Chemoenzymatic Synthesis of Heparins 51

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Abstract

Chemoenzymatic synthesis is a strategy to produce heparin oligosaccharides with clearly defined structures and properties. Chemical synthesis involving protection and deprotection is combined with enzymatic synthesis. Chemical synthesis is used to construct appropriate acceptor and donor molecules, while enzymatic synthesis is used in extension and modification. Recombinant enzymes are prepared based on heparin's biosynthetic pathway in animals and capsular polysaccharide pathways in bacteria. Chemoenzymatic synthesis relies on a deep understanding of enzyme specificity and the application of modified unnatural chemically synthesized donors and acceptors to perform target-based synthesis of small- to intermediate-sized heparins.

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Introduction

Heparin is a linear, long-chain, polydisperse polysaccharide, called a glycosaminoglycan, with a high level of sulfation. It has a variety of biological functions but is widely used as a clinical anticoagulant. Although heparin is structurally heterogeneous, it has a major (60–80 %) trisulfated disaccharide repeating unit of →4) α-D-*N*-sulfo, 6-*O*-sulfo glucosamine (GlcNS6S) (1–4) α-L-2-*O*-sulfo iduronic acid (IdoA2S) (1→. Heparin also contains a number of important minor disaccharide units having various sulfation patterns on saccharide residues, including α-D-glucosamine (GlcN), α-D-*N*-acetylglucosamine (GlcNAc), and β-D-glucuronic acid (GlcA).

Clinically used heparin comes in three main forms: unfractionated heparin (UFH, molecular weight average MW_{avg} ~17,000), low molecular weight heparin (LMWH, MW_{avg} ~8,000), and ultralow molecular weight heparin (ULMWH, MW_{avg} <2,000). This chapter will focus on the chemoenzymatic synthesis of structurally defined LMWH and ULMWH (Xu et al. 2011, 2014).

Of all the heparin used each day in the United States, ~40 % is UFH, used primarily as an *intravenous* drug and in dialysis patients and in hospitalized patients. LMWH, ~55 % of the US market, is principally used subcutaneously for the treatment of deep vein thrombosis. Finally, a small percentage (<5 %) of the heparin used is an expensive, synthetic ULMWH (such as Arixtra) and is used subcutaneously in select applications when the side effect known as heparin-induced thrombocytopenia is anticipated. The worldwide market for heparin is ~4 billion US dollars, with ULMWHs accounting for ~0.5 billion US dollars (Bhaskar et al. 2012).

Both UFH and LMWH are derived from porcine intestinal mucosa, in a process that has in the past led to the presence of impurities and contaminants. LMWH is currently prepared from UFH by controlled chemical or enzymatic depolymerization. ULMWH is chemically synthesized from simple monosaccharides in a long, low-yielding, and expensive process.

Principles

Chemoenzymatic synthesis combines chemical and enzymatic methods for the most efficient process possible. Chemical synthetic schemes give products with structure and sequence that can be readily controlled and allow for the preparation of unnatural substrates of experimental or therapeutic value. Purification of chemically synthesized molecules, relying on standard organic solvents and chromatography, can also be easier, especially for fully protected and hydrophobic sugars. Chemical synthesis of carbohydrates relies on a complex system of protection and deprotection of reactive functional groups to afford complete control over the

regio- and stereo-selectivity. Some syntheses, particularly those involving regio- and stereo-control, can be performed much more efficiently using enzymes. Enzymes can greatly reduce or eliminate protection and deprotection steps, are generally regio- and stereospecific, and are high yielding or even quantitative. They also use "green" aqueous solvents, eliminating the need for harsh conditions and toxic chemical reagents. Chemoenzymatic synthesis integrates chemical reactions into schemes where unnatural substrates are required or to control enzyme reactivity or specificity in the target-based synthesis of oligosaccharides. Enzymes are then used to perform steps requiring exquisite regio- or stereo-control without the use of repetitive protection and deprotection steps (DeAngelis et al. 2013).

The modification enzymes used in the chemoenzymatic synthesis of heparin are identical to those in the biosynthetic pathway by which heparin is produced in the endoplasmic reticulum and Golgi of animal cells. Enzymes involved in the saccharide donor synthesis and the chain extension were identified in capsular bacteria containing heparosan, →4) α-D-GlcNAc (1–4) β-D-GlcA (1→. These enzymes are then cloned and expressed in commercially useful organisms to prepare inexpensive recombinant enzymes as catalysts (Bhaskar et al. 2012). This biomimetic strategy takes advantage of the natural biological pathway to develop a new in vitro reaction pathway.

