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Chemoenzymatic Synthesis of Glycosaminoglycans

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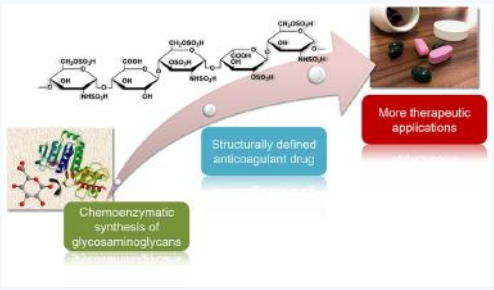
CONSPECTUS: Glycosaminoglycans (GAGs) are a family of structurally complex heteropolysaccharides composed of alternating hexosamine and uronic acid or galatose residue that include hyaluronan, chondroitin sulfate and dermatan sulfate, heparin and heparan sulfate, and keratan sulfate. GAGs display a range of critical biological functions, including regulating cell−cell interactions and cell proliferation, inhibiting enzymes, and activating growth factor receptors during various metabolic processes. Indeed, heparin is a widely used GAG-based anticoagulant drug. Unfortunately, naturally derived GAGs are highly heterogeneous, limiting studies of their structure−activity relationships and even resulting in safety concerns. For example, the heparin contamination crisis in 2007 reportedly killed more than a hundred people in the United States. Unfortunately, the chemical synthesis of GAGs, or their oligosaccharides, based on repetitive steps of protection, activation, coupling, and deprotection, is incredibly challenging. Recent advances in chemoenzymatic synthesis integrate the flexibility of chemical derivatization with enzyme-catalyzed reactions, mimicking the biosynthetic pathway of GAGs, and represent a promising strategy to solve many of these synthetic challenges. In this critical Account, we examine the recent progress made, in our laboratory and by others, in the chemoenzymatic synthesis of GAGs, focusing on heparan sulfate and heparin, a class of GAGs with profound physiological and pharmacological importance.



A major challenge for the penetration of the heparin market by homogeneous heparin products is their cost-effective large-scale synthesis. In the past decade, we and our collaborators have systematically explored the key factors that impact this process, including better enzyme expression, improved biocatalysts using protein engineering and immobilization, low cost production of enzyme cofactors, optimization of the order of enzymatic transformations, as well as development of efficient technologies, such as using ultraviolet absorbing or fluorous tags, to detect and purify synthetic intermediates. These improvements have successfully resulted in multigram-scale synthesis of low-molecular-weight heparins (LMWHs), with some showing excellent anticoagulant activity and even resulting in more effective protamine reversal than commercial, animal-sourced LMWH drugs. Sophisticated structural analysis is another challenge for marketing heparins, since impurities and contaminants can be present that are difficult to distinguish from heparin drug products. The availability of the diverse library of structurally defined heparin oligosaccharides has facilitated the systematic analytical studies undertaken by our group, resulting in important information for characterizing diverse heparin products, safeguarding their quality.

Recently, a series of chemically modified nucleotide sugars have been investigated in our laboratory and have been accepted by synthases to obtain novel GAGs and GAG oligosaccharides. These include fluoride and azido regioselectively functionalized sugars and stable isotope-enriched GAGs and GAG oligosaccharides, critical for better understanding the biological roles of these important biopolymers. We speculate that the repertoire of unnatural acceptors and nucleotide sugar donors will soon be expanded to afford many new GAG analogues with new biological and pharmacological properties including improved specificity and metabolic stability.

INTRODUCTION

What Are Glycosaminoglycans?

Glycosaminoglycans (GAGs) are a family of structurally complex polydisperse (average molecular weights ranging from 10 to over 1000 kDa), hetero-co-polysaccharides composed of the alternating pyranose forms of hexosamine (HexN) and uronic acid (UA) or galatose (Gal) residues.[1] The HexN residue can be glucosamine (GlcN), *N*-acetylglucosamine (GlcNAc, where Ac is acetate), *N*-acetylgalactosamine (GalNAc), or their sulfated derivatives, such as *O*-sulfo HexN or *N*-sulfoglucosamine (GlcNS, where S is sulfo). The UA residues,

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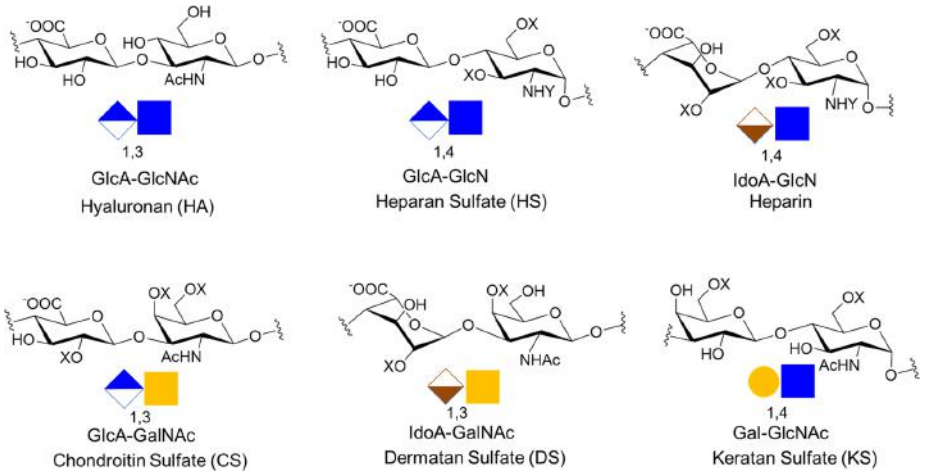

Figure 1. Chemical structures and symbolic representation[2] of the major backbone saccharide units of GAGs are shown. Group X is either the hydrogen or sulfate group, and group Y is either the acetyl (Ac) or sulfo (SO_3^-) group. The major uronic acid epimer found in HS, heparin, and CS is shown.

either glucuronic acid (GlcA) or iduronic acid (IdoA), can be modified as their 2-*O*-sulfo derivatives (GlcA2S or IdoA2S). GAGs are found as both free glycans or attached to core proteins as proteoglycans and control a wide range of physiological and pathophysiological events including cell−cell interactions, cell proliferation, enzyme inhibition, and growth factor receptor activation during various metabolic processes.[1] GAGs are classified into four groups: hyaluronan (HA), chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate (HS)/heparin, and keratan sulfate (KS) based on their different disaccharide repeating units, (i.e., GlcA-GlcNAc, GlcA/IdoA-GalNAc, GlcA/IdoA-GlcNAc/GlcN, Gal-GlcNAc, respectively) glycosidic bonds (i.e., α- or β-, 1→3 or 1→4) and sulfation patterns (i.e., NS, 2S, 3S, 4S, 6S). The structures of the common GAG types are presented in Figure 1.

Why Are Synthetic GAGs Needed?

