CHAPTER 8

Chemoenzymatic Synthesis of Low-Molecular-Weight Heparin and Heparan Sulfate

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8.1 Introduction

8.1.1 What are Heparan Sulfate, Heparin and Heparin-Derivatives?

Heparan sulfate (HS) belongs to the family of linear, highly sulfated anionic polysaccharides known as glycosaminoglycans (GAGs). HS consists of repeating, 1 → 4-linked disaccharide units of alternating α-D-glucosamine with a β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) (Figure 8.1, structures **1** and **2**).[1] HS is widely found on the surfaces of animal cells and in the extracellular matrix and exhibits a wide range of biological functions controlling angiogenesis, inflammatory responses, lipid metabolism, tumor metastasis, and blood coagulation.[2]

Chemical Biology No. 2
Chemical Biology of Glycoproteins
Edited by Zhongping Tan and Lai-Xi Wang

Published by the Royal Society of Chemistry, www.rsc.org

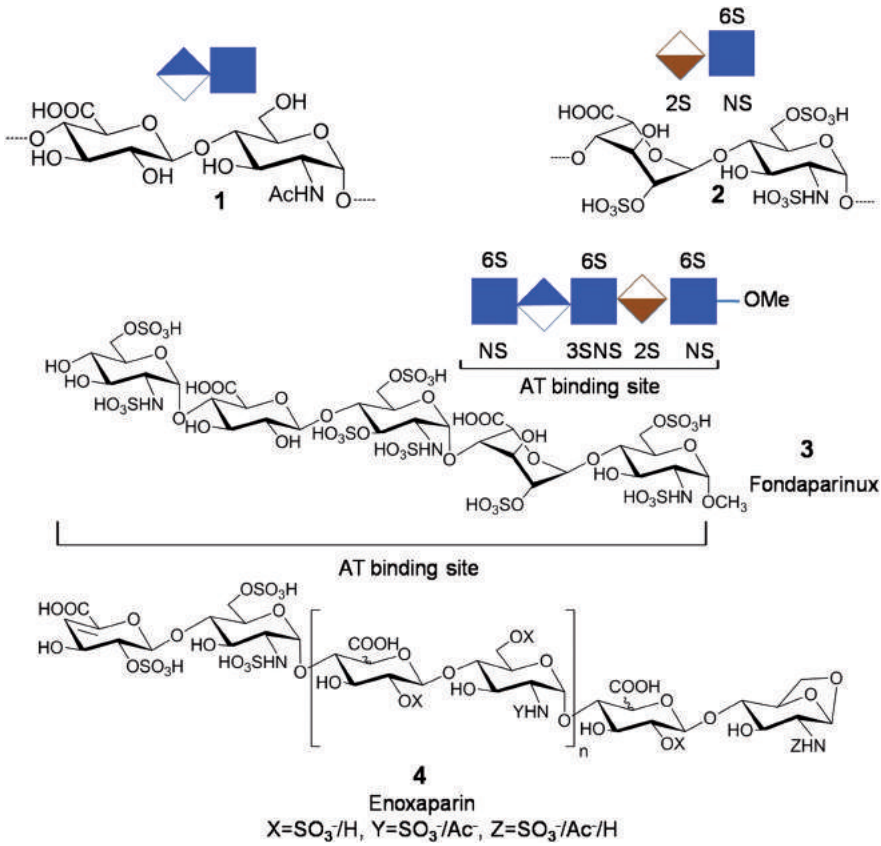

Figure 8.1 Structures of HS, heparin and heparin derivatives. The disaccharide repeating units of HS (primarily **1**) and heparin (primarily **2**), the structure of Arixtra® (fondaparinux) **3**, a ULMWH containing the AT-binding site, and a representative chain of the LMWH, Lovenox® (enoxaparin) **4**, are shown. The chemical structures of the saccharide residues as well as their symbolic representations are shown ref. 1,12.

Heparin is a special form of HS, found within specialized granulated cells, which has a higher level of sulfation, a higher IdoA content, and displays strong anticoagulant activity.[1] There are an average of approximately 2.6 sulfo groups per disaccharide unit in heparin, compared with only approximately 0.6 sulfo groups per disaccharide unit in a typical HS.[1] Approximately 80–90% of the disaccharide units in heparin contain IdoA compared with approximately 20% IdoA in HS.[1] The locations of both the sulfo groups and the IdoA and GlcA residues within heparin and HS play profound role in their activities. A pentasaccharide sequence present within these polysaccharides having the structure, →GlcNS6S → GlcA → GlcNS3S6S → IdoA2S → GlcNS6S → (NS is N-sulfo; Figure. 8.1 **3**, or several other structural variants[3]), is responsible for its specific binding to the serine protease inhibitor (Serpin) antithrombin III (AT).

A diverse collection of HS and heparin oligosaccharides and polysaccharides provide structural leads for developing new therapeutic agents and represent a frontier in medicinal chemistry and in understanding the biology of blood coagulation and other important physiological processes.

Heparin was discovered a century ago by McLean and Howell at Johns Hopkins University, and was soon after introduced as a clinical anticoagulant.[3,4] After 90 years of use, heparin remains the first choice for controlling blood coagulation and treating arterial thrombotic disorders.[5] The Serpin AT binds heparin at a pentasaccharide sequence, containing a central 3-*O*-sulfoglucosamine residue (Figure 8.1, **3**), to form an AT-heparin complex, which inhibits the blood-coagulation proteases to prevent clotting.[6,7] The clinical application of heparin greatly promoted the development of many medical and surgical procedures and in the absence of heparin modern medicine is not possible.

