Chapter 15

ENZYMATIC SYNTHESIS OF GLYCOSAMINOGLYCANS: IMPROVING ON NATURE

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The chemoenzymatic synthesis of glycosaminoglycan (GAG) analogs is described. This chapter is divided into three main sections, each describing the use of one enzyme family in GAG synthesis. First, the polysaccharide lyase enzymes are described. These enzymes play crucial role in obtaining homogenous GAG oligosaccharides. Second, GAG synthases, bacterial enzymes that transfer UDP-monosaccharides to acceptor oligosaccharide, GAG synthases are used to obtain homogenous oligosaccharides and polysaccharides. Finally, GAG sulfotransferases, playing a major role in the biosynthesis, are described for the introduction of sulfo groups to the appropriate positions on the polysaccharide backbone.
1. INTRODUCTION

Glycosaminoglycans (GAGs) are highly charged polysaccharides consisting of repeating 1→3 and/or 1→4 linked hexosamine and uronic acid residues (Figure 1).\(^1,2\) GAGs are polydisperse mixtures of polysaccharide chains of varying lengths with an average molecular weight of 10\(^4\)-10\(^6\) Da.\(^2\) GAGs are synthesized by both prokaryotes\(^3\) and eukaryotes and are found in all animal cells.\(^2\) Pharmaceutical heparin is an example of a structurally complex GAG that is prepared from porcine intestinal mucosa and bovine lung in amounts of 30 metric tons/yr. worldwide.\(^4\)

1.1. GAG-Protein Binding, Biological Activities and Therapeutic Significance.

GAGs are highly charged polyanions that interact with hundreds of different proteins.\(^1,5,7\) GAGs bind to proteins primarily through the interaction of their sulfo and carboxyl groups with basic amino acid residues (i.e., arginine and lysine) present in shallow pockets or on the surface of GAG-binding proteins.\(^1,5,6\) For example, specific sequences in the heparin molecule, bind to antithrombin (AT) inhibiting a number of physiologically important serine proteases.\(^5-10\) In the past twenty years GAGs have been shown to play a central role in the regulation of a large number of important cellular processes including cell growth and cell-cell interaction.\(^6,11,12\) The ability of GAGs, such as heparin and heparan sulfate, to regulate mitogenesis and cell migration primarily results from their interaction with growth factors and chemokines.\(^7,13,14\) The exploitation of specific GAG-protein interactions might lead to important new therapeutic advances. For example, specific oligosaccharide sequences within GAGs, interacting with growth promoting proteins (i.e., fibroblast growth factors (FGF)),\(^14-18\) vascular endothelial growth factor (VEGF),\(^19\) heparin binding growth adhesion molecule (HBGAM),\(^20\) hepatocyte growth factor (HGF),\(^21\) insulin-like growth factor (IGF),\(^22\) bone morphogenic protein (BMP),\(^23\) etc.), might be applied to wound healing/tissue growth\(^24\) or to inhibiting angiogenesis in the eradication of tumors.\(^12,25\) GAG-binding proteins (i.e., annexin,\(^26\) antithrombin (AT),\(^27\) heparin cofactor II,\(^28\) coagulation factors,\(^29\) etc.) are important in the regulation of the biochemical cascades involved in coagulation\(^20\) and complement activation.\(^20\) GAG-binding proteins are frequently found on the surface of pathogens (i.e., malaria circumsporozoite protein,\(^31,32\) hepatitis C virus,\(^33\) herpes simplex virus glycoproteins,\(^34\) human immunodeficiency virus (HIV)\(^35\) and dengue envelope protein\(^36\)) facilitating their attachment to and
Figure 1. GAG structure (R = H or SO$_3^-$, X = COCH$_3$, or SO$_3^-$ or H)
infection of mammalian cells. The structure, physiochemical characterization, and many biological activities of GAGs have been reviewed in detail. While all GAGs show important biological activities, this chapter focuses primarily on heparin and heparan sulfate glycosaminoglycans.