Currently commercially available GAGs (with the exception of HA for which there are microbial sources) are isolated from animal tissues. GAG biosynthesis in vivo results in a variety of chain lengths and modification patterns (again the exception is HA which has a single monotonous disaccharide repeating unit). These modification patterns can be represented, as sequences composed of different sulfo group and C5 UA epimer positions. In GAGs isolated from animal sources, seasonal variations, environmental factors, animal species, organ tissue collected, animal age, animal breed, animal health, and animal feed can contribute to the variability of naturally occurring GAGs,[1] resulting in commercial GAGs being complex, very heterogeneous mixtures of molecules (i.e., HS obtained from animal tissues contains many different sequences and chain lengths). Due to the structural similarity of GAGs, their polydispersity, molecular heterogeneity, and limitations in purification methods and the lack of simple and reliable analytical methods, cross-contamination of GAGs (i.e., the presence of CS in heparin) also frequently occurs.[3,4] Moreover, the supply chain of pharmaceutical GAGs, particularly heparin, has been poorly regulated and also presents safety concerns. A worldwide distribution of contaminated heparin in 2007, caused by its adulteration with a semisynthetic oversulfated chondroitin sulfate contaminant, adversely impacted the purity and safety of animal-sourced unfractionated heparin and low-molecular-weight heparins (LMWHs) and was associated with over 100 deaths in the United States.[4] In addition, keratan sulfate, a possible impurity in CS, may have resulted in negative assessments of CS efficacy in clinical trials on osteoarthritis.[3] Another potential threat is that other bioactive entities (i.e., viruses, prions, growth factors) might remain associated with the GAG chains present in animal extracts.[1] Thus, the cost-effective preparation of structurally defined GAGs from nonanimal sources is highly desirable[5,6] as are reliable structural characterization techniques.

GAGs, with the exception of HA, are among the most structurally complex carbohydrates in nature, having various monosaccharide residues, chain lengths, and modification patterns. Thus, the preparation of homogeneous GAGs relying on purely synthetic chemical methods, based on repetitive steps of protection, activation, coupling, and deprotection, while highly desirable, is incredibly challenging.[7] For example, Arixtra, a U.S. Food and Drug Administration (FDA)-approved pentasaccharide anticoagulant drug, requires as many as 60 chemical steps, and it is produced in an overall yield of 0.1%[8] although there have been recent synthetic improvements.[9] In addition, during its preparation, there are many separation steps needed to remove undesirable isomers adding cost and decreasing overall yields. Thus, chemical synthesis is usually not amenable for the commercial preparation of most GAGs and GAG oligosaccharides. In contrast to chemical synthesis, enzymes have exquisite stereoselectivity (α- or β-glycosidic linkages) and regioselectivity (1 → 3 versus 1 → 4 glycosidic linkages and sulfo group positioning on specific saccharide rings) for GAG synthesis. Purely enzymatic synthesis has its own limitations, since it is currently not possible to control GAG biosynthetic enzymes sufficiently to obtain target chains having a single sequence; thus, a purely enzymatic synthesis of homogeneous GAGs or GAG oligosaccharides cannot yet be achieved. Chemoenzymatic synthesis, however, integrates the flexibility of chemical derivatization with the specificity of enzyme-catalyzed reactions,[10] mimicking the biosynthetic pathway of GAGs, and represents a promising strategy to solve these synthetic challenges, and offers a potential alternative for the preparation of safer, high purity GAGs and homogeneous

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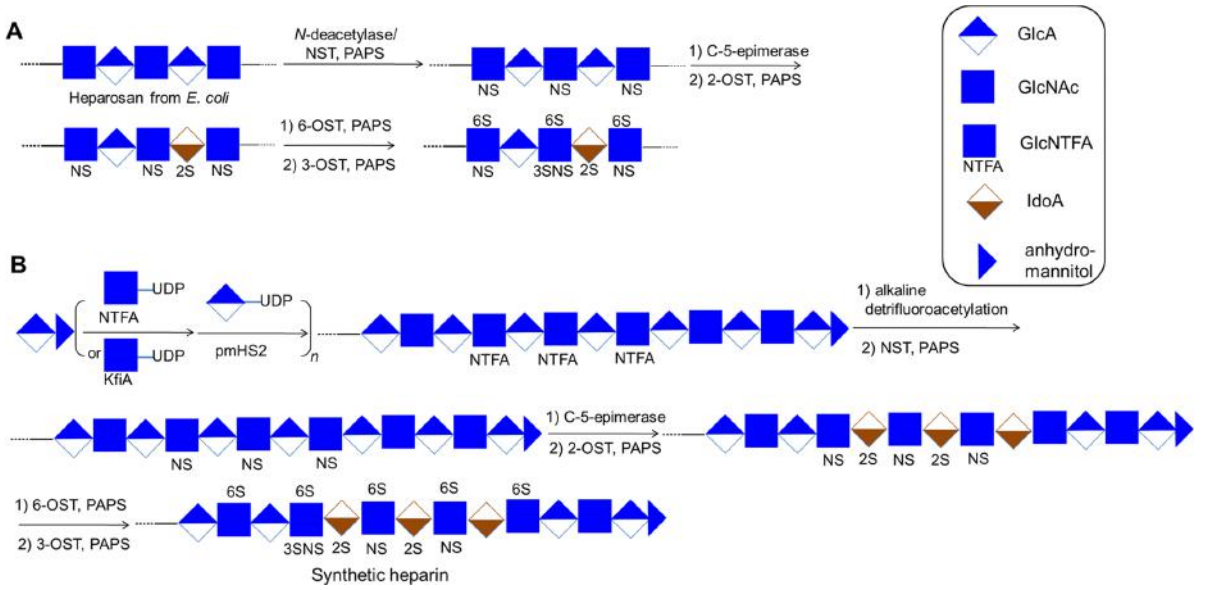

Figure 2. Chemoenzymatic approaches for synthesis of GAGs are shown. (A) Semisynthesis of heparin and heparan sulfate polysaccharides starting from heparosan CPS.[14] (B) Total synthesis of heparin oligosaccharides through the addition of UDP-activated donor sugars to a disaccharide acceptor prepared through the nitrous acid catalyzed depolymerization of *N*-deacetylated heparosan.[15]

