glycosidic bonds and sophisticated protecting group strategies to achieve regioselective sulfation [9–15,16•]. Many syntheses employ a modular, convergent approach in which a core oligosaccharide is assembled from a common disaccharide precursor. A late divergent approach is then exploited to differentially deprotect and regioselectively sulfate the core oligosaccharide and produce compounds with specific sulfation motifs.

A major challenge remains the generation of diverse libraries of GAG oligosaccharides with defined structures. To date, only a small proportion of the theoretically possible structures have been synthesized. However, new methods have emerged that may accelerate the generation of comprehensive collections of defined GAG oligosaccharides. For example, Jacquinet and coworkers developed efficient syntheses of CS tetrasaccharides and hexasaccharides bearing the CS-A, CS-C, CS-D,

Figure 1



The structural diversity of GAGs. **(a)** Representative GAGs heparin/HS and CS, with potential sites of sulfation indicated. Ac = acetyl. **(b)** Bioactive sulfation sequences such as the Arixtra pentasaccharide are found within the heterogeneous structure of GAG polysaccharides.

CS-E, CS-K, CS-L, and CS-M sulfation motifs using a precursor derived from natural CS polysaccharides [17–19]. Their method exploited the controlled hydrolysis of commercially available CS to rapidly and inexpensively obtain a fully protected disaccharide building block. More recently, Seeberger and coworkers reported a solid-phase synthesis of CS oligosaccharides [20]. By attachment of the oligosaccharides to Merrifield resin, many of the arduous purification steps associated with carbohydrate synthesis were avoided. The growing oligosaccharides were elongated by the sequential addition of monosaccharide phosphate donors and were selectively deprotected and sulfated over the course of three days to produce CS-A and CS-C hexasaccharides with an average yield of 86–88% per step. These methods may help expedite the development of much needed structural libraries that encompass a larger proportion of the molecular diversity found in GAGs.

