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Chemoenzymatic synthesis of heparan sulfate and heparin

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Heparan sulfate is a polysaccharide that plays essential physiological functions in the animal kingdom. Heparin, a highly sulfated form of heparan sulfate, is a widely prescribed anticoagulant drug worldwide. The heparan sulfate and heparin isolated from natural sources are highly heterogeneous mixtures differing in their polysaccharide chain lengths and sulfation patterns. The access to structurally defined heparan sulfate and heparin is critical to probe the contribution of specific sulfated saccharide structures to the biological functions as well as for the development of the next generation of heparin-based anticoagulant drugs. The synthesis of heparan sulfate and heparin, using a purely chemical approach, has proven extremely difficult, especially for targets larger than octasaccharides having a high degree of site-specific sulfation. A new chemoenzymatic method has emerged as an effective alternative approach. This method uses recombinant heparan sulfate biosynthetic enzymes combined with unnatural uridine diphosphate-monosaccharide donors. Recent examples demonstrate the successful synthesis of ultra-low molecular weight heparin, low-molecular weight heparin and bioengineered heparin with unprecedented efficiency. The new method provides an opportunity to develop improved heparin-based therapeutics.

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1 Introduction

Heparan sulfate (HS) is a polysaccharide-based natural product, widely expressed on mammalian cell surfaces and in the extracellular matrix. A large body of evidence demonstrates that HS plays essential roles in a number of biological processes, including the embryonic development, inflammatory responses, bacterial/viral infection and blood coagulation.[1] Its wide range of biological functions has attracted considerable interest in the development of new medicines using HS as a structural scaffold. Heparin, a specialized, highly sulfated form of HS, is a widely used anticoagulant, used to prevent and treat arterial and venous thrombosis.[2,3] HS consists of a disaccharide repeating unit of either iduronic acid (IdoA) or glucuronic acid (GlcA), and glucosamine (GlcN) residues, each capable of carrying sulfate groups (Fig. 1). The presence of sulfate groups and the location of the IdoA and GlcA residues dictate the functional selectivity of HS. The challenge in developing HS-based drugs centers on the synthesis of HS oligosaccharides and polysaccharides having desired sizes and sulfation patterns. HS oligosaccharides have been synthesized using a purely chemical approach; however, the synthesis is difficult, due to the required use of complex protecting/deprotecting procedures. The syntheses of diversified HS structures are further complicated, because they require the preparation of a large number of precursor compounds. Therefore, the HS oligosaccharide synthesis can be completed by skilled synthetic chemists in a small number of highly specialized labs.[4–13] In recent years, a chemoenzymatic synthesis has emerged using glycosyltransferases, epimerases and sulfotransferases.[14,15] Compared to the chemical synthesis, the chemoenzymatic approach offers shorter synthetic routes, excellent recovery yields, and uses a few common precursors for the preparation of diverse HS oligosaccharide structures. This article reviews the recent development of the chemoenzymatic synthesis method and its progress towards the synthesis of HS oligosaccharide targets having diverse sulfation patterns.

2 Heparin

Discovered in 1916, heparin has been the drug of choice to treat thrombotic disorders for nearly 100 years.[16] The discovery of heparin contributed significantly to the development of many advanced medical and surgical procedures.[17] Three forms of heparin are approved by the US Food and Drug Administration (FDA): unfractionated heparin (UFH, average molecular weight (MW_{avg}) 16 000 Da), low-molecular weight heparin (LMWH,

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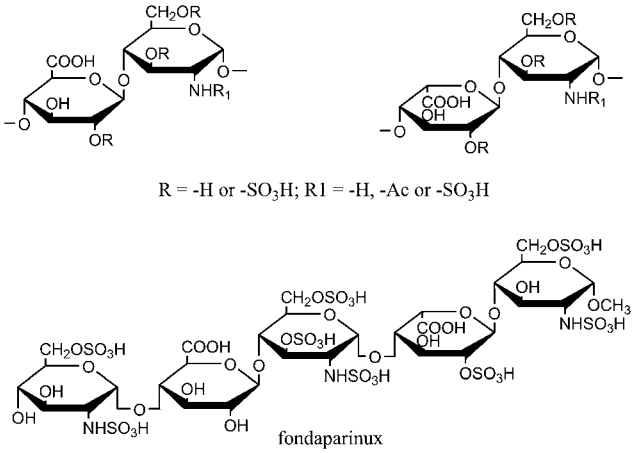

Fig. 1 Structures of the disaccharide repeating units of HS and the structure of fondaparinux.

MW_{avg} 3500–6000 Da) and fondaparinux (MW 1 508 Da). UFH is a safe *intravenous* drug for the treatment of renal-impaired patients and its effects can be reversed using the cationic polypeptide drug protamine;[18] however, it shows a 1–6% incidence of heparin-induced thrombocytopenia (HIT), a life-threatening complication.[19] LMWH is *subcutaneously* administered and has a longer half-life than UFH, permitting its outpatient use and self-administration.[16] However, LMWH can only be used in renal-impaired patients at reduced doses[20] and is incompletely neutralized with protamine, thereby increasing the risk of bleeding. Fondaparinux, a synthetic pentasaccharide, is *subcutaneously* bioavailable and has reduced risks of HIT and osteoporosis.[21] However, it is primarily excreted through the kidneys and, thus, is not suitable for renal-impaired patients, and it lacks an antidote.[20]