Heparin binds to AT, a serine protease inhibitor, which becomes activated and inhibits thrombin and other biologically important serine proteases. Rosenberg et al. and Lindahl et al. deduced the structure of the AT-binding site (Figure 2), by performing a partial depolymerization of heparin, and then purified the products using affinity chromatography on immobilized antithrombin.

The presence of an unusual 3-O-sulfo group on a glucosamine residue was demonstrated by the release of sulfate on the incubation of a pentasaccharide with 3-O-sulfatase and confirmed by NMR studies. Chemical synthesis of a pentasaccharide containing this unique 3,6-di-O-sulfoglucosamine residue substantiated these findings. This chemical synthesis also facilitated detailed structure-activity relationship (SAR) studies on heparin, where numerous analogs were synthesized and their binding properties tested. Such time consuming, and intensive studies, while scientifically valuable, are simply not possible for oligosaccharides corresponding to each of the over 100 proteins that bind heparin.

1.2. Synthesis of Defined GAG Oligosaccharides

Despite recent advances, the total chemical synthesis of GAGs, GAG oligosaccharides and derivatives using current state of the art techniques, has serious limitations due to the heterogeneity of GAG structures, especially in sulfation pattern (Table 1) and the configuration of uronic acids. The multiple steps required to chemically synthesize an intricately substituted carbohydrate, while displaying elegant chemistry, results in a product that simply costs too much. Indeed, much synthetic work has focused on simplifying the target structure (i.e., replacing 2-amino-2-deoxy-D-glucopyranose with D-glucopyranose to prepare active analogs) rather than optimizing the synthesis of the natural product. While recent advances in the chemical synthesis of GAG oligosaccharides by Jacquot, Jan Boons, Seeberger, and others have decreased the number of synthetic steps, increased stereocontrol and enhanced yields, each new target still represents a major research commitment. Despite problems inherent to the chemical synthesis of GAG oligosaccharides, the synthetic AT-binding pentasaccharide Arixtra, has been successfully introduced as a clinical anticoagulant in both the U.S. and Europe. There are several reasons driving the use of this pentasaccharide including: 1) The perceived benefits of a pure, homogenous anticoagulant/antithrombotic agent.
Figure 2. Structure of the AT binding site of heparin/ heparan sulfate
2) The clinical failure of other synthetic non-heparin anticoagulant/antithrombotic agents; and 3) The concern, particularly in Europe, about difficulties establishing that heparin is prion-free.

Table 1. Most encountered sulfation patterns in GAGs

<table>
<thead>
<tr>
<th>Heparin/Heparan sulfate</th>
<th>Chondroitin sulfate</th>
<th>Dermatan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNS6S</td>
<td>GalN6S</td>
<td>GalN4S</td>
</tr>
<tr>
<td>GlcNS6S</td>
<td>GalN4S</td>
<td>GalN4S</td>
</tr>
<tr>
<td>GlcNS3S6S</td>
<td>GalN4,6S</td>
<td></td>
</tr>
<tr>
<td>IdoA2S</td>
<td>GlcA2S</td>
<td>IdoA2S</td>
</tr>
<tr>
<td>GlcA3S</td>
<td>GlcA3S</td>
<td></td>
</tr>
</tbody>
</table>

In the past decade all the heparin biosynthetic enzymes have been isolated, cloned and expressed. These enzymes, while still incompletely characterized, have recently been used to prepare the AT-binding site. This technical achievement resulted in only ~1 µg of AT-binding site and is also limited to the preparation of only naturally occurring structures. A new chemoenzymatic synthetic approach, such as that being developed in our laboratories, affording multimilligram amounts of GAG oligosaccharides with a variety of natural and unnatural structures is needed.

This chapter describes recent advances in chemoenzymatic preparation of GAG analogs. Three enzyme families, GAG lyases, synthases and sulfotransferases, are described for the preparation of natural and unnatural GAG analogues.