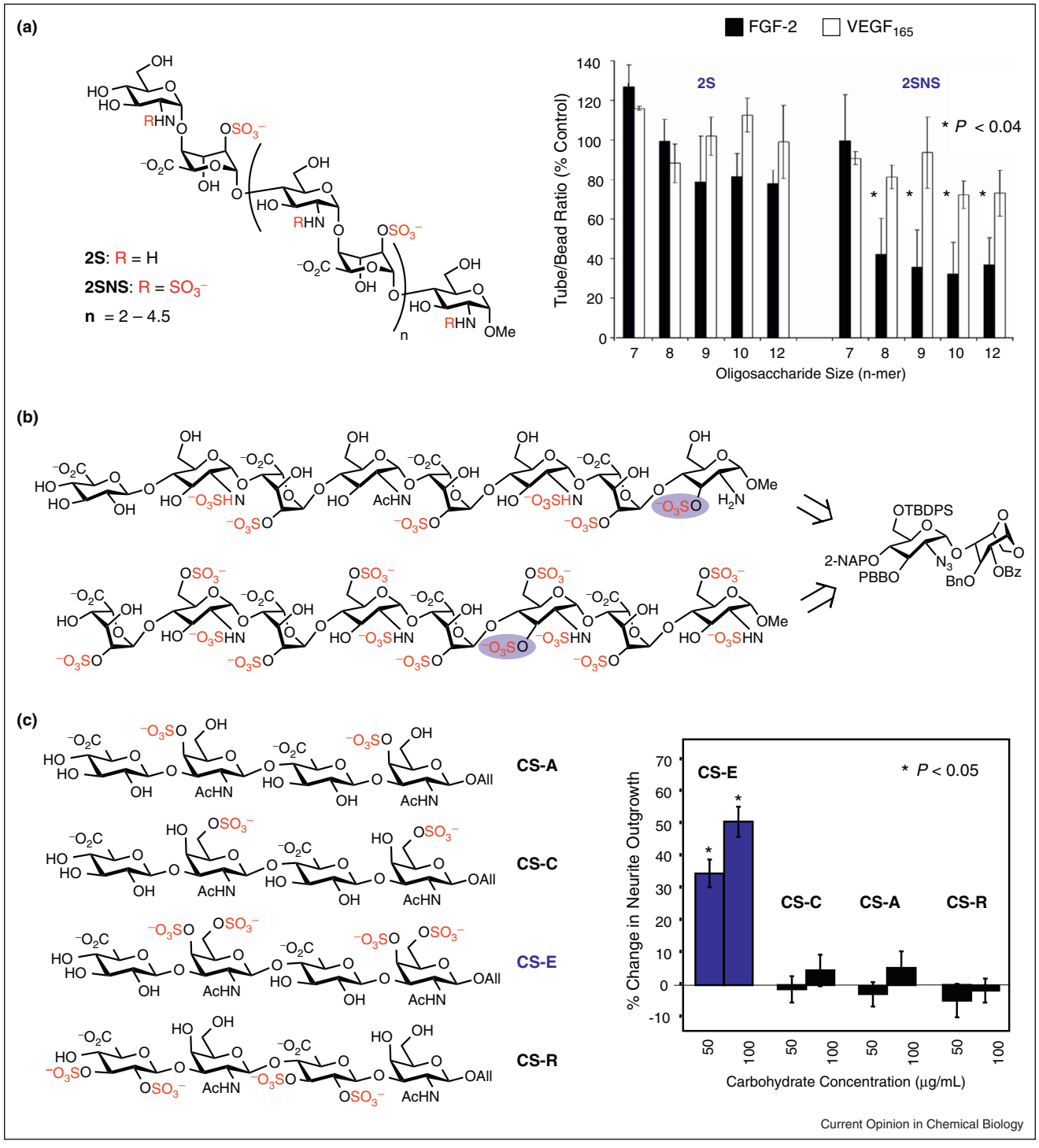
The joint venture of chemical synthesis and biological studies can have an enormous impact on our understanding of GAGs and their roles in physiological processes. The quintessential illustration of the power of this combined approach is demonstrated by the pioneering studies of Petitou, Sinaÿ, Choay, Lindahl and others, whose investigations in the late 1970s and early 1980s led to the discovery of a specific pentasaccharide within heparin that is responsible for its anticoagulant activity (Figure 1b) [21–23]. Their work provided critical insights into heparin's mechanism of action and facilitated the development of the pentasaccharide drug Arixtra (GlaxoSmithKline).

More recently, de Paz *et al.* studied the interactions of various synthetic heparin oligosaccharides with chemokines, a large family of proteins involved in injury, inflammation, and atherosclerosis [24•]. Various monosaccharides, disaccharides, tetrasaccharides, and hexasaccharides with natural and non-natural sulfation motifs were synthesized with amine-terminated linkers to allow for the generation of glycan microarrays. The authors rapidly screened the affinities of various chemokines for 12 oligosaccharides, revealing patterns of binding strengths that depended on individual sulfate groups. Notably, different chemokines displayed preferences for different sulfation patterns. For example, CCL21 exhibited robust binding to tetrasaccharides and hexasaccharides containing at least two sulfate groups per disaccharide, whereas CXCL13 and CCL19 showed weak or no binding to the same structures. This study demonstrates that synthetic oligosaccharides, when combined with glycan microarrays, can be used to rapidly assess the role of individual sulfation patterns in mediating GAG–protein interactions. Further development of comprehensive libraries of synthetic GAGs may lead to elucidation of the exact structural requirements for HS recognition by different chemokines.