Both UFH and LMWH are considered to be animal-sourced products.[22] UFH is isolated from porcine intestines or bovine lungs or intestines, while LMWH is a depolymerized product of heparin from porcine intestines. A worldwide contamination of heparin in 2007 affected the purity and safety of both UFH and LMWH associated with over 200 deaths in the US.[23,24] This incident revealed the fragility of the heparin supply chain. Although fondaparinux is fully synthetic,[11] and its supply does not depend on animal sources, the drug does not display all the clinical benefits of UFH and LMWH. A cost-effective method to prepare synthetic heparins and LMWH that can be manufactured under the FDA's guidelines will eliminate the need for animal-sourced materials.[24] Furthermore, the synthetic heparins could be engineered to introduce additional beneficial pharmacological properties that do not exist in UFH and LMWH from natural sources.[16]

Heparin and HS have similar disaccharide repeating units; however, heparin has higher sulfation levels than HS, containing 2.6 sulfo groups per disaccharide compared to 0.6 sulfo groups in HS. In addition, nearly 90% of the disaccharide units in heparin contain IdoA, while only 20% of the disaccharide units in HS contain IdoA. HS can be isolated from many cell types, whereas heparin is an exclusive product of mast cells (Fig. 1).

3 Enzymes involved in the chemoenzymatic synthesis

The chemoenzymatic synthesis of HS requires several classes of biosynthetic enzymes, some comprised of multiple isoforms.[25] Glycosyltransferases are used to build the HS backbone structure consisting of a disaccharide repeating unit of GlcA and *N*-acetylglucosamine (GlcNAc) (Fig. 2).[26] An epimerase (C_5-epi) is responsible for converting a GlcA residue to an IdoA residue.[27] Sulfo groups are transferred from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the oligosaccharides using specialized HS sulfotransferases. For example, 2-*O*-sulfotransferase (2-OST) specifically transfers a sulfo group to the

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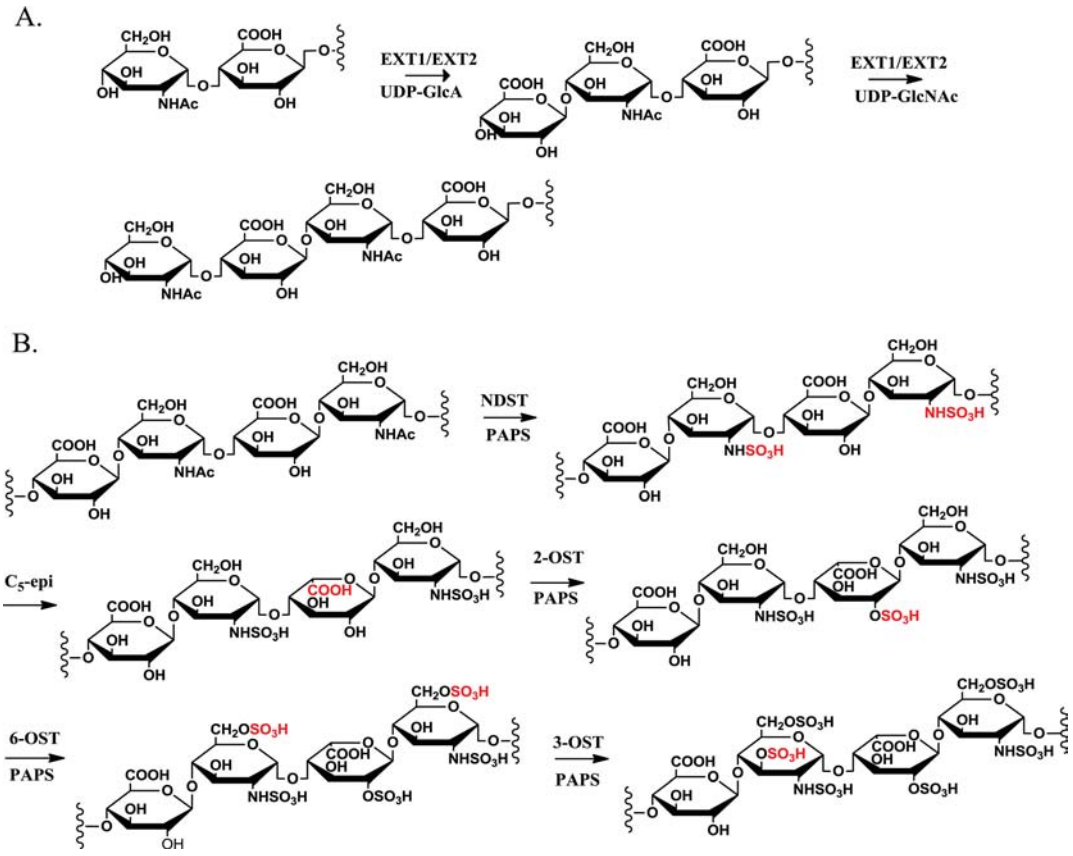

Fig. 2 Enzymes involved in the biosynthesis of HS. Panel A shows the elongation reactions to prepare the nonsulfated and unepimerized saccharide backbone. Both EXT1 and EXT2 proteins are involved in the elongation reactions. Panel B shows the reactions involved in the polysaccharide modifications. The sites of reaction are highlighted in red. Abbreviations: EXT1: exostosin glycosyltransferase 1, EXT2: exostosin glycosyltransferase 2, NDST: *N*-deacetylase/*N*-sulfotransferase, C_5-epi: C_5-epimerase, 2-OST: 2-*O*-sulfotransferase, 6-OST: 6-*O*-sulfotransferase, 3-OST: 3-*O*-sulfotransferase, PAPS: 3′-phosphoadenosine 5′-phosphosulfate.