Table 1. Common Enzymes Utilized in GAGs Synthesis

enzymes	abbreviation	expression system	enzymatic function
N-acetyl-D-glucosaminyl transferase	KfiA	*E. coli* K5	transfers GlcNAc residue to the backbone for HS synthesis
heparosan synthase 2	PmHS2	*P. multocida*	transfers GlcA and GlcNAc residue to the backbone for HS synthesis
hyaluronan synthase	PmHAS	*P. multocida*	transfers GlcA and GlcNAc residue to the backbone for HA synthesis
chondroitin synthase	PmCS	*P. multocida*	transfers GlcA and GalNAc residue to the backbone for CS synthesis
chondroitin synthase	KfoC	*E. coli* K4	transfers GlcA and GalNAc residue to the backbone for CS synthesis
β4-galactosyltransferase-1	β4GalT-1	*Neisseria meningitidis*, *N. gonorrheae*, or *Helicobacter pylori*	transfers Gal residue to the backbone for KS synthesis
β3-*N*-acetylglucosaminyltransferase	β3GnT	*N. meningitides* or *H. pylori*	transfers GlcNAc residue to the backbone for KS synthesis
N-acetyl-glucosamine-1-phosphate uridyltransferase	GlmU	*E. coli* K5	converts a GlcNAc-1-phosphate to an UDP-GlcNAc
C5-epimerase	C5-Epi	*Cricetulus griseus*	converts a GlcA to an IdoA residue for heparin synthesis
HS 2-*O*-sulfotransferase	HS 2OST	*C. griseus*	introduces a sulfo group to the 2-*O*-position of an IdoA or a GlcA residue for heparin synthesis
HS 6-*O*-sulfotransferases	HS 6OSTs	*Mus musculus*	introduces a sulfo group to the 6-*O*-position of a GlcNS(GlcNAc) residue for heparin synthesis
HS 3-*O*-sulfotransferases	HS 3OSTs	*M. musculus*	introduces a sulfo group to the 3-*O*-position of a GlcNS $\pm$ 6S residue for heparin synthesis. Different enzyme isoforms have different substrate specificities.
N-deacetylase/*N*-sulfotransferase-1	NDST-1	*Rattus norvegicus*	converts a GlcNAc to a GlcNS residue.
chondroitin 4-*O*-sulfotransferases	CS 4-OST	*P. pastoris* or insect cells	introduces a sulfo group to the 4-*O*-position of a GalNAc residue for CS synthesis
chondroitin 6-*O*-sulfotransferases	CS 6-OST	*P. pastoris* or insect cells	introduces a sulfo group to the 6-*O*-position of a GalNAc residue for CS synthesis
chondroitin GalNAc 4-*O*-sulfotransferases	CS GalNAc4S6-OST	*P. pastoris* or insect cells	introduces a sulfo group to the 6-*O*-position of a GalNAc4S residue for CS synthesis
chondroitin uronosyl 2-*O*-sulfotransferase	UA2-OST	*P. pastoris* or insect cells	introduces a sulfo group to the 2-*O*-position of a GlcUA residue for CS synthesis

GAG oligosaccharides. In this strategy, chemical reactions are applied to prepare unnatural enzymatic reaction substrates (i.e., unnatural uridine diphosphate (UDP)-sugars or acceptors) to modify the carbohydrate backbone, while enzymes catalyze the reaction with exquisite regioselectivity and stereoselectivity under mild conditions without the need for repetitive protection or deprotection steps.[11,12]

Types of Chemoenzymatic Syntheses of GAGs

The semisynthesis of GAGs employs a naturally occurring polydisperse polysaccharide, obtained through bacterial fer-

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mentation as a starting material, followed by backbone modification by enzymatic or chemical reactions.[13] For example, HS or heparin semisynthesis usually begins with the capsular polysaccharide (CPS), heparosan,[14] which is prepared from the *Escherichia coli* K5 strain that is composed of a [→4) β-D-glucuronic acid (GlcA) (1→4) *N*-acetyl-α-D-glucosamine (GlcNAc) (1→]$_n$ repeating disaccharide unit (Figure 2A). After partial chemical *N*-deacetylation and *N*-sulfation (this step can also be performed enzymatically with a *N*-deacetylase/*N*-sulfotransferase), this polysaccharide is then transformed into heparin or HS using recombinant biosynthetic enzymes (i.e., 2-, 6-, and 3-*O*-sulfotransferases and C5-epimerases), mimicking the in vivo biosynthesis of these GAGs.[14] This approach can avoid redundant steps and is relatively easy to scale, but full control of polymer size (chain length), composition, and sequence are sacrificed.

The total-synthesis of GAGs or GAG oligosaccharides can also be initiated from available monosaccharides or disaccharides by using both backbone elongation and chemoenzymatic modifications.[16] For example, glycosyltransferases (KfiA and PmHS2) utilize UDP-activated sugars as donors and transfer saccharides onto a monosaccharide or disaccharide acceptor to build the HS backbone structure. The nascent HS chain is subsequently taken through a series of modifications involving the action of the bifunctional enzymes, *N*-deacetylase-*N*-sulfo transferases (NDSTs), or in a stepwise fashion involving monofunctional enzyme constructs *N*-deacetylase (ND)/*N*-sulfotransferase (NST), followed by C5-epimerase (C5-epi), 2-*O*-sulfotransferase (2-OST), 6-*O*-sulftransferases (6-OSTs isoforms1−3), and 3-*O*-sulftransferases (3-OSTs isoforms 1−7), resulting in the target HS or heparin structure (Figure 2B).[15]

■ ENZYMES REQUIRED FOR CHEMOENZYMATIC SYNTHESIS

Glycosyltransferases

The biosynthesis of GAGs includes saccharide chain elongation and modification of the saccharide backbone. Enzymes involved in GAG biosynthesis were first identified and prepared through the pioneering work of laboratories headed by Ulf Lindahl, Robert Rosenberg, Koji Kimata, and Paul Weigel.[2] The glycosyltransferases used for chemoenzymatic synthesis come from a variety of different sources that are able to mimic GAG glycosylations in vitro. HS and heparin elongation is usually achieved by recombinantly expressed bacterial glycosyltransferases, KfiA/KfiC and PmHS2[17] (Table 1). KfiA is an *N*-acetylglucosaminyltransferase that incorporates GlcNAc from UDP-GlcNAc and is obtained from the *E. coli* K5 strain. KfiC is a glucuronosyl transferase that incorporates the GlcA residue from UDP-GlcA in vivo in the *E. coli* K5 strain, but it has not been successfully expressed for in vitro synthesis. PmHSs are homologous heparosan synthases from *Pasteurella multocida* (PmHS1 and PmHS2), which can transfer both GlcA from UDP-GlcA and GlcNAc from UDP-GlcNAc (or modified GlcN derivatives in the case of PmHS2) donors to build the HS backbone.[18] KfiA and PmHS are typically used in an alternating fashion during chemoenzymatic synthesis of HS.[19] PmHS2, while able to catalyze the addition of both GlcNAc and GlcA, it is also often used only as GlcNAc transferase in place of KfiA during chemoenzymatic synthesis of HS and heparin.[20]