The effects of HS sulfation pattern and chain length on cytokine-dependent angiogenic functions were examined by Cole *et al.* using a series of synthetic oligosaccharides [25•]. Compounds ranging in length from 7 to 12 sugar units and containing a repeating disaccharide unit of 2-*O*-sulfated iduronate with or without *N*-sulfated glucosamine (2S or 2SNS) were tested for their ability to compete with HS for binding to fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor 165 ($VEGF_{165}$) (Figure 2a). A strong correlation was found between the oligosaccharide structures, their affinity for the cytokines, and their ability to inhibit specific cytokine-dependent functions such as endothelial cell migration and tube formation. The 2SNS decasaccharide and dodecasaccharide showed the most potency overall, whereas the 2SNS heptasaccharide and all 2S oligosaccharides had no significant effect. This work represents one of the most systematic structure–function studies to date using synthetic HS oligosaccharides, and the results suggest that important cytokine-mediated cell functions depend highly on the fine structure of HS.

Hu *et al.* recently studied the role of specific HS sulfation motifs in herpes simplex virus type 1 (HSV-1) infection using synthetic oligosaccharides [26•]. HSV-1 attachment and entry into host cells is mediated by the binding of several viral envelope glycoproteins to HS chains on the host cell surface. One glycoprotein, gD, binds specifically to HS modified with the rare 3-*O*-sulfate motif [27]. In this

Figure 2



Synthetic oligosaccharides for studying the diverse biological functions of GAGs. **(a)** Oligosaccharides used to study the effects of HS sulfation pattern and chain length on cytokine-dependent angiogenic functions. Longer oligosaccharides containing both 2-*O*-sulfation and *N*-sulfation inhibited FGF-2-mediated endothelial tube formation. **(b)** HS octasaccharides containing the rare 3-*O*-sulfate group prevented HSV-1 infection of Vero cells. TBDPS = *tert*-butyldiphenylsilyl, 2-NAP = 2-naphthylmethyl, PBB = *p*-bromobenzyl, Bn = benzyl, and Bz = benzoyl. **(c)** Synthetic CS tetrasaccharides used to demonstrate sulfation sequence-specific effects on the outgrowth of developing neurons. All = allyl.

study, the authors completed an impressive synthesis of two nonrepeating, gD-binding HS octasaccharides that contained the 3-*O*-sulfate epitope at different sites (Figure 2b). Their synthetic route made use of a key disaccharide intermediate to acquire the different building blocks required for oligosaccharide assembly. Both molecules prevented HSV-1 infection of Vero cells in a dosage-dependent manner, presumably by blocking the binding of gD to endogenous HS. In contrast, commercially available HS from natural sources failed to prevent HSV-1 infection. These studies suggest flexibility in designing 3-*O*-sulfate-containing HS oligosaccharides as anti-HSV-1 agents and demonstrate the potential for defined synthetic molecules to reveal insights into the mechanisms of viral–host cell infection.

Like HS, CS has been shown to engage protein receptors and facilitate important cellular signaling events. The first investigations using synthetic oligosaccharides to study the activity of CS sulfation motifs were reported by Gama *et al.* [16•]. A late divergent synthetic strategy was developed to produce tetrasaccharides containing the CS-A, CS-C, and CS-E sulfation motifs, as well as a non-natural, highly sulfated motif (CS-R; Figure 2c). Although the CS-E and CS-R tetrasaccharides contained the same number of sulfate groups, only the CS-E tetrasaccharide modulated the outgrowth of developing neurons. The authors found that the growth-promoting activity was primarily due to the interaction of CS-E with specific growth factors, including brain-derived neurotrophic factor (BDNF) and midkine. Subsequent studies using a combination of glycan microarrays, computational modeling, and cellular assays showed that the CS-E motif is capable of forming a multimeric protein complex between BDNF and its cell surface receptors, thereby modulating neurotrophin signaling pathways [28]. Together, these results demonstrate that the specific sulfation sequence — namely, the precise position of the sulfate groups along the carbohydrate backbone rather than electrostatics alone — can modulate GAG–protein interactions and direct important neuronal signaling events.