Synthetic Methods

Strategy

Target-based chemoenzymatic synthesis of heparin oligosaccharides begins with a carefully designed retrosynthetic scheme. The assembly of the required reagents, substrates, cofactors, and enzymes, many of which are not commercially available, is the first challenge. Synthesis typically involves the saccharide chain extension of an acceptor through its enzymatic or chemical glycosylation, using glycosyl donors. Chemoenzymatic synthesis generally mimics the biosynthetic pathway, building the oligosaccharide in a stepwise fashion from the reducing end to the nonreducing end. Next, modification reactions are undertaken relying on chemical deprotection and modification, and enzymatic modification using sulfotransferases and epimerase. Since half of the mass of heparin is sodium sulfate and the remainder is carbohydrate, a large amount of sulfo donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is required, and as reactions are scaled up, this typically requires cofactor recycling (Burkart et al. 2000; Fig. 1).

Acceptors

Different monosaccharide or disaccharide acceptors used in the chemoenzymatic synthesis of heparin oligosaccharides have different advantages and disadvantages. One commonly used acceptor, a disaccharide with a reducing end anhydromannitol residue, is conveniently prepared from bacterial polysaccharide (Liu et al. 2010;

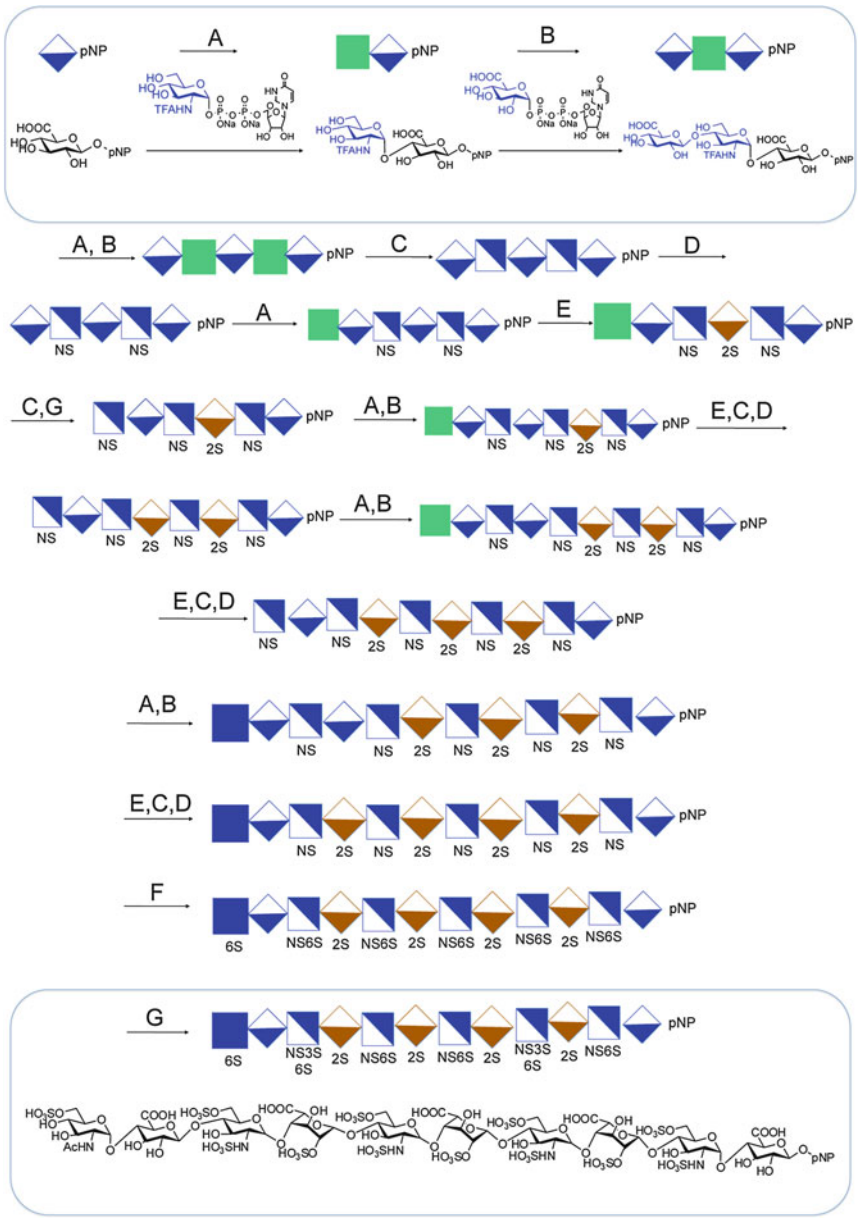

Fig. 1 Chemoenzymatic synthesis of low molecular weight heparins. *A*. KfiA, UDP-GlcNTFA; *B*. PmHS2, UDP-GlcA; *C*. LiOH; *D*. NST, PAPS; *E*. C5-epi, 2-OST, PAPS; *F*. 6-OST isoforms 1 and 3, PAPS; *G*. 3-OST isoforms 1 and 5, PAPS