2. POLYSACCHARIDE LYASES

The Linhardt laboratory prepares polysaccharide lyases from Flavobacterium hep.arinum and Bacteroides stearcoris. These enzymes have been purified to homogeneity, cloned, and expressed in bacteria. Their physical and catalytic properties and specificity have been extensively investigated.

2.1 Mechanism of lyase catalyzed GAG depolymerization

Microorganisms utilize an eliminative mechanism to breakdown GAGs that involves abstraction of the proton at C-5 of the hexuronic acid by a general base and β-elimination of the 4-O-glycosidic bond with concomitant formation.
of an unsaturated C4-C5 bond within the hexuronic acid located at the non-reducing end (Figure 4). The leaving group must be protonated, either by a side chain acting as a general acid, or by proton abstraction from a water molecule. Proton abstraction and β-elimination are expected to proceed in a stepwise as opposed to concerted manner.68, 70

Figure 3. Eliminative cleavage mechanism for chondroitin lyase family. R = GlcAp; R' = GalpNAc; X = H or SO₃⁻ (where E = enzyme; A = an acidic group on the enzyme capable of protonating the glycosidic oxygen; B = a basic residue capable of removing the acidic proton from the C-5 of the GlcAp residue; and “+” a positive amino acid residue or a metal ion capable of favorably interacting with (stabilizing) the negative charge formed at the carboxyl group.

There is an extensive variation in specificity among lyases for different GAG types. Thus, chondroitinase B is specific for cleavage of dermatan sulfate, accepting only an iduronic acid, whereas chondroitinase ABC will accept either glucuronic acid or iduronic acid (Table 2). Extensive biochemical and mutagenesis studies have been carried out on enzymes obtained from Flavobacterium heparinum (Pedobacter heparinus), which produces three heparin lyases61 and two chondroitinases (FlavoAC and FlavoB)60, 71 and on two general specificity chondroitinases from Proteus vulgaris (PvulABCI and PvulABCII).72

The Linhardt laboratory also prepares chondroitin sulfate lyases from F. heparinum. These enzymes have been purified to homogeneity, cloned, expressed, and the physical and catalytic properties and specificity of chondroitin B lyase and chondroitin AC lyase have been extensively investigated.54-68, 71 Chondroitin lyases act on chondroitin sulfates, dermatan sulfate, and hyaluronic acid and can afford large quantities of oligosaccharides for use as inexpensive building blocks.73 Some of the well characterized polysaccharide lyases acting on glycosaminoglycans are listed in Table 2.
**Table 2. Properties of polysaccharide lyases acting on glycosaminoglycans**

<table>
<thead>
<tr>
<th>Name(^a)</th>
<th>Substrates</th>
<th>Linkage specificity(^b)</th>
<th>Action pattern</th>
<th>Mr (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinase I (Fh(^{61, 63, 74, 75}))</td>
<td>Heparin HS</td>
<td>→4)GlcNS6X(1→4)IdoA2S(1→</td>
<td>Endo</td>
<td>42,800</td>
</tr>
<tr>
<td>Heparinase II (Fh(^{61, 63, 74, 75}))</td>
<td>Heparin HS</td>
<td>→4)GlcNY6X(1→4)UA2X(1→</td>
<td>Endo</td>
<td>84,100</td>
</tr>
<tr>
<td>Heparinase II (Bs(^{74, 76}))</td>
<td>Heparin HS</td>
<td>→4)GlcNY6X(1→4)IdoA2S(1→</td>
<td>Endo</td>
<td>100,000</td>
</tr>
<tr>
<td>Heparinase III (Fh(^{61, 63, 74, 75}))</td>
<td>HS</td>
<td>→4)GlcNY6X(1→4)GlcA(1→</td>
<td>Endo</td>
<td>70,800</td>
</tr>
<tr>
<td>Chondroitinase AC (Fh(^{71, 77}))</td>
<td>CS-A (4S) CS-C (6S) HA</td>
<td>→3)GalNAc(or GlcNac)4X,6X(1→4)GlcA(1→</td>
<td>Endo</td>
<td>74,000</td>
</tr>
<tr>
<td>Chondroitinase AC (Aa(^{66}))</td>
<td>CS-A (4S) CS-C (6S) HA</td>
<td>→3)GalNAc(or GlcNac)4X,6X(1→4)GlcA(1→</td>
<td>Exo</td>
<td>79,840</td>
</tr>
<tr>
<td>Chondroitinase B (Fh(^{73}))</td>
<td>DS(CS-B)</td>
<td>→3)GalNAc4X,6X(1→4)IdoA2X(1→</td>
<td>Endo</td>
<td>55,200</td>
</tr>
<tr>
<td>Chondroitinase ABC (Bs(^{73}))</td>
<td>CS-A (4S) CS-C (6S) DS(CS-B)</td>
<td>→3)GalNAc4X,6X(1→4)UA2X(1→</td>
<td>Endo</td>
<td>116,000</td>
</tr>
<tr>
<td>Hyaluronate lyase (Pa(^{73}))</td>
<td>HA CS-A (4S) CS-C (6S)</td>
<td>→3)GalNAc(or GlcNac)4X,6X(1→4)GlcA(1→</td>
<td></td>
<td>85,110</td>
</tr>
</tbody>
</table>