These examples highlight the power of synthetic oligosaccharides to recapitulate the activity of natural GAG polysaccharides in a variety of biological contexts. The ability to access defined sulfation sequences instead of relying on the average, bulk properties of natural polysaccharides enables functions to be assigned to specific sulfation motifs. Such structure–function analyses have provided meaningful mechanistic insights into important biological processes and may provide novel therapeutic opportunities, as exemplified by Arixtra.

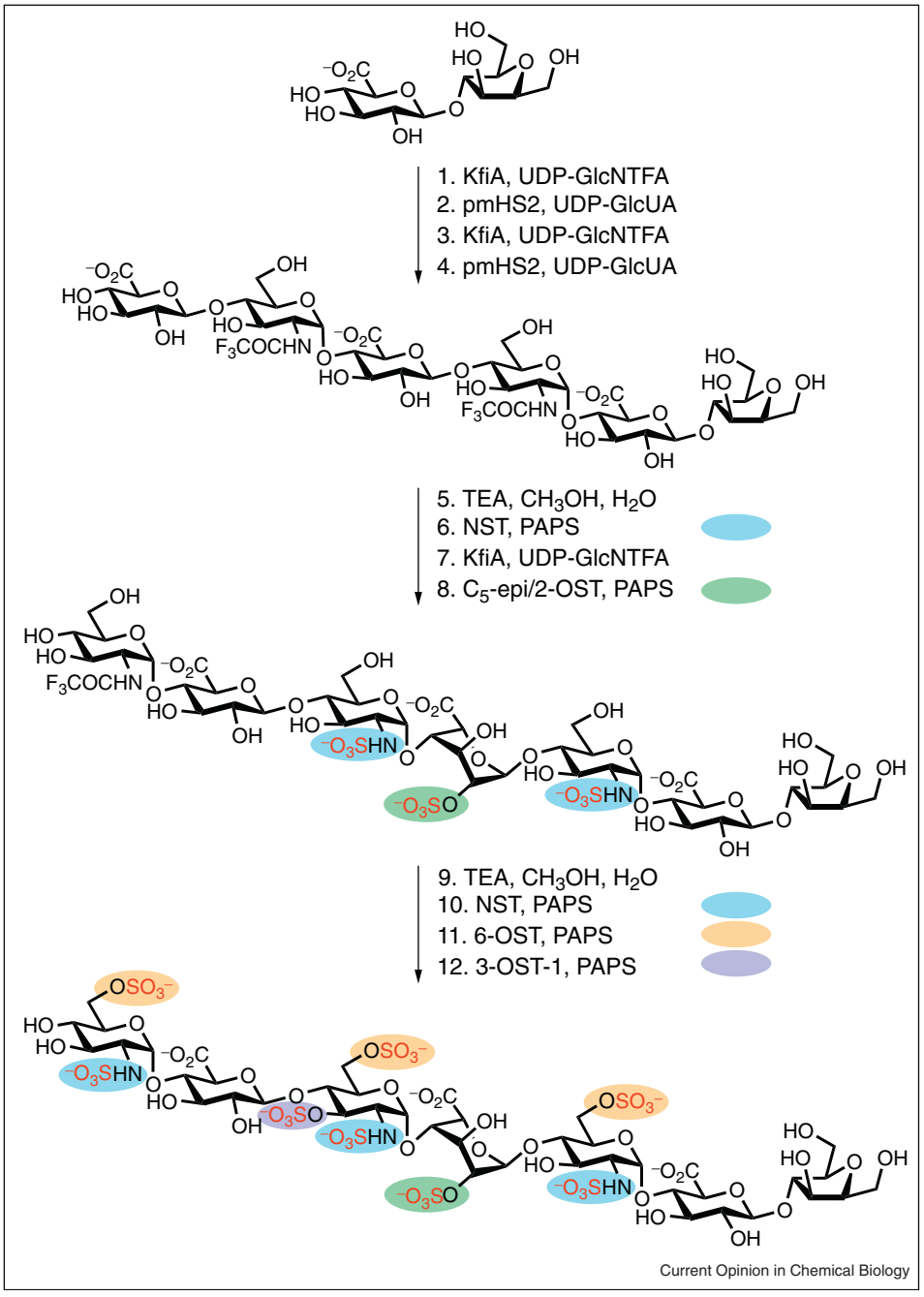
Chemoenzymatic approaches to defined GAG oligosaccharides