The enzymes PmHAS, PmCS that prepared from different serotypes of *P. multocida* are responsible for forming hyaluronan and chondroitin CPSs, respectively.[21] PmHAS catalyzes the formation of HA chains by adding GlcNAc and GlcA monosaccharides from the corresponding nucleotide sugar donors.[22] Moreover, PmHAS can also elongate HA chains using HA oligosaccharides as acceptors in a synchronized non-processive manner. Besides PmCS, the enzyme KfoC,[23] readily expressed through the *E. coli* K4 strain (the K4 strain synthesizes a fructosylated chondroitin capsular polysaccharide in vivo), was recently used to synthesize the chondroitin backbone by transferring both UDP-GalNAc and UDP-GlcA. Chemoenzymatic approaches for KS synthesis are still very limited. A combination of human β4-galactosyltransferase-1 (β4GalT-1) and bacterial β3-*N*-acetylglucosaminyltransferase (β3GnT) has enabled the synthesis of *N*-acetyllactosamine (LacNAc) and its oligomers corresponding to the saccharide backbone of KS.[24,25]

Enzymes for Backbone Modification

In HS and heparin backbone modification, sulfotransferases transfer sulfo groups from 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the natural sulfate donor, to GAG oligosaccharides and polysaccharides with excellent regiospecificity.[26] A bifunctional enzyme *N*-deacetylase/*N*-sulfotransferase (NDST) is responsible for the removal of *N*-acetyl group from a GlcNAc residue and the introduction of *N*-sulfo groups. The expression of an active bifunctional bacterial NDST has been problematic; thus, *N*-deacetylase (ND) and *N*-sulfotransferase (NST) domains have been cloned and produced separately. An alternative method using the unnatural UDP-GlcNTFA donor, has been developed to introduce a GlcNTFA residue that can be easily chemically de-*N*-trifluoroacetylated to GlcN for subsequent chemical or enzymatic *N*-sulfonation.

HS 2-OST transfers a sulfo group from PAPS to the 2-position of either IdoA and GlcA residues, with IdoA residues being the preferred substrate.[27] In humans, there are three HS 6-OST isoforms (6-OST-1, 6-OST-2, 6-OST-3), and seven HS 3-OST isoforms (3-OST-1−7) have been identified and transfer sulfo groups to the 6- and 3- positions of the GlcN residues, respectively. Some of these different isoforms of enzymes exhibit slightly different specificities. For example, HS 6OST-1 and HS 6OST-2 prefer to transfer 6-*O*-sulfo groups to a GlcNS that is next to a GlcA residue and IdoA2S residue, respectively.[28] HS 3-OST-1 catalyzes the introduction of a 3-*O*-sulfo group forming a GlcNS3S6S residue that is linked to a GlcA residue on its nonreducing side, whereas the HS 3-OST-3 enzyme introduces a sulfation to form a GlcNS3S residue that is linked to an IdoA2S residue on its nonreducing side.[29] There is a single C5-epimerase (C5-epi) that is responsible for converting GlcA into its C5-epimer IdoA. C5-epi catalyzes both the forward and reverse reactions, resulting in an equilibrium mixture of GlcA and IdoA residue that can be driven toward IdoA through the action of the single 2-OST preferentially forming IdoA2S.[16] The enzymes for CS backbone modification are summarized in Table 1. These enzymes can be expressed in eukaryotes including *Pichia pastoris* recombinant strains or insect cells using a baculovirus expression approach but are more difficult to express in prokaryotes.[30] However, the use of 6-*O*-sulfotransferases in KS synthesis is limited by their low specific activity after heterologous expression and their poor selectivity for the repeating LacNAc disaccharide of keratan.

Bacterial-expression systems often provide enzymes with limited solubility, stability, and activity, hindering the effectiveness in GAGs synthesis. In contrast, biosynthetic enzymes prepared in eukaryotic systems tend to show improved properties but are expressed at lower levels. Moreover, other

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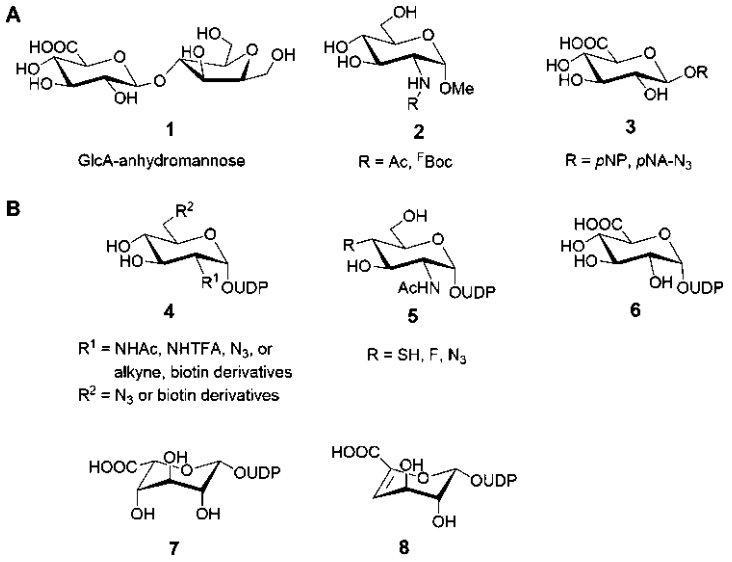

Figure 3. Carbohydrate synthons designed for use in chain elongation reactions. (A) Selected acceptor structures. (B) Selected donor structures.

challenges remain, for example, enzymatic synthesis is typically limited to synthesizing naturally occurring structures and may not be suitable for the preparation of HS oligosaccharides having intricate sulfation patterns due to the promiscuous substrate specificities of many of the biosynthetic enzymes.

BUILDING BLOCKS PREPARED FOR CHEMOENZYMATIC SYNTHESIS

Acceptors

In the total synthesis of GAGs, particularly HS and heparin oligosaccharides, a number of monosaccharide and disaccharide derivatives have been used as the initial acceptors and their use has greatly facilitated the development of chemoenzymatic synthesis. An easily prepared disaccharide unit, composed of glucuronic acid (1→4) anhydromannose (Figure 3A, **1**) was once widely used in chemoenzymatic synthesis.[19] This disaccharide acceptor could be further modified to introduce a "click" reactive azide group or amino group at the reducing end allowing for conjugation.[31] However, the absence of a hydrophobic chromophore on this disaccharide acceptor has limited its widespread application.

A commercially available *β*-glucuronide derivative with an ultraviolet-detectable, hydrophobic tag at its reducing end, such as *p*-nitrophenyl (*p*-NP)[32] and *p*-aminophenyl *N*-(6-azidohexanamidyl) (*p*NA-N_3),[20] has been used by Liu and co-workers to prepare a diverse library of HS oligosaccharides (Figure 3A, **3**). Moreover, GlcA-*p*NP is an excellent acceptor in the chemoenzymatic synthesis of CS[30] and HA.[33] The strong UV absorbance and high C-18 binding affinity of *p*-NP or *p*NA-N_3 enable the easy detection and purification of the resulting oligosaccharides using a C18 resin. In addition, these groups can be further chemically modified with a fluorescent tag or biotin, serving directly as probes or affixed onto chips for glycan microarray analysis,[34] respectively. However, the potential toxicity of the aromatic *p*-nitrophenyl group is a potential concern in clinical applications.