\(^a\) Fh, *Flavobacterium heparinum*; Aa, *Arthrobacter aurescens*; Pv, *Proteus vulgaris*; Bs, *Bacteroides stercoris*; Pa, *Propionibacterium acnes*

\(^b\) The primary sites of action are shown. X=SO\(_3\)Na, Y=SO\(_3\)Na or COCH\(_3\), UA= glucuronic or iduronic acid.
2.2 The use of polysaccharide lyases in preparation of GAG oligosaccharides

Polysaccharide lyases have been used to produce Δ^4,5-uronate containing disaccharides (Figure 3, 1a-1r) and higher oligosaccharides from heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate, hyaluronan, and chemically modified GAGs. 62, 68, 74, 80-82

**Figure 4.** Glycosaminoglycan disaccharides prepared in the Linhardt laboratory that will serve as synthetic building blocks. Structure 1a is the abundant heparin trisulfated disaccharide.

Because both the polysaccharide substrates and enzymes are relatively inexpensive, these oligosaccharides can be prepared in large quantities at a low cost. Indeed, the discovery of new GAGs, such as acharan sulfate 83 as well as *Escherichia coli* polysaccharide K5 (heparosan), 84 can afford further structures in large quantities and at low cost (Figure 1). Currently this approach, for
preparing large quantities of inexpensive GAG-derived oligosaccharides, is limited by the small number of available lyases and their limited specificities. Recently we proposed that these lyase-derived oligosaccharides could be chemically linked together to form larger oligosaccharides with the requisite structure for a wide variety of biological activities. The first objective would be to differentially protect enzymatically prepared desulfated disaccharides and to use these neutral disaccharides to prepare larger target oligosaccharides (Figure 5). The advantages of this approach are 1) disaccharides can be assembled into oligosaccharides with a reduced number of glycosylation reactions and 2) a high level of structural complexity (i.e., stereochemistry, sulfation pattern) is already built into these disaccharides. In addition, we investigated the use of 2,2,2-trifluorodiazoethane as a reagent for sulfo group protection in enzymatically prepared CS disaccharides (Figure 5).

This approach was first used for sulfate ester protection in carbohydrates by Flitsch and co-workers. Once the sulfo groups have been protected, the free hydroxyl and carboxyl groups could be protected in organic solvents used in standard carbohydrate synthesis. This chemistry has been successfully used to selectively protect primary and secondary O- and N-sulfo groups in unprotected sulfated mono- and di-saccharides in high yields.