Exploiting natural GAG processing enzymes to obtain defined structures represents a complementary approach to the chemical synthesis of GAGs. Such chemoenzymatic methods utilize glycosyltransferases to produce oligosaccharides of defined length, followed by sulfotransferases for regioselective sulfation. These approaches can streamline the synthesis by circumventing the requirement for stereoselective glycosylation chemistry and sophisticated protecting group strategies.

A beautiful series of studies by Rosenberg, Liu, Linhardt, and coworkers has illustrated the potential of chemoenzymatic approaches to GAGs. Investigations into the specificity and activity of various HS polymerase, sulfotransferase, and epimerase enzymes have allowed for the synthesis of defined heparin oligosaccharides with potent anticoagulant properties [29–33,34••]. While pioneering methods produced an antithrombin III-binding pentasaccharide on a microgram scale [33], recent work substantially improved on the approach to generate two ultralow-molecular-weight heptasaccharides in 10–12 steps (>95% purity, 38% overall yield) on up to a 50-milligram scale (Figure 3) [34••]. The heptasaccharides exhibited strong anticoagulant factor Xa activity with potencies comparable to the pentasaccharide drug Arixtra, which requires approximately 50 chemical steps and affords only a 0.1% overall yield [35]. Although industrial-scale syntheses must still be developed, these impressive studies demonstrate that the efficient chemoenzymatic synthesis of defined, therapeutically important GAG structures is now possible.

Recent efforts have also focused on chemoenzymatic methods to produce CS oligosaccharides and polysaccharides. Unsulfated chondroitin oligomers were assembled by generating two partial loss-of-function mutants of the bifunctional chondroitin polymerase (K4CP) to isolate the individual glycosyltransferase activities [36]. Alternating application of each enzyme immobilized on agarose beads extended a pyridylamine-functionalized CS trisaccharide substrate to produce an unsulfated CS 16-mer. In another study, wild-type K4CP was used to generate a 10-kDa unsulfated chondroitin polymer starting from a hexasaccharide acceptor substrate, which was then treated with various sulfotransferases (C4ST-1, C6ST-1, GalNAc4S-6ST, and UA2ST) to produce sulfated CS libraries that were more highly enriched in particular sulfation motifs than their natural counterparts [37]. For example, the artificial CS-E polysaccharide contained 88% of the CS-E disaccharide unit, compared to 64% found in purified CS-E enriched polysaccharides. Moreover, the approach generated unsulfated, CS-A, and CS-C polysaccharides, with no detectable amounts of the more highly sulfated motifs that contaminate natural polysaccharides and can complicate structure–function analyses. These chemoenzymatically synthesized polysaccharides, although still only enriched in particular motifs, will provide valuable tools for studying the physiological functions of specific

Figure 3



Chemoenzymatic synthesis of defined heparin therapeutics. A bioactive heptasaccharide mimic of the anticoagulant drug Arixtra was produced in 12 steps on a milligram scale. KfiA = *N*-acetyl glucosaminyl transferase, UDP-GlcNTFA = uridine diphosphate *N*-trifluoroacetylglucosamine, pmHS2 = heparosan synthase-2, UDP-GlcUA = uridine diphosphate glucuronic acid, TEA = triethylamine, NST = *N*-sulfotransferase, PAPS = 3′-phosphoadenosine 5′-phosphosulfate, C_5-epi = C_5-epimerase, 2-OST = 2-*O*-sulfotransferase, 6-OST = 6-*O*-sulfotransferase, and 3-OST-1 = 3-*O*-sulfotransferase isoform 1.