Heparins are classified into three forms, each of which have been approved by the US Food and Drug Administration (FDA): (1) unfractionated heparin (UFH, average molecular weight (MW_{avg}) 17 000 Da); (2) low-molecular-weight heparin (LMWH, MW_{avg} 3500–6000 Da); and (3) ultralow-molecular-weight heparin (ULMWH, MW_{avg} < 2000 Da).[8] UFH is primarily isolated from porcine intestine and represents approximately 40% of all the heparin used in the USA.[9] UFH is an intravenous drug used in surgery and kidney dialysis but prone to result in the development of a life-threatening adverse event known as heparin-induced thrombocytopenia (HIT).[10] LMWH is prepared from UFH by various depolymerization methods and is the most widely prescribed heparin in the USA (approximately 55%) due to its predictable subcutaneous bioavailability, longer half-life and slightly reduced incidence of HIT and bleeding (see Figure 8.1 **4** for an example of a LMWH).[8] ULMW heparins are synthetic, subcutaneously bioavailable products, corresponding to five to ten saccharide units, and because of expense and limited applications still represent a small percentage (less than 5%) of clinically used heparin.[9] ULMWH shows greatly reduced risks of HIT. The only clinically approved ULMWH, Arixtra® (fondaparinux, introduced in 2003), is a pentasaccharide containing the AT-binding region of heparin (Figure 8.1, **3**).[11]

8.1.2 Why are Synthetic Heparins and Heparan Sulfates Needed?

UFH and LMWH are polypharmacological agents, complex mixtures of molecules prepared from animal tissues.[13] The supply chain of pharmaceutical heparin has been poorly regulated and has presented safety concerns. Due to the similar nature of glycosaminoglycans (GAGs), their polydispersity, molecular heterogeneity, limitations in purification methods and the lack of simple and reliable analytical methods, heparin often contains HS, dermatan sulfate (DS) and chondroitin sulfate (CS). Worldwide distribution of contaminated heparin in 2007, caused by its adulteration with a semi-synthetic oversulfated chondroitin sulfate (OSCS) contaminant, adversely affected the purity

and safety of animal-sourced UFH and LMWH, and was associated with over 200 deaths in the USA.[14,15] Another potential threat is that other bioactive entities, such as viruses, prions or growth-modulating factors, may remain associated with the HS chains in animal extracts. Thus, the cost-effective preparation of a more structurally defined heparin from non-animal sources is highly desirable as are reliable structural characterization techniques.[14]

Until now, the preparation of homogeneous ULMW heparin using a purely synthetic chemical method, based on repetitive steps of protection, activation, coupling, and de-protection, is very challenging. For instance, Arixtra® (fondaparinux, Figure 8.1 **3**), a relatively simple pentasaccharide, requires as many as 60 chemical steps, and the overall yield is only 0.1%.[16,17] In addition, during preparation, there are many separation steps needed to remove undesirable isomers (especially the incorrect anomers) as well as the unavoidable failure products that add cost and decrease overall yields. Moreover, complex LMWHs and UFHs are usually not amenable to chemical synthesis, as it is virtually impossible to commercially prepare heparin oligosaccharides longer than five sugar units using currently available chemical approaches.[18]

Chemoenzymatic synthesis, relying on combined enzymatic and chemical methods, mimics the biosynthetic pathway of heparin, and represents a promising strategy to solve these synthetic challenges.[3] In this strategy, enzymatic synthesis is usually performed under mild temperatures (20–60 °C) using "green" aqueous conditions[19] catalyzing the reaction with exquisite regioselectivity and stereoselectivity (α- or β- glycosidic linkages) without the need for repetitive protection or deprotection steps. Chemical reactions are required to prepare unnatural enzymatic reaction substrates (unnatural uridine diphosphate (UDP)–sugars or acceptors), modify the backbone of enzyme-catalyzed heparin products or to control the regioselectivity of the enzymatic modifications taking place along the polysaccharide backbone.[1,18]

8.1.3 Types of Chemoenzymatic Synthesis

The total-synthesis of heparin and HS includes both backbone elongation and saccharide modification and starts from available monosaccharides or disaccharides. Glycosyltransferases (KfiA and pmHS2) utilize UDP-activated sugars (**6**, **7**, **8**) as donors to transfer saccharides onto an acceptor (**5**) to build the HS backbone structure (Figure 8.2A).[5] The nascent HS chain subsequently undergoes a series of modifications through the action of *N*-deacetylase (ND)/*N*-sulfotransferase (NST) (or the bifunctional enzymes NDSTs), C5-epimerase (C5-epi), 2-*O*-sulftransferase (2-OST), 6-*O*-sulftransferases (6-OSTs) and 3-*O*-sulftransferases (3-OSTs) result in the target HS or heparin structure.[8] A number of structurally well-defined HS and heparin oligosaccharides have been prepared and these synthetic heparins can exhibit potent bioactivity.

Semi-synthesis of heparin and HS employs naturally occurring polydisperse polysaccharide, obtained from bacterial fermentation, as starting material.[20] After its partial *N*-deacetylation and *N*-sulfation, this polysaccharide is then transformed into heparin or HS using recombinant biosynthetic enzymes,