The monosaccharide *N*-acetyl-*α*-D-glucosamine-*O*-methyl-glycoside (Figure 3A, **2**)[35] has been used as an acceptor in approaches aimed at the chemoenzymatic synthesis of the commercial Arixtra. While it is sufficiently nontoxic to be a component of the drug Arixtra, it is not ultraviolet detectable and does not facilitate detection and purification. Fluorous tags have also be used to facilitate purification through fluorous solid-phase extraction but their large size can interfere with enzyme recognition and binding.[36]

Donors

Natural UDP−Sugar/Donors. Uridine diphosphate (UDP) monosaccharides are donors commonly transferred to glycosyl acceptors by a glycosyltransferase or synthase in the chemoenzymatic synthesis of GAGs. Donors, including both natural and unnatural UDP-sugars, have been prepared chemically, enzymatically, or chemoenzymatically.[37,38] UDP-GlcNAc and UDP-GlcA are the natural donor substrates required in the chemoenzymatic synthesis of HS and HA. UDP-GlcA is commonly synthesized from UDP-glucose by a NAD^+-dependent oxidation process catalyzed by a UDP-glucose dehydrogenase.[39] The UDP-GalNAc donor is required in CS and DS synthesis and is prepared using *N*-acetylhexosamine kinase (NAHK) and the mammalian GalNAc pyrophosphorylase AGX1.[30,40] UDP-Gal is a donor required in KS synthesis, and it is commonly synthesized by treating UDP-glucose with C4-epimerase.

Unnatural UDP−Sugar/Donors. GlcNAc and GalNAc are the naturally occurring hexosamine residues prevalent in GAGs, and thus, structural analogues of UDP-GlcNAc or UDP-GalNAc are of interest for use in enzymatic reactions of GAG synthesis and represent an excellent approach for understanding the mechanism of GlcNAc/GalNAc-related pathways.[41] In addition, by chemically modifying the monosaccharide prior to its addition as a UDP moiety, the fine structure of the resulting oligosaccharide or polysaccharide can be manipulated offering another level of structural control.

Since the bacterial expression of an active NDST has been problematic, the regiselective (or regiospecific) N-sulfonation of glucosamine in backbone modification becomes a major roadblock in chemoenzymatic synthesis of HS and heparin. UDP-GlcNTFA (*N*-trifluoroacetylglucosamine), an unnatural

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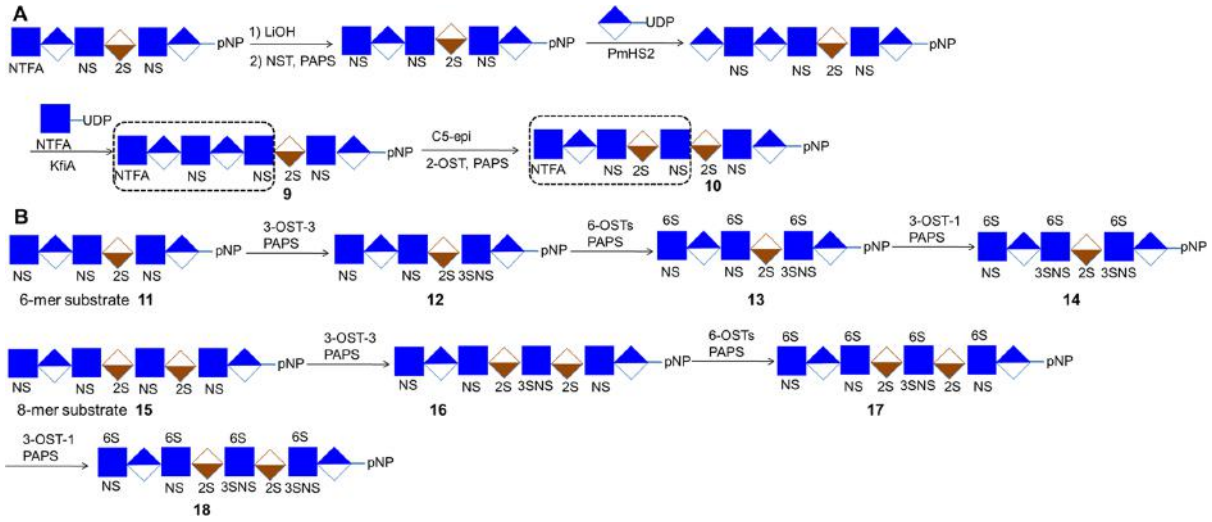

Figure 4. Controlled chemoenzymatic synthesis of HS/heparin oligosaccharides. (A) Examples for C5 epimerization.[32] (B) Examples for 3-O-sulfation using different isoforms of sulfotransferases.[29] Symbols are defined in Figure 2.

UDP-sugar, was introduced to solve this problem. UDP-GlcNTFA is an excellent substrate for glycosyltransferase KfiA or PmHS2 and the resulting GlcNTFA residue can then be easily converted to GlcNS by removing the trifluoroacetyl group under mild alkaline conditions followed by the introduction of *N*-sulfo groups either using NST or through chemical N-sulfonation. The use of UDP-GlcN*t*Boc (*N*-*t*Boc protected glucosamine), an alternative approach in which the *t*Boc protecting group be removed under mild acid conditions, has been less widely applied as it is not as good of a KfiA or pmHS2 substrate.

Azide-derivatized UDP sugars have become exciting targets due to their well-established and unique reactivity in biological systems. Chen and co-workers[42] reported a one-pot three-enzyme system to produce UDP-6-N_3GlcNAc, and we[43] prepared C4-modified UDP sugars, UDP-4-N_3GlcNAc and UDP-4-N_3GalNAc, and tested their incorporation into GAGs synthesis. Fluoride C4-modified UDP sugars have also been developed by us[44] as terminators of chain polymerization. Wang and co-workers have made systematic efforts toward the synthesis of unnatural UDP-sugars. These studies rely on a platform that combines chemical synthesis and enzymatic synthesis for the facile preparation of a library of unnatural UDP-sugars modified with diverse bioorthogonal reactive groups.[45] However, very limited examples of syntheses involving UDP-uronic acid derivatives have been reported with the exception of UDP-IdoA, which failed to serve as a donor in heparin synthesis.[46]

PRODUCT CONTROL THROUGH SEQUENTIAL ENZYMATIC MODIFICATION

The chemoenzymatic synthesis of GAGs involves a series of chemical and enzymatic reaction steps, requiring a careful design of the sequence of these reactions.[47] An ideal reaction order can greatly impact the enzyme activity giving high yields of the desired products, while an improper sequence often results in poor yields and product mixtures. For example, C5-epimerase can only be used after the introduction of *N*-sulfo groups during conversion from compound **9** to **10**, as it will only act on a GlcA residue that is flanked by two GlcNS residues[32] (Figure 4A). Since the GlcA residue is thermodynamically favored over IdoA C5-epimerization works best when used in tandem with 2-OST to form IdoA2S, which cannot be converted by C5-epimerase to GlcA2S.[20] Moreover, careful design in chemoenzymatic synthesis can afford GAG chains with different domain motifs exhibiting distinctive signaling activities.[48]