CS sulfation motifs. Additionally, this work suggests that the success of chemoenzymatic approaches to defined heparin/HS oligosaccharides also should be amenable to CS.

Glycomimetic GAG polymers

Despite significant advances in the synthesis of GAGs, access to longer oligosaccharides of single, defined structure and varied sulfation sequence remains a central

challenge. Longer oligosaccharides are often required for full biological activity [1,22], and comprehensive collections of GAG oligosaccharides that represent all of the theoretically possible sulfation sequences cannot be obtained using current technologies. Overcoming these challenges will be required to understand fully the specificity of GAG interactions and to develop selective GAG-targeting agents. To address these issues, recent studies have exploited polymerization techniques to generate synthetic glycopolymers that present short, defined sulfation motifs within multivalent frameworks to recapitulate the activity of longer oligosaccharides.

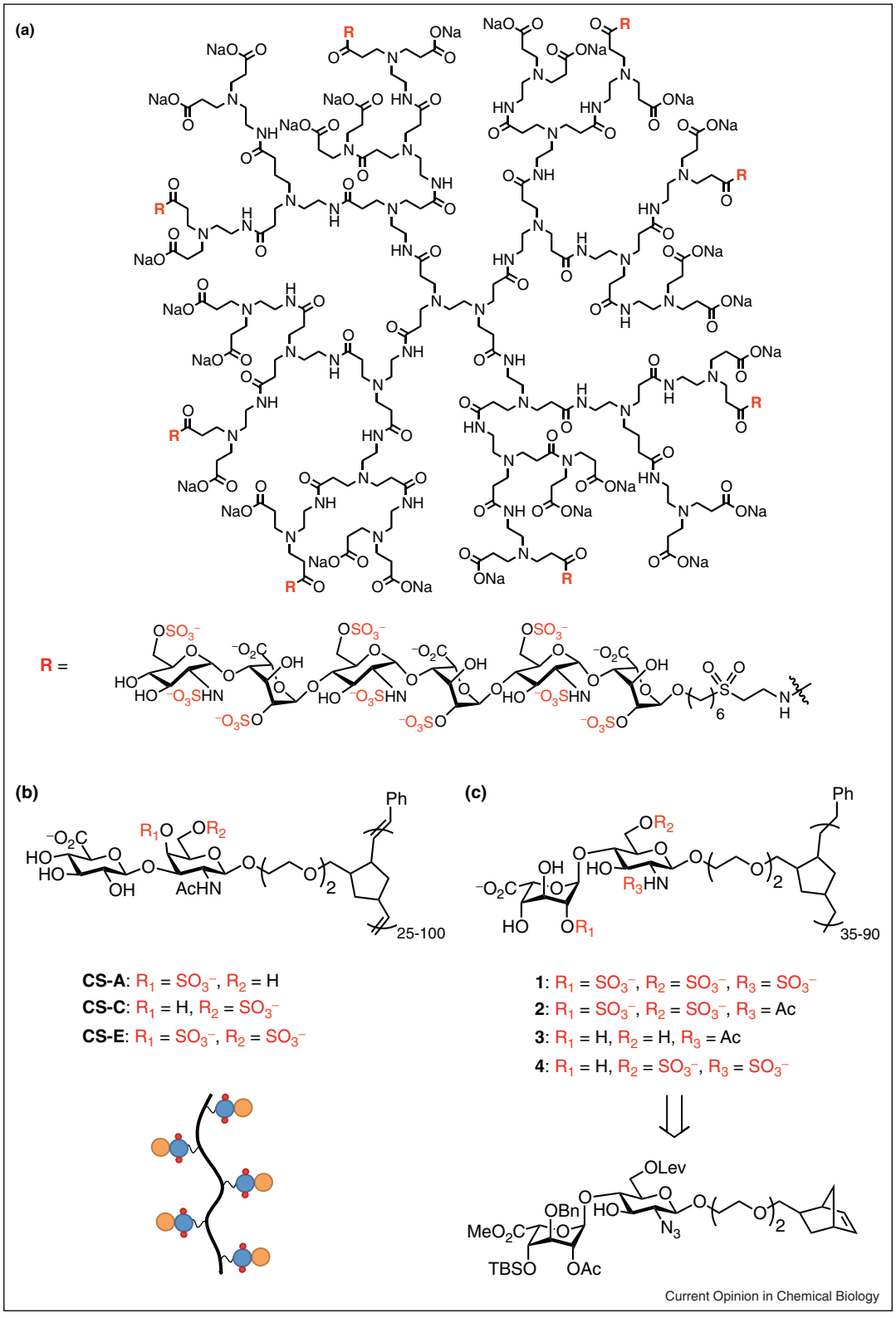
Seeberger and coworkers have functionalized starburst polyamidoamine (PAMAM) dendrimers containing 32 terminal carboxylate groups with amine-terminated heparin monosaccharides, disaccharides, and hexasaccharides (Figure 4a) [38]. Twenty-five percent loading (eight saccharide monomers appended) was achieved, which was attributed to steric hindrance or electrostatic repulsion between the negatively charged saccharides and PAMAM cores. Nonetheless, the glycodendrimers competed with heparin for binding to FGF-2 and stimulated downstream extracellular signal-regulated kinase (ERK) phosphorylation better than the corresponding monovalent hexasaccharide. In another study, de Paz *et al.* showed that the same hexasaccharide glycodendrimer inhibited CCL21-mediated chemotaxis, whereas the monomeric hexasaccharide had no significant effect [24•]. Interestingly, the monomeric hexasaccharide containing three sulfates per disaccharide exhibited stronger binding to CCL21 when compared to other sulfation epitopes. These findings underscore the importance of both the sulfation sequence and multivalency in eliciting HS-mediated responses.