3. GLYCOSAMINOGLYCAN SYNTHASES

Most described glycosyltransferase enzymes catalyze the transfer of only one specific type of monosaccharide to an acceptor molecule. In contrast, the various glycosyltransferases (GAG synthases) that produce the GAG polymer backbone transfer two distinct monosaccharides (HexNAc and GlcA) to the growing chain in a repetitive fashion. In all known organisms, the enzymes synthesize the alternating sugar repeat backbones utilizing UDP-sugar precursors and metal cofactors (e.g., magnesium and/or manganese ion) near neutral pH according to the overall reaction:

\[ n\text{UDP-GlcA} + n\text{UDP-HexNAc} \rightarrow 2n\text{UDP} + [\text{GlcA-HexNAc}]_n \]  

(Eq. 1)

where HexNAc is GlcNAc (N-acetylglucosamine) or GalNAc (N-acetylgalactosamine). Depending on the specific GAG and the particular organism or tissue examined, the degree of polymerization, \(n\), ranges from \(10^{34}\). The GAG synthase enzymes are found in a variety of organisms including all animals from hydras to humans, certain pathogenic bacteria, and at least one virus. However, with respect to chemoenzymatic synthesis of
Figure 5. Preparation of desulfated and sulfoprotected disaccharide starting materials for the synthesis of CS/DS oligosaccharides.
carbohydrates, the enzymes from the Gram-negative bacteria *Pasteurella multocida* are extremely useful because: (a) the catalysts for the production of all three uronic acid-containing GAGs are present in various serotypes of this microbe, (b) the isolated recombinant enzymes may be produced in soluble forms that either (c) rapidly form long polymer chains *in vitro* or (d) readily extend exogenously supplied GAG oligosaccharides *in vitro*. In contrast, most of the other known enzymes are not as easy to use (membrane proteins obtained in poor yields), exhibit low polymerization activity after isolation, and/or cannot elongate existing polymers.

### 3.1 *Pasteurella* Glycosaminoglycan Synthases

The native *Pasteurella* bacterial GAG glycosyltransferases are associated with the cell membranes; this localization makes sense with respect to synthesis of polysaccharide molecules destined for the cell surface to form the sugar coating known as a capsule. It appears that these enzymes associate with other membrane components of the Gram-negative capsular polysaccharide transport apparatus and, fortunately, are themselves not integral membrane proteins. The *Pasteurella* native sequence catalysts possess very good sugar transfer specificity (*i.e.* will use only the authentic UDP-sugar precursors) but will accommodate a variety of acceptor oligosaccharides (*i.e.* certain non-cognate GAG sugars may be elongated; discussed later). All the enzymes can be produced in a functional state by utilizing the appropriate expression plasmid in most laboratory strains of *Escherichia coli*.

The first *Pasteurella* GAG synthase to be identified was the 972-residue HA synthase from Type A strains, PmHAS (*Table 3*). This single polypeptide transfers both sugars, GlcNAc and GlcA, to form the HA disaccharide repeat. UDP-Glc or UDP-GalNAc do not serve as substrates for this enzyme. The chondroitin chain is chemically identical to HA except that its HexNAc residue is GalNAc instead of GlcNAc. The 965-residue Type F enzyme, PmCS, which has ~90% identity at the gene and protein level to PmHAS, polymerizes unsulfated chondroitin chains. This enzyme uses UDP-GalNAc, but not the C4 epimer UDP-GlcNAc, as a donor.

The 617-residue Type D *Pasteurella* heparosan synthase, PmHS1, and the 651-residue Type A,D, and F *Pasteurella* cryptic heparosan synthase, PmHS2, are not very similar at the protein level to either PmHAS or PmCS. Both of these enzymes produce unsulfated heparosan chains. The PmHS enzyme, however, resembles a fusion of the *E. coli* K5 KfiA and KfiC proteins.
Table 3. *Pasteurella* Glycosaminoglycans and Synthases

<table>
<thead>
<tr>
<th>Polysaccharide*</th>
<th>Repeat Structure</th>
<th>P. multocida</th>
<th>enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA, Hyaluronan</td>
<td>[\beta1,4 \text{GlcA}-\beta1,3 \text{GlcNAc}]</td>
<td>Type A</td>
<td>PmHAS</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>[\beta1,4 \text{GlcA}-\beta1,3 \text{GalNAc}]</td>
<td>Type F</td>
<td>PmCS</td>
</tr>
<tr>
<td>Heparosan</td>
<td>[\beta1,4 \text{GlcA}-\alpha1,4 \text{GlcNAc}]</td>
<td>Type D</td>
<td>PmHS1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type A,D,F</td>
<td>PmHS2</td>
</tr>
</tbody>
</table>