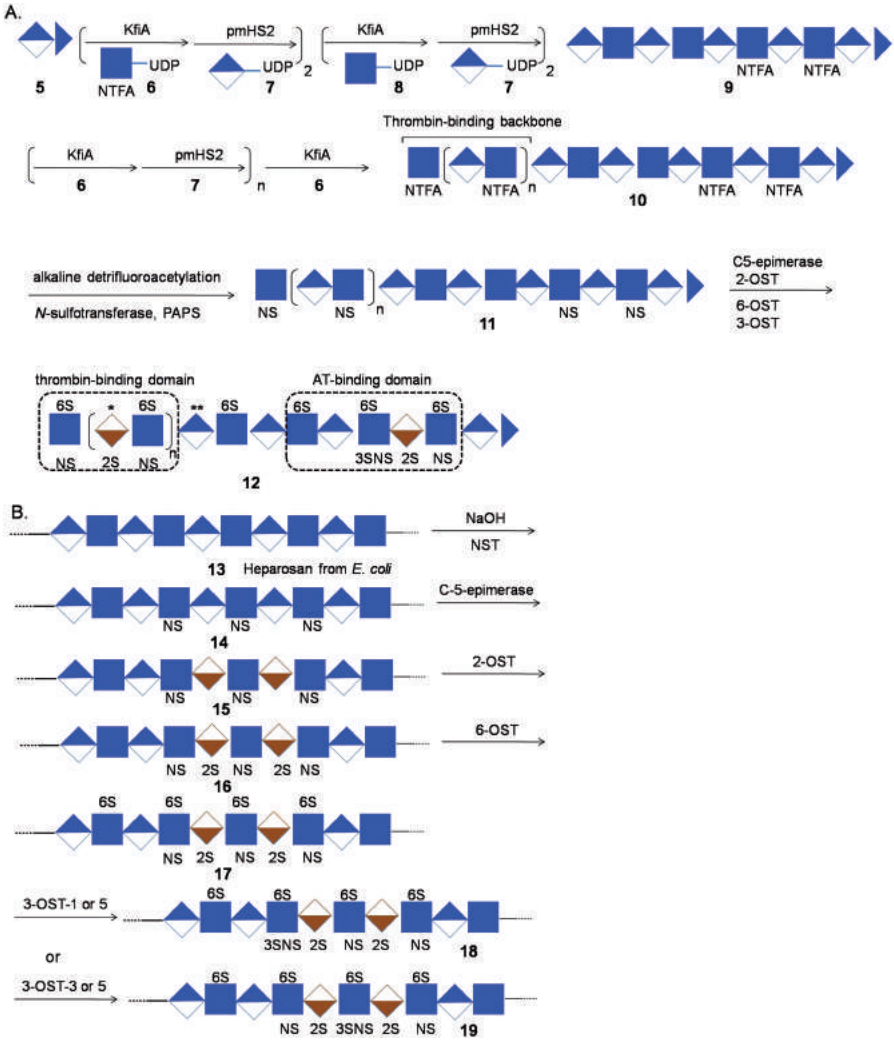

Figure 8.2 Chemoenzymatic synthesis of heparins and heparan sulfates and their oligosaccharides. (A) Total synthesis of heparin and heparan sulfate oligosaccharides (**12**) through the addition of UDP-activated donor sugars to a disaccharide acceptor prepared through the nitrous-acid-catalyzed depolymerization of *N*-deacteylated heparosan. The residue at the position indicated with * can also be an IdoA or a GlcA residue and the residue at the position indicated with ** can be an IdoA residue. (B) The semi-synthesis, starting from heparosan (**13**) of heparin and heparan sulfate (**19**) using a chemoenzymatic approach. NTFA: *N*-trifluoroacetyl.

mimicking the *in vivo* biosynthesis of these GAGs (Figure 8.2B).[21] Rosenberg and colleagues[22] first reported the small-scale semi-synthesis of AT-binding-site-containing HS using cloned enzymes mimicking those present in cells and clearly demonstrated the feasibility of an enzymatic approach to synthesize HS. The heparosan polysaccharide is comprised of a [→4) β-D-glucuronic acid (GlcA) (1 → 4) *N*-acetyl-α-D-glucosamine (GlcNAc) (1→]$_n$ repeating disaccharide units (unsulfated heparan sulfate, Figure 8.1 structure **1**). The large-scale preparation of heparosan from *Escherichia coli* K5 strain has been described.[22] Compared with total synthesis of heparins, the semi-synthetic approach is more easily scaled and provides a cost saving by not requiring the use of purified UDP–sugars, but the polymer size and compositional control are sacrificed.

8.2 Enzymes Required for Chemoenzymatic Synthesis

The enzymes used for chemoenzymatic synthesis of heparin and HS come from a variety of different sources. The glycosyltransferases, pmHS2 and KfiA, are bacterial enzymes from *Pasteurella multocida* and *E. coli* K5, respectively.[23,24] The mammalian Golgi NDST is a large bifunctional enyme that has not been actively expressed, but the ND and NST domains can be separately expressed in their active forms as fusion proteins from *E. coli*.[21,25] The C5-epi and the specialized *O*-sulfotransferases are also mammalian Golgi enzymes and have been cloned and expressed as fusion proteins in *E. coli*. Since all these mammalian enzymes are glycoproteins, recent efforts have focused on their expression in glycosylation-competent systems such as yeast and baculovirus-infected insect cells.[26] These biosynthetic enzymes are described in Table 8.1.

8.2.1 Glycosyltransferases

The biosynthesis of HS includes chain elongation and modification of the sugar backbone. *In vivo*, the HS chain is elongated by the Exostosin gene products, Ext1 and Ext2.[27] This process can be mimicked *in vitro* due to the availability of two recombinant expressed bacterial glycosyltransferases, KfiA and pmHS2. KfiA, prepared from *E. coli* K5 strain, is an *N*-acetylglucosaminyltransferase polymerizing UDP–GlcNAc (or the unnatural UDP–glucosamintrifluoroacetate (UDP–GlcTFA)) donor to a monosaccharide or disaccharide acceptor in a controlled, stepwise manner until the desired oligosaccharide length is reached. The UDP–GlcNAc can be produced by uridyltransferases such as GlmU, which allows the synthesis of some unnatural UDP–sugars for introduction into heparin oligosaccharides.[28] PmHS2 is a homologous heparosan synthase readily obtained from *P. multocida*, which can transfer UDP–GlcA donor to build the HS backbone. KifiA and pmHS2 are usually used in an alternating fashion during chemoenzymatic synthesis.

Table 8.1 Enzymes utilized in heparin and HS synthesis.

Enzyme	Abbreviation	Expression system	Enzymatic function
N-acetyl-D-glucos-aminyltransferase	KfiA	*E. coli* K5	Transfers a GlcNAc (or a GlcNTFA) residue to the backbone
Heparosan synthase 2	PmHS2	*P. multocida*	Transfers a GlcA and a GlcNAc (or a GlcNTFA) residue to the backbone
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* (CHO cell)	Converts a GlcA to an IdoA residue
2-*O*-sulfotransferase	2-OST-1	*C. griseus*	Introduces a sulfo group to the 2-OH position of an IdoA or a GlcA residue
6-*O*-sulfotransferase 1	6-OST-1	*Mus musculus*	Introduces a sulfo group to the 6-OH position of a GlcNS(GlcNAc) residue
6-*O*-sulfotransferase 3	6-OST-3	*M. musculus*	The same as 6OST-1
3-*O*-sulfotransferase 1	3-OST-1	*M. musculus*	Introduces a sulfo group to the 3-OH position of a GlcNS ± 6S residue that is linked to a GlcA (or an IdoA) on the non-reducing end
3-*O*-sulfotransferase 5	3-OST-5	*M. musculus*	Has both 3-OST-1 and 3-OST-3A substrate specificities
3-*O*-sulfotransferase 3	3-OST-3	*M. musculus*	Introduces a sulfo group to the 3-OH position of a GlcNS ± 6S residue that is linked to an IdoA2S on the non-reducing end
N-deacetylase/*N*-sulfotransferase-1	NDST-1	*Rattus norvegicus*	Converts a GlcNAc to a GlcNS residue