Hsieh-Wilson and coworkers have utilized ring-opening metathesis polymerization (ROMP) chemistry to synthesize both CS and heparin/HS glycopolymers [5,39,40,41••,48]. Fully protected CS-E disaccharides and tetrasaccharides containing *cis*-cyclooctene or norbornene groups were polymerized using the fast-initiating ruthenium catalyst $(H_2IMes)(Py)_2(Cl)_2Ru{=}CHPh$ to generate a series of CS polymers, which were subsequently deprotected (Figure 4b) [5,39,40]. Varying the amount of catalyst allowed for excellent control of the degree of polymerization, affording polymers with 25–100 sugar units attached. Notably, the glycopolymers displayed robust activity comparable to natural CS-E polysaccharides in neurite outgrowth assays [39]. The activity was polymer length-dependent, while monovalent disaccharides and tetrasaccharides had minimal activity, highlighting the importance of multivalency and the tunability of the synthetic polymers. Lee *et al.* later showed that glycopolymers made from norbornenyl-functionalized CS-E disaccharides could be end-functionalized with a biotin moiety to afford a handle for microarray and surface plasmon resonance studies [40]. More recently, the approach was expanded by Brown *et al.* to generate glycopolymers displaying different sulfation motifs (CS-A, CS-C, and CS-E) for structure–function analyses [5]. Only glycopolymers containing the CS-E motif inhibited the outgrowth of adult sensory neurons and induced growth cone collapse. These results, combined with additional biological studies, linked the specific CS-E sulfation epitope to the inhibition of axon regeneration after spinal cord and other neuronal injuries. Indeed, blocking the CS-E structure with an antibody raised against a synthetic CS-E tetrasaccharide promoted optic nerve regeneration *in vivo*, suggesting a new potential strategy for neuronal repair.