In addition, different isoforms of sulfotransferases can have distinct and unique substrate requirements. For example, Liu and co-workers[29] have synthesized a library of 3-O-sulfated oligosaccharides that comprising the -IdoA2S-GlcNS3S- or -IdoA2S-GlcNS3S6S- disaccharide units by rearranging the enzymatic modification sequences. They found that the 3-OST-1 enzyme sulfated oligosaccharide substrates that carry 6-O-sulfation, such as conversion from compound **13** to **14**, or **17** to **18**, while displaying very low reactivity toward the oligosaccharide substrates that lack 6-O-sulfation (Figure 4B). 3-OST-3 preferentially sulfates oligosaccharides that do not carry 6-O-sulfation, such as preparing compound **12** and **16**, respectively. These conclusions were also supported by kinetic analysis, showing that 3-OST-3 has higher catalytic efficiency for oligosaccharide substrates without 6-O-sulfation as determined by the values of k_{cat}/K_m.

NOVEL CHEMOENZYMATIC SYNTHESIS

One-Pot Reactions

In some instances, it might be more efficient to transform complicated multiple-enzyme-catalyzed reaction sequences into one-pot reactions, where oligosaccharides or polysaccharides are prepared without the required isolation or purification of intermediates. Chen and co-workers[39] developed an efficient one-pot multienzyme (OPME) system utilizing a series of unnatural UDP-sugars to synthesize heparin-like oligosaccharides. This strategy relies on in situ production of a nucleotide sugar from a simple monosaccharide or its derivative, which is used as the donor for a glycosyltransferase in the same pot for the synthesis of desired carbohydrates without the isolation of intermediates. For example, glycosyl donor UDP-GlcNTFA **21** was first synthesized using a one-pot, three-enzyme reaction containing NAHK_ATCC55813, PmGlmU, and PmPpA using

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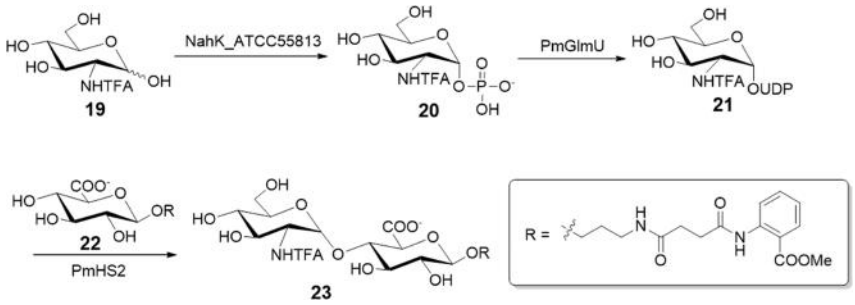

Figure 5. One-pot multienzyme approach to access heparin oligosaccharides.[39]

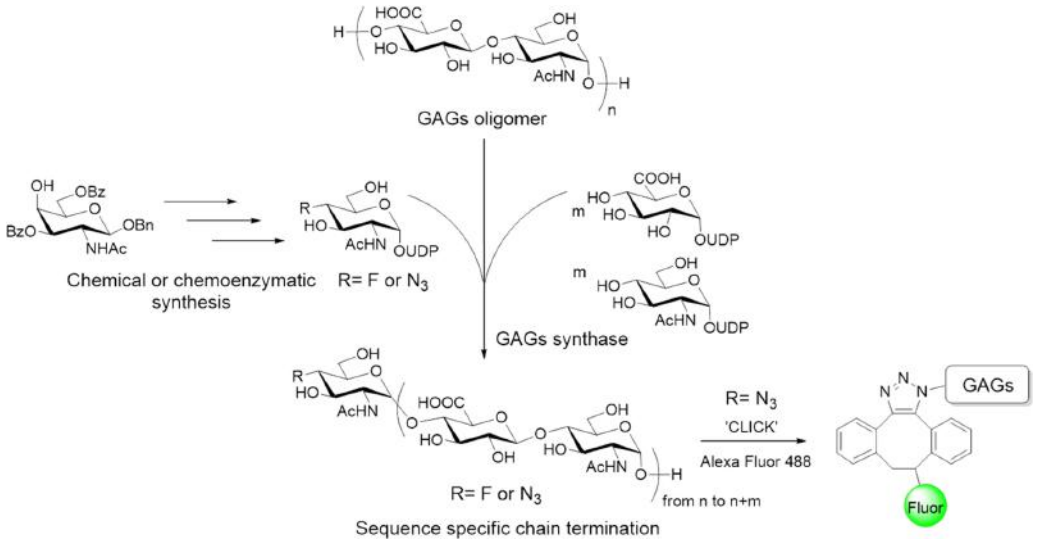

Figure 6. Unnatural UDP-sugars are used for the synthesis and incorporation into GAGs.[43,44]

GlcNTFA **19** as the donor precursor. GlcA derivative acceptor **22** was glycosylated with donor **21** using PmHS2 to produce disaccharide **23** (Figure 5).

Unnatural UDP–Sugar Donors for the Synthesis of Functionalized GAGs

UDP-sugars could be transferred to the nonreducing end of carbohydrate chains by glycosylation reactions with glycosyltransferases in the GAGs biosynthetic pathway. We[44] have synthesized UDP-4-FGlcNAc using both chemical and chemoenzymatic syntheses relying on GlmU. The resulting unnatural donor could be accepted by PmHS1 and subsequently serve as a chain terminator,[49] offering an approach to synthesize HS/heparin oligosaccharides of defined sequence length (Figure 6). The unnatural donor, UDP-4-N_3GlcNAc,[43] was also used to add 4-N_3GlcNAc to the nonreducing end of the sugar chain, serving as a chain termination substrate in hyaluronan and heparosan synthesis. The resulting 4-N_3GlcNAc-terminated polysaccharides were then successfully conjugated with Alexa Fluor 488 DIBO alkyne, demonstrating that this approach is generally applicable for labeling and detection of GAGs (Figure 6).

Compared to the chemical method to introduce azido groups into polysaccharides, this enzymatic technique provides a "green" approach to access regioselectively, azide-functionalized GAGs, which would bring better control of the final structure and composition of these macromolecules. These resulting unnatural GAG oligosaccharide analogs might be resistant to catabolism by blocking the action of heparanase and lysosomal degradative enzymes, increasing the biological half-life of the oligosaccharides.