2-position of an IdoA residue and, to a lesser extent, to the 2-position of a GlcA residue.[28,29] The 6-*O*-sulfotransferase (6-OST) and the 3-*O*-sulfotransferase (3-OST) transfer sulfo groups to the 6- and 3-positions of the glucosamine residues, respectively.[29,30] The *N*-deacetylase/*N*-sulfotransferase (NDST) converts a GlcNAc residue to a *N*-sulfoglucosamine (GlcNS) residue.[31] NDST is unique displaying two activities, the *N*-deacetylase activity removing the *N*-acetyl group from a GlcNAc residue, and the *N*-sulfotransferase activity transferring a sulfo group to a GlcN residue, forming a GlcNS residue.[32,33]

The enzymes used for the chemoenzymatic synthesis of heparin are predominantly mammalian proteins, presenting challenges for making these recombinant proteins accessible in large quantities. Currently, most of these HS biosynthetic enzymes, with the exception of NDST, EXT-1 and EXT-2, have been expressed in *E. coli* with sufficient efficiency for a high milligram-scale synthesis.[34] Only the *N*-sulfotransferase (NST) domain of NDST can be effectively expressed in *E. coli* and, thus, this protein lacks the *N*-deacetylase activity. Two bacterial glycosyltransferases, known as KfiA (an *N*-acetylglucosaminyltransferase from *E. coli* K5 strain) and pmHS2 (heparosan synthase 2 from *Pasteurella multicida*), are used as substitutes for EXT1 and EXT2 to build the HS backbone. Both enzymes, KfiA and pmHS2, can be readily expressed in *E. coli*.[35,36]

4 Design of sugar nucleotides for the chemoenzymatic synthesis of HS

The chemoenzymatic synthesis of HS requires a cascade of enzymatic modifications. The *N*-sulfation of glucosamine is essential for the down-stream enzymatic modifications to occur.[25] The synthesis of the HS backbone containing GlcNS residues represented a major roadblock due to the lack of efficient recombinant NDST. An unnatural sugar nucleotide,[37,38] UDP-GlcNTFA (uridine diphosphate *N*-trifluoroacetylglucosamine), was introduced to overcome this problem. UDP-GlcNTFA was first reportedly used in the synthesis of carbohydrates of an *O*-linked glycoprotein using a "core-2" GlcNAc transferase with modest yield (56%).[39] For the HS backbone synthesis, UDP-GlcNTFA appears to be an excellent substrate for KfiA with a nearly quantitative conversion in the preparation of the heparin oligosaccharide backbone.[40] The GlcNTFA residue is then converted to a GlcNS residue by removing the trifluoroacetyl group under mild alkaline conditions followed by the *N*-sulfotransferase modification (Fig. 3). The use of UDP-GlcNTFA provides a means of introducing a GlcNS residue at precisely the desired position within an oligosaccharide. This strategy is now used to synthesize structurally

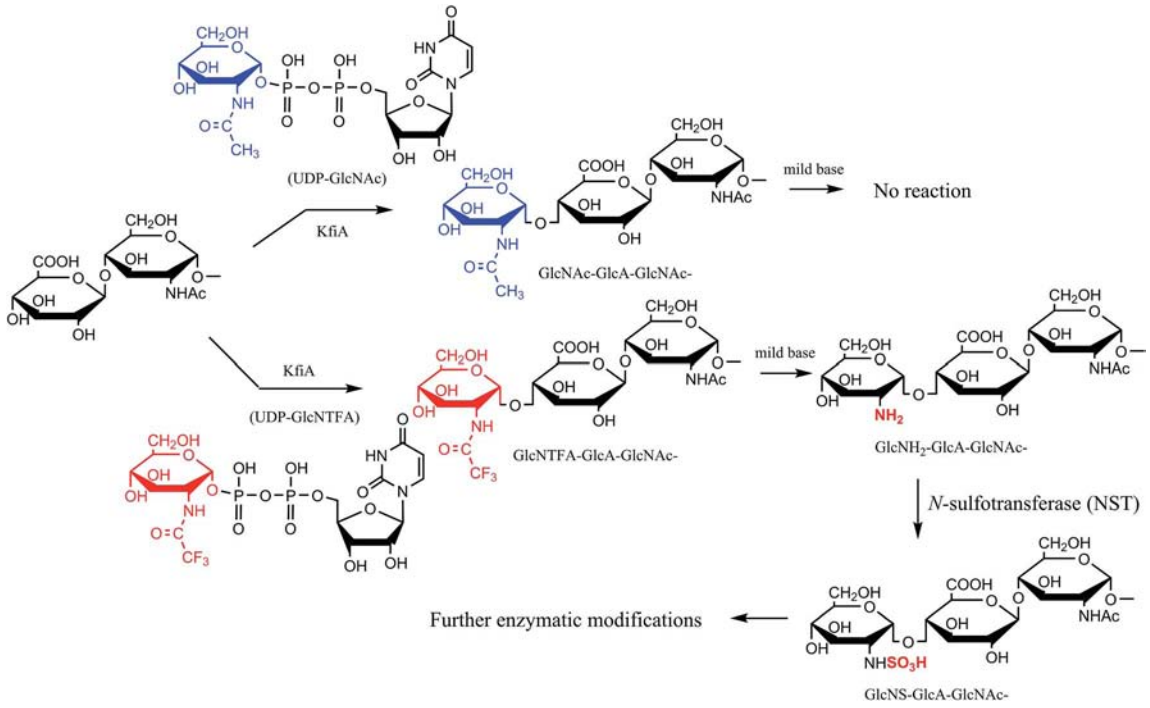

Fig. 3 Building the HS backbone containing GlcNS residues using a non-natural sugar nucleotide. KfiA transfers a GlcNAc residue to the primer substrate, where the sugar nucleotide donor is UDP-GlcNAc, a natural substrate for KfiA. KfiA also transfers a GlcNTFA residue to the primer substrate, where the sugar nucleotide donor is UDP-GlcNTFA, an unnatural sugar nucleotide. The trifluoroacetyl group can be removed under mild basic conditions to form an *N*-unsubstituted glucosamine (GlcN) residue. The GlcN residue is then converted to GlcNS using the *N*-sulfotransferase (NST). The removal of the trifluoroacetyl group can be achieved using triethylamine and methanol at room temperature, or with 0.1 M LiOH at 4 °C. The synthesis of UDP-GlcNTFA is completed in a two-step synthesis using *N*-acetylhexosamine 1-kinase and glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU).