8.2.2 Sulfotransferases and C5-Epimerase

Several sulfotransferases involved in heparin biosynthesis have been expressed and characterized. These sulfotransferases transferred sulfo groups from 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the natural sulfate donor, to heparin and HS oligosaccharides with excellent regiospecificity.[29] The introduction of *N*-sulfo groups is catalyzed by *N*-deacetylase/*N*-sulfotransferase (NDST), a bifunctional enzyme having two active sites. The *N*-deacetylase removes the *N*-acetyl group from a GlcNAc residue and the *N*-sulfotransferase activity transfers a sulfo group to the resulting GlcN residue. The expression of an active bacterial NDST has been problematic. This has resulted in the development of an alternative method to position GlcNS residues using the unnatural UDP–GlcNTFA donor to introduce a GlcNTFA residue that can be easily chemically de-*N*-trifluoroacetylated to GlcN for subsequent

N-sulfation. The *N*-deacetylase/*N*-sulfotransferase (NDST) is believed to be the first enzyme to modify an intact heparosan chain during biosynthesis, and its action is crucial to all subsequent sulfation reactions.[21]

The 2-OST is only present in one isoform, transferring a sulfo group to the 2-position of both IdoA and GlcA residues, with a preference for IdoA.[30] Thus, IdoA2S is commonly found in heparin and HS but GlcA2S is rarely observed.[31] Three 6-OST isoforms (6-OST-1, 6-OST-2, 6-OST-3) and seven 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.[32,33] The different isoforms of enzymes exhibit slightly different specificities. For example, 6-OST-1 and 6-OST-2 prefer to transfer 6-*O*-sulfo groups to a GlcNS that is next to a GlcA residue and an IdoA2S residue, respectively.[13] The 3-OST-1 isoform transfers a sulfo group to a GlcNS that is linked to a GlcA or IdoA at the non-reducing end, while the 3-OST-5 isoform prefers a GlcNS that is linked to an IdoA2S, GlcA, or IdoA.[34]

C5-epimerase is responsible for converting GlcA into its C5-epimer, IdoA. Although both the natural UDP–GlcA and the unnatural UDP–IdoA have been tested as donors, only the GlcA residue is incorporated.[35] Thus, C5-epi is indispensable in preparing heparin and HS containing IdoA residues. C5-epi catalyzes both the forward and reverse reaction, generating an equilibrium mixture of GlcA and IdoA residues. Since the GlcA residue is thermodynamically favored, the IdoA residue is only generated in large amounts in the presence of 2-OST resulting in the formation of IdoA2S, which cannot be converted by C5-epi to GlcA2S.[36]

8.3 Building Blocks Prepared for Chemoenzymatic Synthesis

8.3.1 Acceptors

In the total synthesis of HS and heparin oligosaccharides, a number of monosaccharide and disaccharide acceptors have been developed that have greatly facilitated the chemoenzymatic synthesis (Figure 8.2A **5** and Figure 8.3A **20**, **21**, **5**). The addition of an ultraviolet-detectable, hydrophobic tag to the acceptor synthesis has improved both detection and purification. The *p*-nitrophenyl (*p*-NP), *p*-nitrobenzyl (*p*-NPM), *p*-methoxybenzyl (*p*-MPM) and *p*-pivaloylaminobenzyl (*p*-PAB) β-glucuronides **20**, represent a series of important commercially available acceptors.[37,38] These aromatic groups are UV-visible and hydrophobic and they can be reversibly bound to reversed-phase chromatography resins, enabling the easy purification of the resulting oligosaccharides using a C18 resin. In addition, these tags can be easily removed or modified using standard chemical methods. For example, by reducing the *p*-NP group to a free amine followed by amidation with fluorescent tag, Alexa Fluor 488 dye, the resulting complex could be used as a sensitive probe in live or fixed cells.[39] However, the potential toxicity of *p*-NP is a concern, especially in clinical applications.

Compared with the *p*-NP tagged acceptor, a monosaccharide or disaccharide acceptor with a reducing end *α*-*O*-methyl glycoside (Figure 8.3 **21**), is

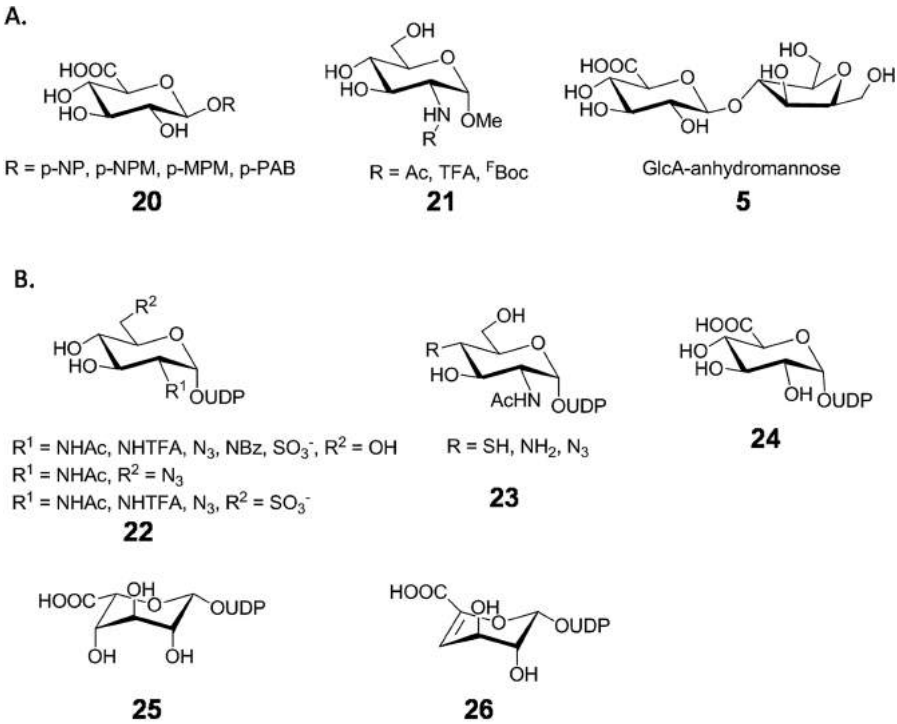

Figure 8.3 Carbohydrate synthons designed for use in chain elongation reactions. (A) Selected acceptor structures. (B) Selected donor structures.