Xu et al. 2011). This disaccharide acceptor is difficult to modify or remove, and because it lacks a chromophore or hydrophobic group, its detection and purification is difficult.

Another acceptor, *p*-nitrophenyl (*p*-NP) β-glucuronide, is commercially available, can be removed or modified, and contains a hydrophobic chromophore (Cai et al. 2013; Xu et al. 2014). This ultraviolet-detectable tag allows easy chromatographic purification of oligosaccharide intermediates. One disadvantage is the risk of toxicity of the (*p*-NP) in clinical applications.

A third acceptor is a monosaccharide or disaccharide with a reducing end α-*O*-methyl glycoside, similar to that found in the commercial ULMWH, Arixtra. This small aglycone gives the acceptor a nearly natural structure, is unlikely to be toxic, and prevents anomeric mixtures since the *O*-methyl group locks the reducing end in an α-conformation. Drawbacks to this acceptor are that it requires chemical synthesis and that it has no hydrophobic chromophore to aid in its detection and purification, although removal functionality such a fluorous tag might be used (Cai et al. 2014).

Donors

The common donors for enzymatic glycosylation of heparin are uridine diphosphate (UDP) monosaccharides. These can be synthesized chemically, enzymatically, or chemoenzymatically and can be either natural or unnatural UDP-sugars. Natural UDP-sugars required in heparin synthesis are UDP-GlcNAc and UDP-GlcA. In heparin biosynthesis, the introduction of functional domains is dependent on the placement of GlcNS, GlcNAc, and GlcN residues by the large bifunctional *N*-deacetylase/*N*-sulfotransferase (NDST) enzyme. While there are four NDST isoforms, none have been cloned and produced in an active form in a bacterial expression system. Thus, this limited availability has impacted the understanding of their specificity and control. Instead, chemical methods, using unnatural UDP-sugar donors, are currently the most reliable way of positioning GlcNS, GlcNAc, and GlcN residues to control the placement of structural domains within heparin. Useful unnatural donors, such as UDP-*N*-trifluoroacetylglucosamine (UDP-GlcNTFA) and UDP-*N*-*t*-Boc-glucosamine (UDP-GlcNtBoc), are accepted as substrates for some bacterial glycosyltransferases. The advantage of these unnatural donors is that the residues can be used as temporary protecting groups, as chemical tags, or as foundations for further modifications before their selective removal under mild base or acid conditions, respectively (Liu et al. 2010; DeAngelis et al. 2013).

Reactions

The important reactions in chemoenzymatic synthesis are glycosylation/extension and modification, and each of these can be performed using either chemical or enzymatic methods. Chemical glycosylation is usually performed (if at all) at the

beginning of the synthesis, to prepare an appropriate acceptor having a precise and often unnatural structure. Acceptor synthesis can be long and inefficient, due to a large number of protection and deprotection reactions required, and along with chemical glycosylation can result in purification issues and low yields.

Enzymatic glycosylation to prepare heparin oligosaccharides generally relies on the bacterial glycosyltransferases PmHS2 (heparosan synthase-2 from *Pasteurella multocida*) and KfiA (heparosan synthase from *Escherichia coli*). These enzymes are used in an alternating fashion: UDP-GlcNTFA (or UDP-GlcNAc) donor is added to the acceptor using KfiA, followed by UDP-GlcA donor added by PmHS2, to synthesize the basic repeating disaccharide backbone, and so on until the desired oligosaccharide length is reached (DeAngelis et al. 2013). These glycosyltransferases afford excellent regio- and stereo-control and quantitative product yields, resulting in no partial extensions and thus requiring little if any product purification between steps.