homogeneous ultra-low molecular weight heparins, low-molecular weight heparins[14,15] and heterogeneous heparin-like polysaccharides.[41,42]

A novel strategy to use a series of unnatural sugar nucleotides to synthesize a heparin-like oligosaccharide was recently proposed by Chen and coworkers.[43] Here, the 6-hydroxyl group of GlcN was substituted by an azido group to synthesize sugar nucleotides, including UDP-[6-azido]GlcNAc or UDP-[6-azido]-GlcNTFA. Chen and coworkers demonstrated the synthesis of oligosaccharides, which carry azido groups at the 6-position of the GlcN residues, in excellent yields using the pmHS2 enzyme. The azido group was then converted to an amino group followed by *N*-sulfation to generate 6-*N*-sulfo GlcN residues. Ultimately, HS-analog oligosaccharides, containing *N*-sulfate groups at both the 2- and the 6-positions of GlcN, were synthesized, mimicking the 2-*N*-sulfo-6-*O*-sulfo GlcN residues in natural HS and heparins. As this synthesis only involves the use of a glycosyltransferase and the chemoselective *N*-sulfation to produce sulfated saccharides, this approach eliminates the potential complexity of introducing a sulfate group at a desired site by relying on the substrate specificity of the *O*-sulfotransferases. Because of the unnatural 6-*N*-sulfation, these products might be resistant to heparanase and lysosomal degradation, increasing the biological half-life of these oligosaccharides.

5 Design of the sequence of enzymatic modifications

The chemoenzymatic synthesis of heparin requires a series of enzymatic modification steps. The design of an appropriate modification sequence is closely related to the substrate specificities of the biosynthetic enzymes. *In vivo*, the HS biosynthetic pathway is responsible for installing multiple layers of sulfation on the polysaccharide backbone by following a specific order. The cells probably use this modification sequence to control the HS polysaccharide structure. The HS biosynthetic enzymes have evolved to only react with selected substrates having unique sulfation patterns, while other saccharide substrates, lacking these sulfation patterns, remain unmodified. Such a feature assures that the biosynthesis of HS follows a preprogrammed modification sequence.[44] For example, the 2-*O*-sulfation step, catalyzed by 2-OST, occurs only after the *N*-sulfation step, but prior to the 6-*O*-sulfation step. The ternary crystal structure of 2-OST reveals that three amino acid residues, Arg-80, Lys-350 and Arg-190, from 2-OST directly interact with the *N*-sulfo group of the GlcNS residue flanked on both ends by IdoA residues. The mutation of any of those three amino acid residues, that interact with the *N*-sulfo group, resulted in the loss of the sulfotransferase activity. Likewise, substituting the *N*-sulfo group with an *N*-acetyl group or hydrogen on the GlcN residue of the