more similar to that in the commercial ULMWH Arixtra® (Figure 8.1 **3**).[40] The disadvantages of an aglycone are that it is not ultraviolet-detectable and does not facilitate detection and purification. A fluorous tag (Figure 8.3 **21**),[40] while not ultraviolet-detectable might facilitate purification by fluorous solid-phase extraction (FSPE) but is larger than a methyl group and might interfere with enzymatic recognition in both chain elongation and sulfation.

Another commonly used acceptor is a disaccharide unit comprised of glucuronic acid (1 → 4) anhydromannose (Figure 8.3 **5**), which is conveniently obtained through the nitrous-acid-treatment of *N*-deacetylated heparosan. Disaccharide **5** is contained in commercial LMWHs and, therefore, should be considered relatively non-toxic.[41] This disaccharide acceptor can also be modified to introduce a 'click' reactive azide group or amino group at the reducing end, for diverse conjugation.[42] The main drawback of this disaccharide acceptor is the lack of a hydrophobic chromophore.

8.3.2 Donors

8.3.2.1 Natural UDP–Sugars

Uridine diphosphate (UDP) monosaccharides are common donors, being transferred to glycosyl acceptors by a glycosyltransferase or synthase in

the chemoenzymatic synthesis (Figure 8.3B **22**–**26**). These donors include both natural and unnatural UDP–sugars, and can be prepared in the laboratory chemically, enzymaticlly, or chemoenzymatically.[43] UDP–GlcNAc and UDP–GlcA are major natural donor substrates required in HS synthesis. UDP–GlcA is commonly synthesized from UDP–Glc by a NAD^+-dependent oxidation process catalyzed by a UDP–glucose dehydrogenase (Ugd).[44]

8.3.2.2 Unnatural UDP–Sugars

GlcNAc and GlcA are naturally occurring saccharide residues, which are prevalent in heparin and HS and play essential roles in biological processes. The use of unnatural GlcNAc/GlcA analogs together with chemical or enzymatic transformations represents a novel approach for the controlled preparation of specific natural as well as unnatural sequences found within heparin and HS. Wang and colleagues[45] reported the enzymatic synthesis of UDP–GlcNAc analogs using recombinant *E. coli* *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU). Chen and colleagues[46,47] reported a one-pot three-enzyme synthesis of UDP–GlcNAc derivatives and subsequently developed improved one-pot multi-enzyme (OPME) systems for synthesizing UDP–GlcA. This improved system avoided the use of nicotinamide adenine dinucleotide (NAD^+)-cofactor in the UDP–Glc dehydrogenase-dependent process and can be broadly applied to the preparation of various GlcA-containing heparins. In addition to the pure enzymatic approach, Linhardt and colleagues[28] reported the chemical synthesis of a series of different GlcNAc-1-phosphate analogs and tested their recognition by the GlmU uridyltransferase enzyme. They found that analogs having an amide linkage at the C-2 nitrogen are critical for acceptance by GlmU. The unaccepted analogs could be converted to UDP–sugar donors by chemical synthesis.

8.3.2.2.1 UDP–GlcNTFA. While most of the HS biosynthetic enzymes have been efficiently prepared *via E. coli*, only an active *N*-sulfotransferase (NST) domain of NDST can be easily expressed.[25] Moreover, it is still unclear how the regioselectivity of the NDST isoforms, expressed in eukaryotic cells, can be controlled. Thus, the regioselective *N*-sulfonation of glucosamine in HS backbone modification has become a major roadblock in chemoenzymatic synthesis.[1] Uridine diphosphate–*N*-trifluoroacetylglucosamine (UDP–GlcNTFA), an unnatural UDP–sugar, was introduced to solve this problem.[25] UDP–GlcNTFA is an excellent substrate for KfiA to elongate the HS backbone with high yield. The resulting GlcNTFA residue can then be easily converted to GlcNS by removing the trifluoroacetyl group under mildly alkaline conditions followed by the introduction of *N*-sulfo groups using either NST or chemical *N*-sulfonation. This strategy for the regioselective synthesis of GlcNS residues has been successfully applied in the preparation of LMWHs and ULMWHs.[41,48,49]

8.3.2.2.2 Other Unnatural UDP–Sugars. The application of unnatural donors can also play an important role in synthesizing unnatural heparins, as temporary protecting groups, as chemical tags, or as a precursor for further modifications before their selective removal under mild basic or acidic conditions. Developing various unnatural UDP–sugars is of great interests, and many novel donors have been proposed. Chemical synthesis is still very important in preparing unnatural UDP–sugars and can still require a many steps. Linhardt and colleagues[35] have prepared UDP–IdoA and UDP–hexuronic acid (HexA) as potential glucuronosyltransferse substrates. Chen and coworkers[50] successfully used a 6-azide substituted UDP–GlcNAc derivative to synthesize a heparin-like oligosaccharide. The resulting 6-azide-GlcNAc residue can be either converted to a special moiety 6 *N*-sulfo or form an oligosaccharide cluster using click chemistry. We anticipate that many additional unnatural UDP–sugars, such as thiol-, azide- or amine-substituted donors, will one day be designed and synthesized. These resulting unnatural HS oligosaccharide analogs might be resistant to catabolism by blocking the action of heparanase and lysosomal degradative enzymes,[51,52] increasing the biological half-life of the oligosaccharides prepared using these donors.