* no bacterial polymer is known to be naturally sulfated.

responsible for making heparosan in this human pathogen. Even though HA and heparosan polysaccharides have identical sugar compositions, the basic enzymology of their synthases must differ. All UDP-sugar precursors are alpha-linked. HA is an entirely beta-linked polymer, therefore, only inverting mechanisms are utilized during the sugar transfer process. In contrast, heparosan contains alternating alpha- and beta-linkages suggesting that both a retaining and an inverting mechanism are involved in synthesis. The production of the two types of anomeric glycosidic bonds probably requires distinct catalytic sites.

3.1.1 Domain Structures of *Pasteurella* GAG Synthases

Three-dimensional structures are not yet available for any GAG or polysaccharide synthase, but the PmHAS and PmCS enzymes have been shown to contain two independent glycosyltransferase sites by biochemical analysis of various mutants (Figure 6).93, 94 The HexNAc-transferase or the GlcA-transferase activities of the Pasteurella enzyme can be assayed separately in vitro by supplying the appropriate acceptor oligosaccharide and only one of the UDP-sugar precursors (as in Equations 2 or 3). Two tandemly repeated sequence elements are present in PmHAS and PmCS. Each element contains a short sequence motifs containing aspartate-glycine-serine or aspartate-x-aspartate. Mutation of the aspartate residue in any one motif of PmHAS or PmCS converts the dual-action synthase into a single-action glycosyltransferase.93, 94 The newly described PmHS1 and 2 also appear to possess two domains in our ongoing studies.
pmHAS

<table>
<thead>
<tr>
<th>DGS DCD WGGED</th>
<th>DGS DSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc-Tase</td>
<td>GlcA-Tase</td>
</tr>
</tbody>
</table>

pmCS

<table>
<thead>
<tr>
<th>DGS DCD WGGED</th>
<th>DGS DSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalNAc-Tase</td>
<td>GlcA-Tase</td>
</tr>
</tbody>
</table>

Figure 6. Schematic of PmHAS and PmCS Domains.
The truncated, soluble recombinant enzymes contain two distinct sugar transfer domains. If the aspartates (D) are mutated in one domain, then that glycosyltransferase activity is destroyed, but the other domain functions normally. The PmHS1 and 2 domain structures are still being delineated.

3.1.2 Elongation Activity of Recombinant Pasteurella Synthases

The E. coli-derived recombinant PmHAS, PmCS, and PmHS1,2 will elongate exogenously supplied GAG-oligosaccharide acceptors in vitro; these virgin enzymes lack a nascent GAG chain because the typical host utilized for expression lacks the required UDP-GlcA precursor. The Pasteurella GAG synthases add sugars to the non-reducing terminus of the linear polymer chain as determined by testing defined acceptor molecules. Therefore, the reducing termini of the acceptor sugar may be modified without compromising elongation. We have found that various tagged (e.g., radioactive or fluorescent) or immobilized (e.g., on plastic or glass surfaces) acceptor molecules are elongated by PmHAS, PmCS, or PmHS1 (U.S. Patent 6,444,447).

Experiments with recombinant PmHAS demonstrated that single sugars are added to the growing chain sequentially; the intrinsic fidelity of each transfer step assures the production of the GAG repeat structure. Another potential mechanism, the simultaneous addition of a disaccharide unit to the nascent chain, does not occur. The other synthases, PmCS, PmHS1 and 2, behave in a similar fashion adding saccharides one at a time.