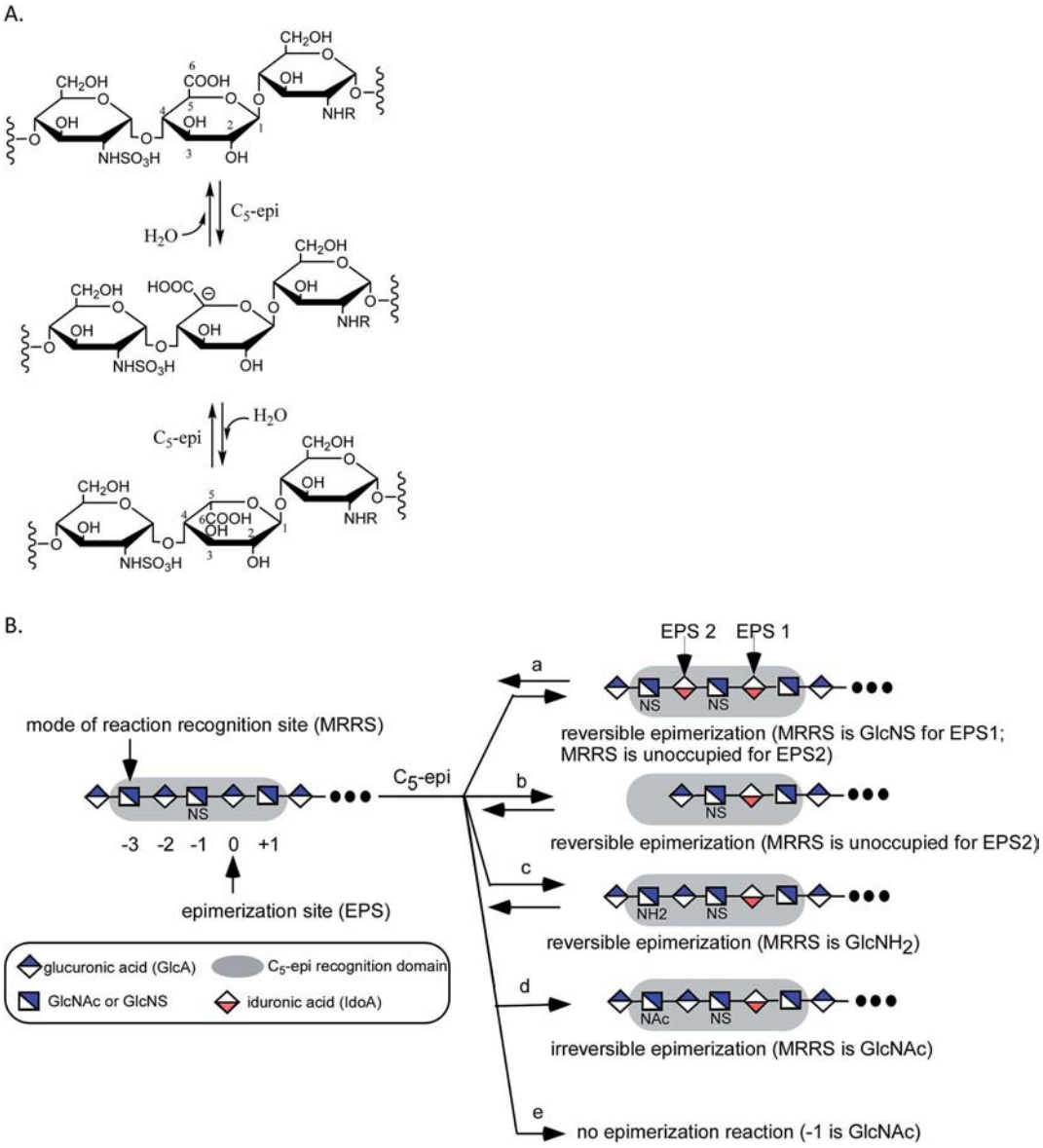

Fig. 4 The reaction catalyzed by C_5-epi and its substrate specificity. (A) shows the reaction catalyzed by C_5-epi. A trisaccharide segment is shown. C_5-epi removes the proton from C_5 of the GlcA residue to form a putative carbanion intermediate. Conversely, C_5-epi can catalyze the reverse reaction, converting an IdoA residue to a GlcA residue. (B) shows the substrate specificity of C_5-epi. The designated epimerization site (EPS) is at residue 0. Residue −1 must be a GlcNS residue to serve as an EPS. If residue −1 is a GlcNAc residue, the site is not reactive to C_5-epi (see reaction e). The *N*-substitution status at the mode of reaction recognition site (MRRS) determines the mode of reaction of C_5-epi. If the MRRS (residue −3) is a GlcNS or GlcN residue or unoccupied, C_5-epi displays a reversible reaction mode (see reactions a, c and b). If a GlcNAc residue is at the MRRS, C_5-epi displays an irreversible reaction mode (see reaction d). The grey shaded box indicates the pentasaccharide domain recognized by C_5-epi.

substrate abolishes its reactivity towards 2-OST modification.[44] In contrast, two amino acid residues, Tyr-173 and Pro-82, near that active site of 2-OST, are probably used by the enzyme to exclude residues with a 6-*O*-sulfo group, suggesting that the 6-*O*-sulfation occurs after the 2-*O*-sulfation in the process of biosynthesizing highly sulfated domains consisting of -IdoA2S-GlcNS6S-, which are commonly found in heparin. The structural and biochemical evidence clearly demonstrates the essential roles of the *N*-sulfation and the 6-*O*-sulfation in regulating the 2-*O*-sulfation in the HS biosynthetic pathway.

6 Synthesis of oligosaccharides with repeating -IdoA2S-GlcNS- units

The synthesis of a domain with repeating -IdoA2S-GlcNS- disaccharide units is critically important for preparing heparin and the high sulfation domains of HS. In heparin, more than 80% of the disaccharides are composed of -IdoA2S-GlcNS6S-.[3] Attempts to synthesize a domain with more than one -IdoA2S-GlcNS- motif results in a complex mixture.[45] Complex product mixtures are acceptable when preparing bioengineered

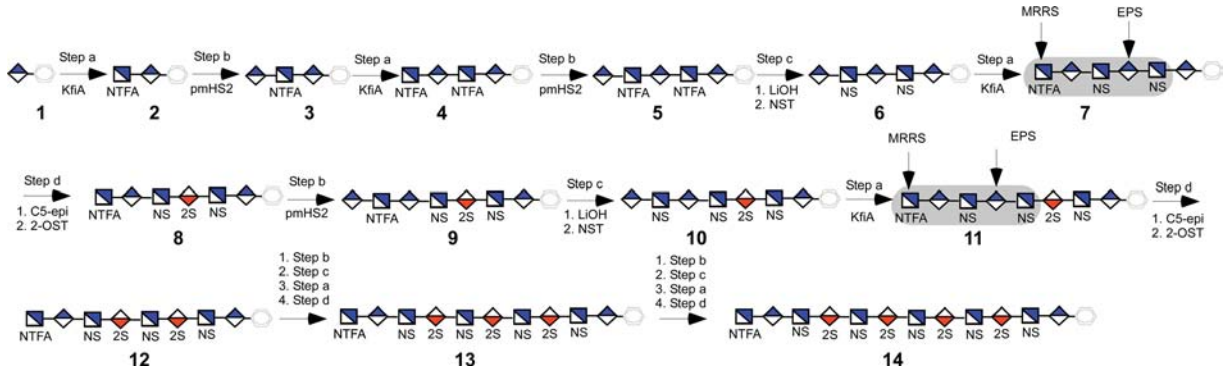

Fig. 5 Route to synthesize oligosaccharides with -IdoA2S-GlcNS- repeats. The key idea in this strategy is to construct a C_5-epi recognition pentasaccharide module utilizing the irreversible reaction. The C_5-epi recognition pentasaccharide module is highlighted in a grey box. The synthesis is initiated from GlcA-pnp (**1**, *p*-nitrophenyl glucuronide). The monosaccharide is converted to a pentasaccharide **5** in four enzymatic steps. Hexasaccharide **7** contains the C_5-epi recognition pentasaccharide module. The synthesis of **8** from **7** is achieved *via* two enzymatic steps (step d) using both C5-epi and 2-OST. Hexasaccharide **8** contains a single -IdoA2S-GlcNS- disaccharide unit. The construction of another C_5-epi recognition pentasaccharide module involves four enzymatic steps, b, c, a and d (transformation of **8**–**12**). Repeating those steps can deliver **13** and **14**.