8.3.2.2.3 Challenges for Unnatural Donors. Unnatural UDP–sugars have been successfully applied in synthesis of heparin analogs, establishing a platform for developing new heparin-based therapeutics and unraveling mechanisms underlying sugar-related biological processes. However, a pure chemical approach requires a long and tedious synthesis and the yields of UDP–sugar are generally low.[18] Enzymatic or cell-based syntheses of unnatural donors need to be developed for their inexpensive and large-scale preparation.[53] The ability of natural synthases and glycosyltransferases to accept novel unnatural UDP–sugars may be limited, thus, protein engineering strategies may be required to make these enzymes more tolerant of unnatural UDP–sugar donors.[18]

8.3.3 Polysaccharide and Oligosaccharide Backbone

Polysaccharides serve as the building blocks in semi-synthesis of HS and heparin. In particular, heparosan, prepared from fermentation of *Escherichia coli* K5, and its derivatives are promising substrates for the preparation of heparin-based therapeutics from non-animal sources. Heparosan is typically modified sequentially through chemical *N*-deacetylation and *N*-sulfonation steps (due to difficulties in the bacterial expression of NDST). The subsequent actions of C5-epi, and 2-OST, 6-OST-1, 6-OST-3, and 3-OST-1 result in bioengineered heparin.[8] A lower level of enzymatic modification or the use of alternative sulfotransferase isoforms can lead to a wide variety of bioengineered HS polysaccharides with differing biological activities.

Chemically synthesized oligosaccharide building blocks can also provide flexibility in the preparation of structurally defined HS oligosaccharides with desired biological activities. Huang, Liu, and colleagues[54] used the

pre-activation-based one-pot approach to chemically prepare a single hexasaccharide precursor, which was further enzymatically extended to produce a series of oligosaccharides with different sulfation patterns and sizes, indicating that interfacing chemical and enzymatic synthesis could be a powerful technique to elucidate the structure and activity relationship of HS.

8.4 Control of Product Through Sequential Enzymatic Modification

The chemoenzymatic synthesis of bioengineered heparin mimics the HS biosynthetic pathway, involving of a series of enzymatic reaction steps, and requiring careful reaction scheme design, especially an appropriate modification sequence. The most effective schemes are those closely related to natural heparin synthesis. The ideal reaction order can greatly affect the enzyme activity, while an improper sequence may result in poor yield and purity. For example, the introduction of 2-*O*-sulfo group into the IdoA residue can only occur following the introduction of *N*-sulfo group in adjacent GlcN residues and prior to the introduction of 6-*O*-sulfo groups into GlcN residues.[1] The critical order of these steps has been demonstrated from ternary crystal structural data.[1,55] C5-epi works best when used in tandem with 2-OST, as the introduction of a 2-*O*-sulfo group can block the reversible activity of C5-epimerase, possibly due to the steric-hindrance. Moreover, C5-epi must be used after the introduction of *N*-sulfo group, because C5-epi only acts on a GlcA residue that is between two GlcNS residues.

ULMWHs **30** and **35** are chemoenzymatically synthesized oligosaccharides that contain the AT-binding domains of porcine and bovine heparin (Figure 8.4A).[41] Disaccharide **5**, prepared from a heparosan disaccharide acceptor, was elongated by two bacterial glycosyltransferases (PmHS2 and KfiA) in alternating fashion to produce the heptasaccharide **27**. In this synthesis, selective C5-epimerization/2-*O*-sulfation takes place on residue **D** but not residue **B**, because residue **D** is flanked by two GlcNS residues. Similarly, as residue **C** is flanked by a GlcA residue at its non-reducing end, 3-OST-1 selectively adds a 3-*O*-sulfo group to residue **C** rather than residue **E** to generate ULMW heparin **30**. The structure of oligosaccharide **35** is a little different from that of **30**, which should introduce a GlcNS6S residue to the non-reducing end. The GlcNTFA residue is critical because it not only can prevent the action of C5-epi and 2-OST on the GlcA but also act a precursor of GlcNS. Finally, the synthesis of oligosaccharide **35** was achieved by rearranging the sequence of the modification and elongation steps without employing additional enzymes, thus demonstrating that both structural control and target diversification are possible in chemoenzymatic synthesis.

It is known that C5-epi is a two-way catalyst, transferring a GlcA to an IdoA residue and also converting an IdoA to a GlcA residue, which results in a final product containing a mixture of GlcA and IdoA.[36] This unique property of C5-epimerase makes the synthesis of a structurally defined LMWH