Thus other possible synthetic reactions include:
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\[ \text{UDP-GlcA} + [\text{HexNAc-GlcA}]_n \rightarrow \text{UDP} + \text{GlcA-[HexNAc-GlcA]}_n \]  \hspace{1cm} (\text{Eq. 2})

\[ \text{UDP-HexNAc+[GlcA-HexNAc]}_n \rightarrow \text{UDP+HexNAc-[GlcA-HexNAc]}_n \]  \hspace{1cm} (\text{Eq. 3})

An interesting feature of the Pasteurella GAG synthases is that they appear to elongate the acceptors in a non-processive fashion \textit{in vitro} with chain release between sugar addition steps. As noted later, this property facilitates chemoenzymatic synthesis reactions \textit{in vitro}.

3.1.3 Synthesis of Monodisperse GAG polymers

The Pasteurella GAG synthases add sugars onto the non-reducing terminus of an existing GAG chain in a rapid fashion, but if no acceptor is present, the enzymes will spontaneously initiate new chain formation \textit{de novo}. This initiation rate is slower than the elongation rate. The DeAngelis Laboratory found it is possible to accelerate and to synchronize GAG chain production by adding an acceptor molecule to the reaction mixture. The synchronized non-processive polymerization of all chains in concert results in the production of product with low polydispersity \((M_W/M_N \approx 1)\) (Figure 7). In nature, GAG polymers (especially HA) are rather polydisperse \((M_W/M_N \approx 2-5)\) populations. Remarkably, polymers of up to 0.5 MDa are made with polydispersity values in the range of \(\approx 1.02\) and those up to 2 MDa have values of \(\approx 1.2\). \((M_W/M_N = 1\) for a monodisperse polymer).

The size of the final GAG polymer is controlled by the stoichiometric ratio of the acceptors to the UDP-sugars. If the same finite amount of precursors is used in two parallel reactions, then a small amount of acceptor spawns a few long chains, while in contrast, a large amount of acceptors results in many short chains. PmHAS and PmCS catalysts are routinely used in synthesis and development of the analogous PmHS1 system is in progress. The availability of defined, uniform polymer preparations is important because in vertebrate biology, the size of the GAG chain (especially for HA) often dictates its effect on cells or tissues.

3.1.4 Synthesis of Defined, Monodisperse Oligosaccharides

The DeAngelis laboratory has developed the chemoenzymatic synthesis of monodisperse GAG oligosaccharides using single-action mutants. Potential medical applications for HA oligosaccharides \((n = 3-10)\) include
Figure 7. Monodisperse HA preparations versus natural HA and DNA standards.
This agarose gel with Stains-All detection depicts the narrow size distribution of the HA made with synchronized reactions (left 2 lanes, a mixture of 5 reactions ranging from 1.5 MDa top to 27 kDa bottom) in comparison to HA from bacteria or chicken (middle 4 lanes). The monodisperse HA bands rival the DNA bands (right lane) which are all composed of a single molecular species.

killing cancerous tumors\cite{96} and enhancing wound vascularization.\cite{97} The Pasteurella HA synthase, a polymerizing enzyme that normally elongates HA chains rapidly (~1 to 100 sugars/second) as in Equation 1, was converted by mutagenesis into two single-action glycosyltransferases. The resulting GlcA-transferase and GlcNAc-transferase are appropriate for performing reactions in Equations 2 or 3, respectively. For convenience, soluble forms of mutant pmHAS truncated at the carboxyl termini were purified and immobilized onto beads for utilization as solid-phase catalysts.\cite{3} Similar operations with PmCS create an immobilized GalNAc-transferase. The immobilized enzyme-reactors were used in an alternating fashion to produce quantitatively desirable sugars in a controlled, stepwise fashion without purification of the intermediates (Figure 8). The PmHAS and PmCS enzymes are also relatively insensitive to the concomitant UDP byproduct accumulation after many reactor steps. This technology platform is also amenable to the synthesis of tagged (fluorescent-, medicant- or radioactive-labeled) oligosaccharides for biomedical testing.
Figure 8. Mass Spectra of HA22 formed with Immobilized Enzyme reactors. This MALDI-ToF MS shows the product formed by extending a HA4 tetrasaccharide starting sugar with 18 reactor steps (9 with GlcNAc-Tase and 9 GlcA-Tase in alternating fashion). No intermediary purifications were performed before the final desalting step.