heparin[46] as a generic replacement for animal-sourced UFHs, since these products are polydisperse and microheterogenous.[3] The challenge comes in synthesizing structurally defined LMWH or ULMWH, containing repeating -IdoA2S-GlcNS6S- motifs. The difficulty in installing repeating -IdoA2S-GlcNS6S units, stems from a unique property of C_5-epi. Unlike other HS biosynthetic enzymes, C_5-epi is a two-way catalyst: it transforms a GlcA to an IdoA residue, and also converts an IdoA to a GlcA residue (Fig. 4A).[47] Therefore, the final product contains a mixture of GlcA and IdoA after C_5-epi modification. A recent study revealed that C_5-epi is a one-way catalyst towards substrates having a unique distribution of GlcNAc and GlcNS residues.[48] The results indicate that C_5-epi can display both, two-way and one-way catalytic modes, by recognizing a sequence motif designated as the C_5-epi recognition pentasaccharide domain (Fig. 4B).[48] This pentasaccharide domain

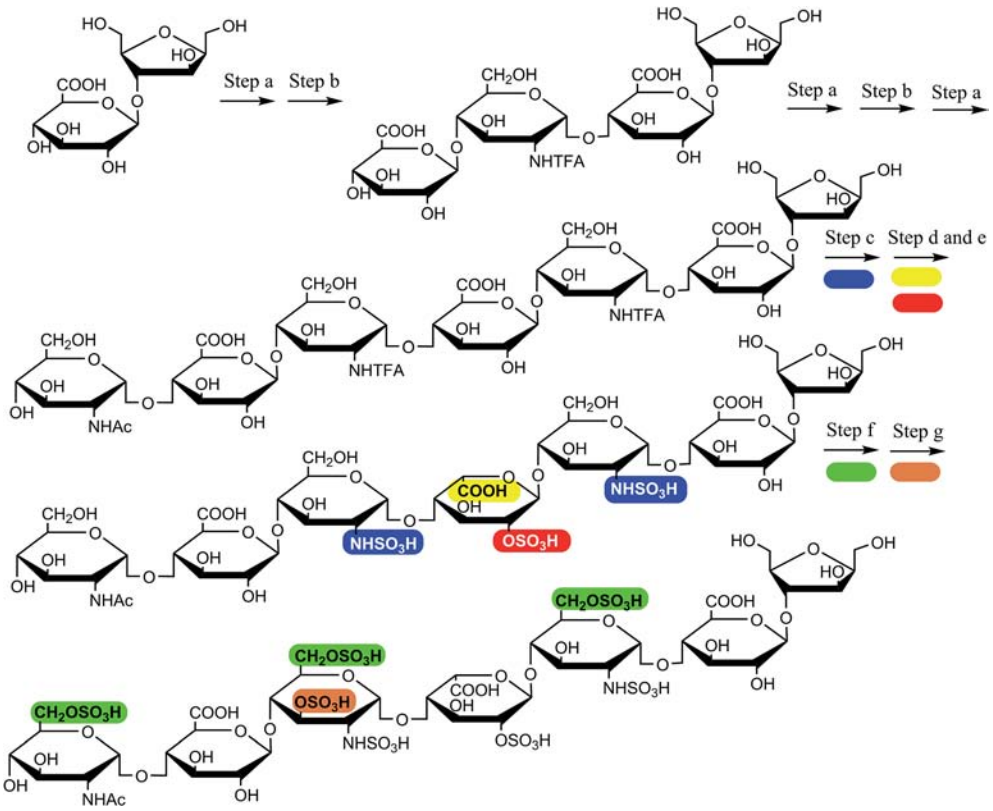

Fig. 6 Route for the chemoenzymatic synthesis of an ultra-low molecular weight heparin.

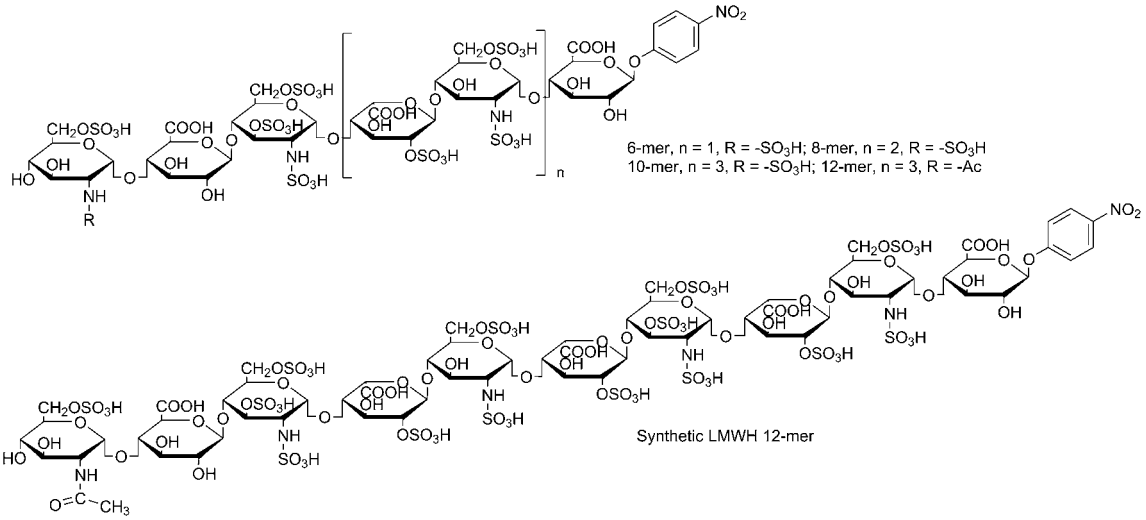

Fig. 7 Structure of a synthetic low-molecular weight heparin. The synthetic LMWH 12-mer has two 3-*O*-sulfo groups.