In addition to providing functional insights, glycomimetic polymers have shown promise as agents for modulating GAG activity, which may be therapeutically beneficial in many contexts. For example, heparin possesses potent anti-inflammatory activity in models of asthma, chronic dermatitis, and ulcerative colitis. However, it is not recommended as an anti-inflammatory agent in clinical practice due to its anticoagulant activity [42]. Sheng *et al.* synthesized a variety of differentially sulfated HS glycopolymers from a core disaccharide precursor using ROMP chemistry (Figure 4c) [41••]. A specific glycopolymer bearing a trisulfated HS epitope antagonized the proinflammatory chemokine RANTES and blocked RANTES-induced cell migration with potencies comparable to heparin. Remarkably, the glycopolymer, which lacked the 3-*O*-sulfate modification, failed to inhibit key serine proteases in the blood coagulation cascade. Thus, controlling the positioning of sulfate groups within the glycopolymer enabled the anti-inflammatory properties of heparin/HS to be dissected from its anticoagulant properties. Given the pleiotropic functions of heparin/HS, such a strategy may allow for the development of more selective GAG-based therapeutic agents with fewer off-target side effects.

More recently, anticoagulant heparin-based glycopolymers were generated containing a tetrasulfated disaccharide epitope designed from Arixtra (Oh *et al.* [48]). The ROMP-generated glycopolymers exhibited potent anti-factor Xa and antithrombin activity and prolonged blood-clotting times in human plasma with efficacies similar to those of the clinical anticoagulants heparin, low-molecular-weight heparin (LMWH), and Arixtra. Whereas short polymers (15 disaccharide units) displayed *ex vivo* clotting times similar to Arixtra, longer glycopolymers (30 and 45 disaccharide units) displayed unique hybrid properties: their activated partial thromboplastin times were similar to LMWH and Arixtra, while their prothrombin times were similar to heparin. These studies not only suggest glycopolymers as a potential new anticoagulant therapeutic agent, but also raise the possibility that fine-tuning the polymer structure may produce novel agents with

Figure 4



Glycomimetic GAG polymers. **(a)** A PAMAM dendrimer functionalized with a synthetic HS hexasaccharide interacted with FGF-2 and stimulated ERK signaling. **(b)** CS polymers generated by ROMP allow for presentation of multiple sulfation epitopes and chain length control. The CS-E glycopolymers showed activities comparable to natural CS-E polysaccharides in both neurite outgrowth and growth cone collapse assays. **(c)** ROMP-based HS polymers used to study the role of sulfation in modulating chemokine activity. Trisulfated polymer **1** selectively inhibited the chemokine RANTES without targeting proteins in the blood coagulation cascade. TBS = *tert*-butyldimethylsilyl and Lev = levulinoyl.

desirable properties distinct from both synthetic and natural GAGs.

Conclusions and future directions

Synthetic GAG oligosaccharides and glycomimetic polymers have provided mechanistic insights into important biological processes, and an understanding of the structure–function relationships of this class of polysaccharides has begun to emerge. Through the use of defined synthetic molecules, specific sulfation motifs have been linked to diverse physiological outcomes. The participation of GAGs in myriad processes, ranging from inflammation and axon regeneration to viral infection, underscores the biological significance of these molecules and their future potential as therapeutic targets.

The continued development of novel methods to synthesize GAG oligosaccharides and polymers will be essential to delineate further the structure–function relationships and develop ways to selectively target specific GAG-mediated events. In particular, the construction of GAG libraries that encompass a larger portion of the sulfation sequence space will be important and may be facilitated in the future by automation and solid-phase methods [43,44]. Advances in pathway bioengineering [45] and directed evolution [46] may enable further improvements in the chemoenzymatic synthesis of GAGs, allowing for streamlined syntheses, larger scale production, as well as microbial factories [47] for producing collections of defined GAG structures. Finally, the development of simplified GAG mimetics capable of targeting specific GAG–protein interactions *in vivo* may be possible in the future through the systematic exploration of different polymer architectures. Ultimately, this synergistic combination of organic chemistry and biology will provide new opportunities to elucidate important physiological processes and to exploit this knowledge for diverse therapeutic applications.

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