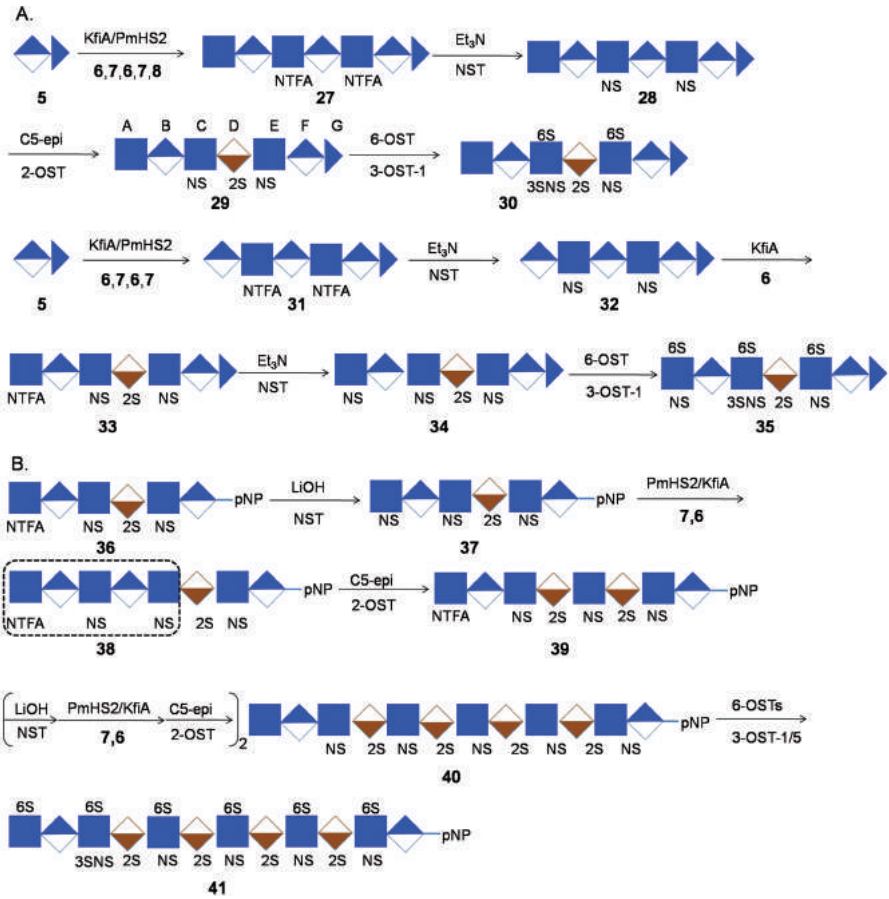

Figure 8.4 Controlled chemoenzymatic synthesis of synthetic heparin HS oligosaccharides. (A) ULMWH **30** and **35** are chemoenzymatically synthesized on acceptor **5**. (B) LMWH **41** is chemoenzymatically synthesized from *p*-NP–hexasaccharide acceptor **36**..

or ULMWH challenging, particularly one containing repeating -IdoA2S–GlcNS6S- units. However, a unique distribution of GlcNAc and GlcNS residues of the substrate can force C5-epi to act as a one-way catalyst. Linhardt, Liu and colleagues[49] have designed an alternative strategy, placement of a pentasaccharide domain GlcNTFA–GlcA–GlcNS–GlcA–GlcNS, for synthesizing repeating -IdoA2s-GlcNS6S- motifs without incomplete conversion (Figure 8.4B). The desired pentasaccharide domain of compound **38** was synthesized from known hexasaccharide **36** through *N*-detrifluoroacetylation, NST and two glycosyl transferase steps. Then C5-epimerization of GlcA was performed and followed by the introduction of a 2-*O*-sulfo group, generating IdoA2S, removing further C5-epi modification. Repeating these steps several times it is possible to obtain repeating -IdoA2S–GlcNS6S- units.

The resulting compound **40** was then converted to the target LMWH **41** after the introduction of *N*-sulfo, 6-*O*-sulfo and 3-*O*-sulfo groups.

8.5 Novel Chemoenzymatic Synthesis

8.5.1 One-Pot Multienzyme System

Glycosyltransferases are widely used in the initial steps of reaction sequences followed by other enzymes such as C5-epi and OSTs (Figures 8.2A). 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 colleagues[50] have developed an efficient OPME system utilizing a series of unnatural sugar nucleotides to synthesize heparin-like oligosaccharides (Figure 8.5A). Using GlcNTFA as the donor precursor, glucuronide GlcAβ2AA with a 2-aminoacridone (2AA) fluorescent label as the acceptor substrate **43**, disaccharide GlcNTFAα1-4GlcAβ2AA **44** was synthesized *via* a one-pot four-enzyme reaction containing NahK_ATCC55813, PmGlmU, PmPpA and PmHS2. The resulting disaccharide **44** was used in a one-pot three-enzyme GlcA activation and transfer system to produce the trisaccharide **46**. In this scheme, Glc-1-P **45** and UTP were coupled using *E. coli* glucose-1-P uridylyltransferase EcGalU to generate UDP–Glc. UDP–glucose dehydrogenase, PmUgd, in the presence of the coenzyme nicotinamide adenine dinucleotide

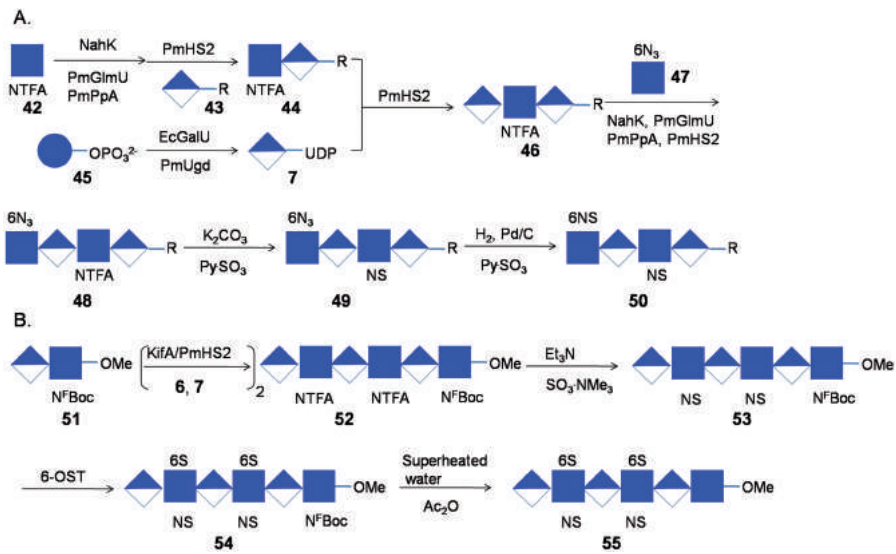

Figure 8.5 Novel approaches to chemoenzymatic synthesis of heparan sulfate oligosaccharides. (A) One-pot multi-enzyme approach where R is 2-aminoacridone. (B) Fluorous-assisted chemoenzymatic synthesis where ^{F}Boc is a fluorinated Boc protecting group.

(NAD^+) oxidized the C6 position of Glc in UDP–Glc forming UDP–GlcA **7** and pmHS2 catalyzed the synthesis of trisaccharide **46**. The trisaccharide **46** obtained was used as a substrate in the one-pot four-enzyme $GlcNAc6N_3$ activation and transfer system to form tetrasaccharide **48**. The *N*-TFA group and 6-N_3 group were converted into free amino groups and sequential *N*-sulfonation generated the HS tetrasaccharide analog **50**. This HS tetrasaccharide analog may have improved therapeutic potential as it should be more resistant to catabolism by heparanase.