Heavy Heparin

A stable isotope label (SIL) is one of the most promising ways to investigate the roles of biomacromolecules in metabolic pathways to track their utilization as these stable isotope-enriched analogs of biomolecules are easily distinguished from their endogenously biosynthesized counterparts when analyzed by mass spectrometry. These biomacromolecules are labeled with stable isotopes such as carbon (^{13}C), nitrogen (^{15}N), deuterium (^{2}H), or sulfur ($^{33/34}$S) and deuterium-labeled analogs reportedly exhibit improved pharmacological properties, such as increased half-lives, and have even been developed as FDA-approved pharmaceuticals.[50] The first SIL version of heparin having ^{13}C and ^{15}N labeling was chemoenzymatically synthesized in minute quantities over a decade ago in our lab.[51] We[52] have recently successfully synthesized perdeuteroheparin (heavy heparin), in sufficient amounts for in vivo studies, from biosynthetically enriched heparosan precursor obtained from microbial culture in deuterated medium (Figure 7). Subsequent chemical de-N-acetylation using aqueous NaOH and N-sulfonated with $(CH_3)_3N{\cdot}SO_3$, enzymatic epimerization and sulfation with recombinant heparin biosynthetic enzymes,

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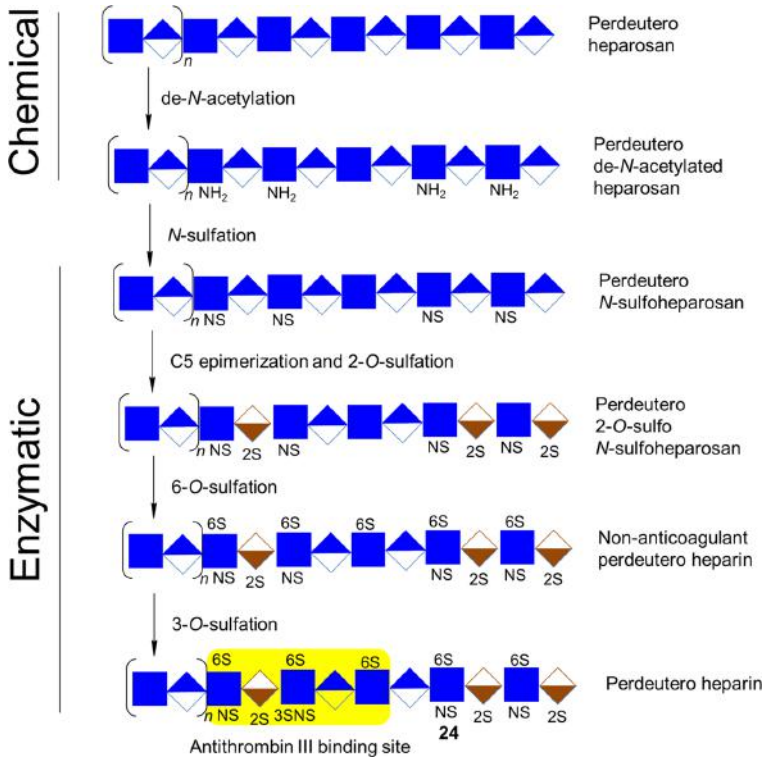

Figure 7. Biosynthetic isotopic enrichment of chemoenzymatically prepared perdeuteroheparin.[52] A single representative chain of one length and one sequence is shown for illustrative purposes. Symbols are defined in Figures 1 and 2.

afforded perdeuteroheparin **24** comparable to pharmaceutical heparin. A series of applications for heavy heparin and its heavy biosynthetic intermediates were demonstrated, including generation of stable isotope labeled disaccharide standards, development of a nonradioactive NMR assay for glucuronosyl-C5-epimerase, and background-free quantification of in vivo half-life following administration to rabbits. We anticipate that this approach can be extended to produce other isotope-enriched GAGs.

Smith and Alkaline Degradation

Current chemoenzymatic synthetic strategies have demonstrated that they are capable of generating a series of GAG oligosaccharides in good overall yield. One limitation of this synthetic approach is that an unnatural acceptor, *p*-nitrophenyl glucuronide (GlcA-*p*NP) or its derivatives has been largely indispensible, so the resulting target molecules contain an unnatural structural feature at the reducing end of the carbohydrate chain (Figure 8A). The selective removal of the GlcA-*p*NP moiety from highly sulfated oligosaccharides containing a uniformly ^{13}C-labeled internal 2-sulfoiduronic units is possible using either Smith or alkaline elimination (Figure 8B).[53,54] A selective sodium periodate oxidative-cleavage of vicinal diol of glucuronic acid residues from the both ends of the carbohydrate chain, followed by either acid or base treatment, afforded the target natural-like heparin oligosaccharides. This was the first time that these two degradation approaches have been applied to the preparative synthesis of an oligosaccharide as they are usually used for the micro scale analysis of polysaccharides relying on mass spectrometry (MS). More importantly, no desulfation was detected during the synthesis. This is a good complement to the current chemoenzymatic approach, which could be able to access not only natural-like heparin but also other GAGs. This resulting ^{13}C-labeled compound was subsequently intravenously administered to septic and nonseptic mice, and results of this study suggested that circulating heparan sulfates are rapidly cleared from the plasma during sepsis and selectively penetrate the hippocampus, where they may have functional consequences.

Solid Phase Synthesis

Oligosaccharide synthesis is considered to be much more complicated than peptide synthesis mainly due to the requirement of multistep transformations involving repeated protection−glycosylation−deprotection reaction sequences and chromatographic purification of intermediates at each stage of the synthesis. Such preparations would greatly benefit from developments in polymer-supported and chemoenzymatic oligosaccharide synthetic strategies, which could greatly simplify purification and afford exquisite stereoselectivity and regioselectivity. The enzymatic solid-phase synthesis of oligosaccharides involves two basic strategies, with either the saccharide or the enzymes bound on the solid phase. Although promising, to date there are few examples of solid-phase chemoenzymatic synthesis of GAGs was reported. HS immobilized onto the surface of the dextran modified gold channel microfluidic surface plasmon resonance sensor chip and on streptavidin-modified magnetic nanoparticles in a digital microfluidic device, called and artificial Golgi, has served as a substrate for subsequent 3-OST-1 modification.[55] Moreover, the multiple enzymes involved in heparin synthesis, including C5-epi, 2-OST, and