consists of an epimerization site (EPS) and a mode of reaction recognition site (MRRS). The MRRS is located three residues away from the non-reducing end side of the EPS (Fig. 4B). The *N*-substitution status of the MRRS residue, controlled in the biosynthesis by NDST,[49] dictates the mode of action of C_5-epi. A GlcNAc residue directs C_5-epi to display an irreversible reaction mode, while a GlcNS or GlcN residue (or the MRRS site being unoccupied) drives C_5-epi to display a reversible reaction mode. The presence of GlcNS at the −1 residue is essential for displaying the oligosaccharide's susceptibility to C_5-epi modification.

An improved knowledge of the substrate specificity of C_5-epi offers an alternative strategy for synthesizing -IdoA2S-GlcNS- repeating units avoiding the formation of by-products (Fig. 5). Seven steps (Fig. 5, steps a, b, a, b, c, a, and d, transformation of **1–8**) are required to synthesize the first IdoA2S residue from a

Table 1 Summary of synthesized HS oligosaccharides using the chemoenzymatic approach

Compounds	Structure	Comments	Reference
16–19	GlcA-GlcNR$_1$-GlcA-GlcNR$_2$-GlcA-GlcNAc-GlcA-AnMan (**16:** $R_1 = R_2 =$ Ac, **17:** $R_1 = SO_3H$, $R_2 =$ Ac, **18:** $R_1 =$ Ac, $R_2 = SO_3H$, **19:** $R_1 = R_2 = SO_3H$)	Demonstration of the control of size and *N*-sulfation	40
20 and **21**	GlcA-GlcNS6S-(GlcA-GlcNS6S)$_n$-GlcA-AnMan (**20:** $n = 3$, **21:** $n = 4$)	Demonstration of the control of complete 6-*O*-sulfation	40
22 and **23**	GlcNR′6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-AnMan (**22:** R′ = SO_3H, **23:** R′ = Ac)	Demonstration of the synthesis of a complete heparin fragment	14
24	GlcNAc6S-GlcA-GlcNS6S-IdoA2S-GlcNS6S-GlcA-AnMan	Demonstration of the application in a 3-OST-1 crystal structure study	53
25 and **26**	GlcA-GlcNAc-GlcA-GlcNR′6S-GlcA-AnMan (**25:** R′ = SO_3H, **26:** R′ = Ac)	Demonstration of the control of partial 6-*O*-sulfation	56
27–30	GlcNS-(GlcA-GlcNS)$_m$-(GlcA-GlcNAc)$_2$-(GlcA-GlcNS)$_2$-GlcA-AnMan (**27:** $m = 2$, **28:** $m = 3$, **29:** $m = 4$, **30:** $m = 5$)	Demonstration of the synthesis of a large oligosaccharide up to the heneicosaccharide	45
31–36	GlcA-GlcNR$_1$6S-GlcA-GlcNR$_2$6S-GlcA-GlcNR$_3$6S-GlcA-GlcNR$_4$6S-GlcA-pNP (**31:** $R_1 = R_2 = R_3 = R_4 = SO_3H$, **32:** $R_1 =$ Ac, $R_2 = R_3 = R_4 = SO_3H$, **33:** $R_1 = R_2 =$ Ac, $R_3 = R_4 = SO_3H$, **34:** $R_1 = R_4 =$ Ac, $R_2 = R_3 = SO_3H$, **35:** $R_1 = R_2 = R_3 = SO_3H$, $R_4 =$ Ac, **36:** $R_1 = R_2 =$ Ac, $R_2 = R_2 = SO_3H$)	Demonstration of the synthesis of a small library of *N*-sulfated and 6-*O*-sulfated nonasaccharides	57
37–40	GlcNR-GlcA-GlcNS-(IdoA2S-GlcNS)$_m$-GlcA-pNP (**37:** R = –SO_3H, $m = 1$, **38:** R = –SO_3H, $m = 2$, **39:** R = –SO_3H, $m = 3$, **40:** R = –Ac, $m = 4$)	Synthesis of oligosaccharides with multiple -IdoA2S-GlcNS- repeats	15
41–44	GlcNR6S-GlcA-GlcNS3S6S-(IdoA2S-GlcNS6S)$_m$-GlcA-pNP (**41:** R = -SO_3H, $m = 1$, **42:** R = –SO_3H, $m = 2$, **43:** R = –SO_3H, $m = 3$, **44:** R = –Ac, $m = 4$)	Synthesis of low-molecular weight heparins	15
45	GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	Synthesis of low-molecular weight heparins	15
46–48	GlcNR$_1$6R-GlcA-GlcNS6R-GlcA2S-GlcNS6R-GlcA-pNP (**46:** R = R_1 = –H, **47:** R = –H, R_1 = –SO_3H, **48:** R = R_1 = -SO_3H)	Synthesis of GlcA2S-containing oligosaccharides	58