8.5.2 Fluorous-Tagging Techniques

Fluorous chemistry is a new tool for solution-phase high-throughput organic synthesis, developed in the late 1990s.[56] Based on the high affinity of perfluoroalkyl chains toward fluorous surfaces and solvent, fluorous separation techniques can significantly facilitate the isolation and purification of intermediates and products. Tagging organic compounds with a light fluorous group such as a perfluorooctyl (C_8F_{17}) or a perfluorohexyl (C_6F_{13}) group followed by product purification using fluorous solid-phase extraction (FSPE) has found increasing synthetic uses.[57] These advances in fluorous chemistry have been successfully applied by Pohl and coworkers[58,59] to the chemical synthesis of oligosaccharides. The combination of fluorous methodology with chemoenzymatic approaches can further enhance synthetic and purification processes.

Linhardt and colleagues[40] have successfully applied a temporary fluorous *tert*-butyl dicarbonate ^{F}Boc linker and FSPE technique in the chemoenzymatic synthesis of HS oligosaccharides (Figure 8.5B). The synthetic fluorous-tagged disaccharide acceptor **51** was repetitively elongated with glycosyltransferase KfiA and UDP–GlcNTFA, PmHS2 and UDP–GlcA, to form the hexasaccharide **52**. With the HS backbone constructed, base-catalyzed deprotection of the TFA group was followed by chemical *N*-sulfation with $SO_3{\cdot}MeN_3$ to generate the *N*-sulfate hexasaccharide **53**. Then incubation of **53** with 6-OSTs and PAPS was used to obtain the 6-*O*-sulfo hexasaccharide **54**. Finally, superheated water, a green solvent,[19] was employed to remove the ^{F}Boc tag and was followed by *N*-acetylation to obtain the HS hexasaccharide **55**. This effort reveals that the fluorous tag did not interfere with the action of these glycosyltransferases and the FSPE technique was suitable for the purification of HS oligosaccharides.

Chen and colleagues[60] have combined the OPME system with fluorous solid-phase extraction cartridges purification to highly efficiently synthesize a series of sialosides and galactosides. This method may be applied to more complex HS oligosaccharides synthesis in the future.

8.5.3 Solid-Phase Synthesis

Since Frechet and colleagues[61] initiated the evolution of solid-phase synthesis of oligosaccharides on a polymer supports in 1971, a number of advances, particularly by the Seeberger group[62,63] have led to the automated chemical synthesis of more complex targets such as glycosaminoglycan

oligosaccharides. This approach, could greatly simplify purification while at the same time affording exquisite stereoselectivity and regioselectivity. Since chemoenzymatic synthesis is performed in aqueous solvents, it is critical that the solid support be hydrophilic to allow good swelling and that the appropriate length of linker, between the acceptor saccharides and the solid support, is used to make the acceptor sites enzyme-accessible. Although promising, to date there have been few examples of solid-phase chemoenzymatic synthesis of carbohydrate reported. Linhardt and colleagues[64] have immobilized HS substrate 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 an artificial Golgi, for subsequent 3-OST-1 modification. While demonstrating proof-of-concept, the multi-step, solid-phase, chemoenzymatic synthesis of more complex targets still need to be demonstrated.

8.5.4 Immobilized Enzymes

While enzymes are conveniently employed as highly efficient biocatalysts under mild reaction conditions they often lack long-term operational stability and often present a technically challenging recovery process.[65,66] Moreover, difficulty in reusing enzyme catalysts has hampered their widespread industrial application. Enzyme immobilization, first reported in 1916 and commercialized in the 1960s, represents a chemical engineering approach to improve enzyme utilization in biotechnological processes.[67] Immobilized enzymes are physically confined catalysts, localized in a defined region of space, which retain catalytic activity, allowing repeated and continuous use. Thus, immobilization provides for the facile separation of enzyme from products, and results in a remarkable reduction of processing costs, and is expected to be of great utility in chemoenzymatic synthesis of heparins. Linhardt and colleagues[68] reported the successful immobilization of the multiple enzymes involved in heparin synthesis, including C5-epi, 2-OST, and arylsulfotransferase-IV. These recombinant heparin biosynthetic enzymes were immobilized on amino-linked agarose gel beads at a loading of 20 mg per ml of gel and showed enhanced thermal stability while still maintaining over 80% of activity after their immobilization. One particular challenge is that these immobilized enzymes need to act in concert to transform a complex substrate into a much more complex product, while also recycling a required cofactor and preventing product inhibition.

8.5.5 Immobilized Enzyme Cofactors

The successful use of immobilized enzyme cofactors, such as NAD^+–NADH, has been demonstrated in oxidation–reduction reactions.[69,70] An immobilized NAD^+ might be applied to the chemoenzymatic synthesis of heparin oligosaccharides described by Chen and colleagues,[50] to oxidize UDP–Glc to UDP–GlcA using PmUgd (Figure 8.5A). It seems fair to speculate that other enzyme cofactors might also prove important in the chemoenzymatic synthesis of heparins.

For example, in the semi-synthesis approach it might be possible to immobilize a tethered PAPS that can be regenerated using aryl sulfotransferase (AST)-IV and an inexpensive sacrificial sulfo group donor, such as *p*-nitrophenylsulfate (PNPS). In the total synthesis approach it might also be possible to tether UDP–sugars and regenerate these *in situ*, greatly reducing synthetic costs.

8.6 Conclusion and Future Perspectives

HS and heparins are important glycosaminoglycans that perform numerous physiological and pharmacological functions. The chemoenzymatic synthesis of LMWH and ULMWH has demonstrated a novel efficient approach to prepare structurally defined heparin oligosaccharides that have been difficult to prepare by traditional chemical synthesis approaches. Over the past five years, there has been a steady improvement in the chemoenzymatic synthesis of such HS and heparin oligosaccharides. These improvements have included higher yields, reduced numbers of steps, greater purities, easier purification, and larger and more structurally complex targets. The advances leading to these improvements have included better enzyme expression, better enzymes resulting from protein engineering, optimization of the order of enzymatic transformations, increases in reaction scale, higher efficiency use of enzyme co-factors, and a better understanding of the substrate specificities of the HS biosynthetic enzymes.

Many challenges remain in this young field. Large-scale chemoenzymatic synthesis for the anticoagulant drug market remains the major challenge, requiring up to a 1 000 000-fold scale-up from micrograms to kilograms. The promises of this technology, however, are great, offering new, safer, and improved drugs for clinicians as well as oligosaccharides for research scientists, leading to a more complete understand of heparin and HS structure–activity relationships.

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