The selective modification of heparin oligosaccharide backbones is mainly done using *O*-sulfotransferases, which transfer a sulfo group to a desired position, and C5-epimerase (C5-epi), which converts GlcA into its C5-epimer IdoA. The *O*-sulfotransferases show excellent regio-specificity, allowing the introduction of 2-, 3-, and 6- *O*-sulfo groups into the heparin oligosaccharide. These enzymes require the expensive cofactor PAPS and large-scale synthesis can necessitate cofactor recycling (Burkart et al. 2000). Another complication of O-sulfotransferases is that with the exception of 2-*O*-sulfotransferase (2-OST), all of these enzymes have multiple isoforms [three 6-*O*-sulfotransferases (6-OSTs) and seven 3-*O*-sulfotransferases (3-OSTs)] having differing selectivity. For example, 6-OST isoform 1 prefers to transfer a 6-*O*-sulfo group to a GlcNS that is next to an unsulfated GlcA residue, while 6-OST isoform 2 prefers one that is next to IdoA2S residue (Bhaskar et al. 2012).

The selectivities of these modification enzymes provide careful control over products and also require careful reaction scheme design. The most effective schemes are those that follow the reaction order found in natural heparin synthesis. Careful study of ideal reaction order and enzymatic activity has shown that C5-epimerase works best when used in tandem with 2-OST. NST must be used before C5-epi/2-OST, because C5-epimerase only works on a GlcA residue that is between two GlcNS residues (Liu et al. 2010). C5-epimerase catalyzes both reversible and irreversible epimerizations, based on the identity of the residue that is three residues in the nonreducing direction from the epimerization site. If this particular residue is GlcNS or GlcN, the reaction will be reversible. If this residue is GlcNAc (or GlcNTFA), the reaction is irreversible (Sheng et al. 2012).

Chemical modification is generally performed early in a reaction sequence using protection and deprotection strategies to create the desired acceptor before glycosylation. However, some chemical modifications can take place later in the reaction sequence, often following the use of enzymatic steps. For example, an oligosaccharide with GlcNTFA residues can undergo a mildly basic reaction to remove the TFA groups, giving free amine groups for further modification. In addition, any tags placed on the acceptor before extension (such as the *p*-NP mentioned above) can be removed chemically once the desired oligosaccharide has been synthesized.

Table 1 Chemoenzymatically or chemically synthesized heparin oligosaccharide targets. *Activity* anti-factor Xa inhibition (IC_{50}, ng/ml), *nd* not determined

Oligosaccharide structure	Activity	References
Arixtra (GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-OMe)	23	Xu et al. (2011)
GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-GlcNS6S-GlcA-(anhydromannitol)	22	Xu et al. (2011)
GlcNS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-GlcNS6S-GlcA-(anhydromannitol)	23	Xu et al. (2011)
GlcA-GlcNTFA-GlcA-R	nd	Chen et al. (2014)
GlcA-GlcNAc($6N_3$)-GlcA-R	nd	Chen et al. (2014)
GlcA-GlcNAcN_3-GlcA-R	nd	Chen et al. (2014)
GlcNS(6NS)-GlcA-GlcNS-GlcA-R	nd	Chen et al. (2014)
GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	14	Xu et al. (2014)
GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	17	Xu et al. (2014)
GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	15	Xu et al. (2014)
GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-pNP	18	Xu et al. (2014)
GlcNAc6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-GlcNS3S6S-IdoA2S-GlcNS6S-GlcA-pNP	21	Xu et al. (2014)

Recently, one-pot enzymatic synthesis has been explored for the preparation of certain heparin oligosaccharide targets (Chen et al. 2014).

Purification of synthesized oligosaccharides is often required at most reaction steps, either chemical or enzymatic. Protected monosaccharides and disaccharides can be subject to flash chromatography on a silica gel column, but this tends to work best with smaller and more hydrophobic compounds. Larger, unprotected oligosaccharides are more easily purified by gel filtration, although these do not easily separate larger amounts of products or oligosaccharides of similar chain lengths, which can often require the use of reverse-phase or ion-exchange chromatography. In addition, careful consideration must be made of hydrophobic tags and other unnatural groups that may affect the quality of purification (Table 1).

Summary

Chemoenzymatic strategies appear to be the next step in the development of efficient syntheses of heparin oligosaccharides having up to 20 saccharide units. These schemes include natural and unnatural sugar residues as glycosyl donors

and acceptors and use enzymes from biosynthetic processes for extension and modification. Several problems remain to be solved, including ideal enzymatic modification order, optimization of chemical reactions, and purification of larger-scale enzymatic reactions. Exploration of these synthetic methods allows the production of useful oligosaccharides with defined structures and properties and the potential for clinical use.

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