monosaccharide starting material. Each repetition of these steps adds an additional IdoA2S residue, transforming **8** to **12**, **13** and **14**, containing two, three and four -IdoA2S-GlcNS- repeats, respectively. The construction of a C_5-epi recognition pentasaccharide module with the structure of GlcNTFA-GlcA-GlcNS-GlcA-GlcNS- is the centerpiece of this strategy (Fig. 5, compound **7**). This pentasaccharide module allows C_5-epi to exhibit the irreversible reaction mode, leading to a single product. The resultant IdoA residue in the pentasaccharide domain is immediately 2-*O*-sulfated to form an IdoA2S residue (Fig. 5, compound **8**). The subsequent construction of the C_5-epi recognition pentasaccharide module involves three steps (Fig. 5, steps c, b and a) to introduce an additional -IdoA2S-GlcNS- motif. For example, compound **8** will be elongated to an octasaccharide with a structure of *GlcNTFA-GlcA-GlcNS-GlcA-GlcNS*-IdoA2S-GlcNS-GlcApnp (compound **11**), containing the C_5-epi recognition pentasaccharide domain (highlighted in italics). The modifications using C_5-epi and 2-OST (steps d and e) will result in an octasaccharide of GlcNTFA-GlcA-GlcNS-IdoA2S-GlcNS-IdoA2S-GlcNS-GlcApnp (compound **12**). The 2-*O*-sulfation, to form an IdoA2S residue, is essential because it prevents further action of C_5-epi on this residue and accelerates the C_5-epimerization reaction.[47] These steps can be further repeated to form a deca- (**13**) and dodecasaccharide (**14**) introducing three and four repeats of the disaccharide -IdoA2S-GlcNS-, respectively, as described in a recent publication.[15]

7 Using the chemoenzymatic method to design heparin drugs

Two specific examples for the application of the chemoenzymatic approach to prepare heparin oligosaccharides with an anticoagulant activity, have been recently demonstrated. In the first example, this method was used to synthesize a homogenous heptasaccharide (Fig. 6). The synthesis, initiated from a disaccharide (GlcA-AnMan, AnMan represents 2,5-anhydromannitol), was completed in nine enzymatic steps with 43% recovery yield.[14] The synthesis includes the elongation of the starting disaccharide to a heptasaccharide using KfiA and pmHS2. The heptasaccharide backbone is then converted to the final product using a series of reactions, including the conversion of the GlcNTFA residue to GlcNS (Fig. 6, step c), the epimerization and 2-*O*-sulfation (Fig. 6, step e and f), the 6-*O*-sulfation (Fig. 6, step g) and the 3-*O*-sulfation (Fig. 6, step h). The product has a very similar anticoagulant potency to that of fondaparinux as measured by *in vitro* and *ex vivo* experiments. The synthesis of fondaparinux using a purely chemical approach requires a much longer route to completion.[11,50]

The second example is the design of LMWH structures with desired pharmacological properties (Fig. 7). Desirable properties of LMWHs include: a homogeneous structure prepared from non-animal ingredients, safety for kidney-impaired patients, and a reversible anticoagulant activity. Toward these goals, five different oligosaccharides were synthesized.[15] Among these were a 6-mer ULMW heparin target compound, very similar to fondaparinux, as well as a 10-mer and 12-mer that were in the size range of LMWHs. Because of the size and structural complexity of LMWHs, a more efficient chemoenzymatic method was required. A commercially available aglycone, 1-*O*-(*para*-nitrophenyl) glucuronide (GlcA-pNP), was used as the starting material. The presence of the pNP motif provides a UV chromophore for monitoring the product during the synthesis and purification. Non-sulfated oligosaccharides, carrying this pNP motif, bind to conventional C_{18}-columns, allowing easy purification of the intermediates from the proteins and unreacted co-factors. Moreover, this pNP group can be removed at the end of the synthesis or reduced to afford an amino group[51] for attaching the resulting oligosaccharide to a support. The unique enzymatic modification sequence was applied to the GlcA-pNP to synthesize the different oligosaccharides, consisting of the repeating disaccharide unit -IdoA2S-GlcNS-, with high purity (Fig. 5). The final products were purified using anion exchange chromatography to obtain a purity of >98%. In a mouse model, oligosaccharides larger than the 10-mer were metabolized in the liver, suggesting that these synthetic LMWHs will be safe for the use in renal-impaired patients. Most interestingly, the anticoagulant activity of the synthetic LMWH 12-mer was reversed by protamine, an FDA approved drug to neutralize unfractionated heparin. The protamine reversibility of this 12-mer could potentially reduce the risk of bleeding in its clinical use.

8 Conclusions

Over the past five years, there has been steady improvement in the chemoenzymatic synthesis of HS and heparin oligosaccharides, focused on making the synthesis more flexible for the preparation of different targets with increased purity and increased synthetic scale. To date, more than 32 heparin oligosaccharides have been synthesized (Table 1). The scale of synthesis has increased from 10 μg, as reported in 2003,[52] to 1 g, a 100 000-fold increase.[15] This improvement in synthesis can be attributed to improved enzyme expressions, the higher efficiency in the synthesis of enzyme co-factors and an improved understanding of the substrate specificities of the HS biosynthetic enzymes. The availability of these newly synthesized compounds has improved the heparin and HS research. For example, synthetic heparin oligosaccharides, prepared using the chemoenzymatic synthesis, were used to solve the co-crystal structure of 3-OST-1/PAP/heptasaccharide and 2-OST/heptasaccharides/3′-phosphoadenosine 5′-phosphate.[44,53] These crystal structures helped us to understand how sulfotransferases distinguish between specific saccharide sequences to exhibit substrate specificity. The results from these studies will, in turn, further improve the synthesis of oligosaccharides using these enzymes.

The next challenge in the anticoagulant drug development is to synthesize homogeneous LMWHs that meet all of the current clinical needs. The chemoenzymatic synthesis of the LMWH 12-mer required 22 synthetic steps with an overall yield of about 10%. The chemoenzymatic synthesis is significantly shorter than the chemical synthesis of fondaparinux that requires 50 steps.[54] Fondaparinux is now synthesized on a kg-scale, and has

been a profitable drug for ten years.[55] Further development of the chemoenzymatic approach should result in cost-effective products, accelerating the modernization of LMWH drugs, and also result in defined HS oligosaccharides to promote the field of biomedical research.

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