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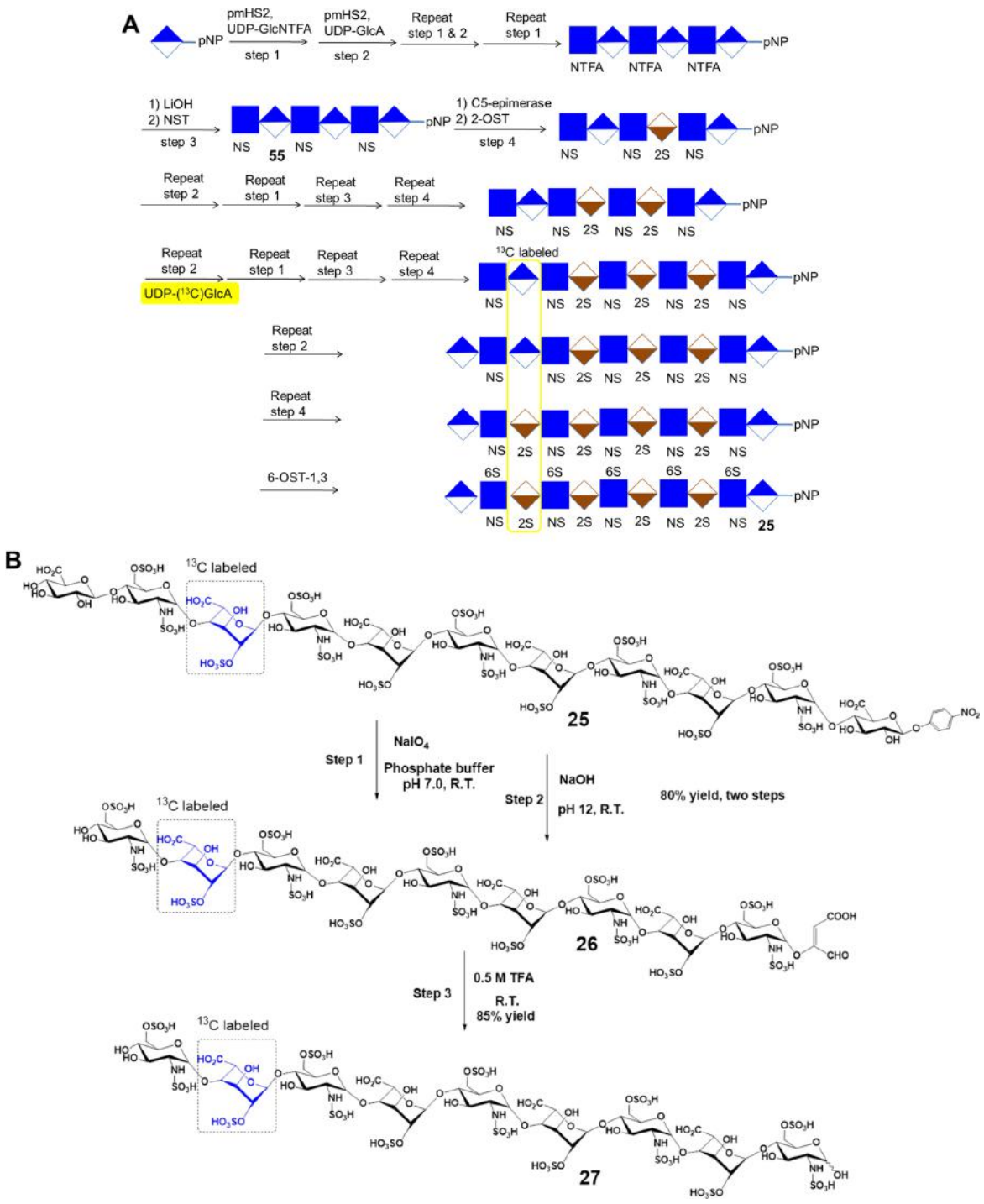

Figure 8. (A) Symbolic structures to show the chemoenzymatic synthesis of the key intermediate undecasaccharide **25**. The (^{13}C) IdoA2S residue is highlighted in yellow. (B) Chemical structures to show the cleavage of undecasaccharide **25** at its flanking GlcA residues and the formation of nonasaccharide **27** composed of Tri-S disaccharide repeating units containing an internal (^{13}C) IdoA2S residue.[53] Symbols are defined in Figures 1 and 2.

arylsulfotransferase-IV, have been successfully immobilized.[56] Immobilized enzymes retain catalytic activity allowing repeated and continuous use, thus providing for the facile separation of enzyme from products and resulting in a remarkable reduction of processing costs. Although solid phase chemoenzymatic synthesis is conceptually possible, it is much less well developed

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than the chemical approach. More complex target synthesis using a multistep, solid-phase, chemoenzymatic approach is needed in the future.

HISTORICAL AND FUTURE PERSPECTIVES

GAGs display a range of physiological and pharmacological functions, involving cell−cell interactions, cell proliferation, enzyme inhibition, and growth factor receptors during various metabolic processes. The chemoenzymatic approach is an efficient approach to prepare structurally defined GAG oligosaccharides that have been difficult to prepare by traditional chemical synthesis approaches, and it is promising for development as the next generation of safer, reliable, and affordable synthetic heparin-based drugs.[57] Over the past 5 years, there has been a steady improvement in the chemoenzymatic synthesis of GAG polysaccharides, including higher yields, greater purities, easier purification, larger scale and more structurally complex targets, and a better understanding of the substrate specificities of the GAG biosynthetic enzymes. The ready availability of recombinant enzymes, an improved understanding of their specificity, the improved analytical methods, and a strategic perspective developed from the past work by protein and nucleic acid chemists are largely responsible for the success of chemoenzymatic GAG synthesis. Remarkable advances in the metabolic engineering of both eukaryotic[58] and prokaryotic[59] cells suggests that one day even GAGs might even be prepared through fermentation. However, even when this day arrives, there will be a place for chemoenzymatic synthesis of GAGs having unique and unnatural structures for specialized biological and pharmacological applications. Although there are many challenges remaining in this young field, such as it is far from enough to scale up to kilograms that fulfill the commercial market, the promises of this technology are great, offering new, safer, and improved drugs for clinicians.

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Notes

The authors declare no competing financial interest.

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Lei Lin received a B.Sc. from Beijing Normal University and Ph.D. from Tsinghua University under the supervision of Prof. Jinghong Li. She moved to a postdoctoral research project on glycosaminoglycans analysis and bioengineered heparin synthesis at Rensselaer Polytechnic Institute under the supervision of Prof. Robert J. Linhardt in 2015. Recently, she was appointed as an associate professor in Nanjing Normal University (China), focusing on analysis of environmental pollutants.

He Huang is currently the dean of School of Food Science and Pharmaceutical Engineering in Nanjing Normal University (China). He received his Ph.D. in biochemical engineering from Purdue University in 2002. His research focuses on industrial microbial technology development and has authored over 200 research articles and holds over 100 patents. He has received multiple honors, including Scholar of the Yangtse River, National Outstanding Young Investigator Award, and National Technology Invention Awards.

Robert J. Linhardt received his Ph.D. in chemistry from Johns Hopkins University in 1979 and did his postdoctoral studies at Massachusetts Institute of Technology. He is currently the Anne and John Broadbent, Jr. '59 Senior Constellation Chair in Biocatalysis and Metabolic Engineering at Rensselaer Polytechnic Institute. His research focuses on glycoscience, and he is an expert on glycosaminoglycans and their synthesis, biology, and analysis. He has received multiple honors, including the National Academy of Inventors (NAI) Fellow, American Chemical Society Horace S. Isbell, Claude S. Hudson, and Melville L. Wolfrom Awards, the AACP Volwiler Research Achievement Award, the Society of Glycobiology Karl Meyer Award, and the Scientific American 10.

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