4. GLYCOSAMINOGLYCAN SULFOTRANSFERASES

Glycosaminoglycan sulfotransferases carry out the sulfonation of the polysaccharides provided with various backbone structures to biosynthesize the highly sulfated polysaccharides, glycosaminoglycans (GAGs). The enzymes transfer the sulfo group from a universal sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to the NH2- or OH positions of the carbohydrate unit of the GAG. Glycosaminoglycan sulfotransferases are Golgi sulfotransferases and include chondroitin sulfate, keratan and heparan sulfate sulfotransferases, which are involved in the biosynthesis of chondroitin sulfate, keratan sulfate, and heparan sulfate, respectively (Table 4). The enzymes are highly selective toward the acceptor sites by recognizing the saccharide backbone structures as well as the surrounding detailed saccharide sequences. The recent availability of GAG sulfotransferases has offered additional tools for investigating the relationship between the sulfated saccharide sequences and physiological roles of GAGs and the mechanism of the biosynthesis of GAGs.58
Among various glycosaminoglycans, the biosynthesis of heparan sulfate (HS) has been studied extensively. The biosynthesis of HS occurs in the Golgi apparatus, and is involved in four classes of sulfotransferases: N-deacetylase/N-sulfotransferases (NDST) removes the acetyl group from the N-acetylated glucosamine (GlcNAc) unit and sulfonates the resultant unsubstituted glucosamine unit (GlcNH2) to form N-sulfo glucosamine; 2-O-sulfotransferase (2-OST) sulfonates the 2-OH position of glucouronic acid (GlcA) or iduronic acid (IdoA) units; 6-O-sulfotransferase (6-OST) sulfonates the 6-OH position of N-sulfo- or N-acetylated glucosamine units; and 3-O-sulfotransferase (3-OST) sulfonates the 3-OH position of the N-sulfo or N-unsubstituted glucosamine units. NDST was the first reported HS sulfotransferase. Whether all these enzymes are required to act in a precisely sequential manner to generate the fully modified HS is not completely clear. However, a large amount of evidence demonstrated that the generation of N-sulfo glucosamine unit (by NDST) is the very first modification step followed by epimerization and 2-O-sulfonation.

Figure 9. The substrate specificities of 3-OST-1, 3-OST-3, and 3-OST-5.

3-OST-1 sulfonates the glucosamine unit that is linked to a nonsulfonated glucuronic acid at the nonreducing end; 3-OST-3 sulfonates a glucosamine unit that is linked to 2-O-sulfo iduronic acid at the nonreducing end; and 3-OST-5 sulfonates both type of disaccharides. R represents –H or –SO₃H.
Table 4. Glycosaminoglycan sulfotransferases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heparan sulfate sulfotransferases</strong></td>
<td></td>
</tr>
<tr>
<td>N-deacetylase/N-sulfotransferase⁹⁸, ⁹⁹</td>
<td>Removal of the acetyl group and sulfation of NH position of GlcNAc</td>
</tr>
<tr>
<td>2-O-sulfotransferase⁹⁹</td>
<td>Sulfation of 2-OH position of GlcA or IdoA</td>
</tr>
<tr>
<td>6-O-sulfotransferase⁹¹, ⁹²</td>
<td>Sulfation of 6-OH position of GlcNS or GlcNAc</td>
</tr>
<tr>
<td>3-O-sulfotransferase⁹³</td>
<td>Sulfation of 3-OH position of GlcNS or GlcNH₂</td>
</tr>
<tr>
<td><strong>Keratan sulfotransferase</strong></td>
<td></td>
</tr>
<tr>
<td>Keratan sulfate Gal-6-sulfotransferase⁹⁴</td>
<td>Sulfation of 6-OH position of Gal</td>
</tr>
<tr>
<td><strong>Chondroitin sulfate sulfotransferase</strong></td>
<td></td>
</tr>
<tr>
<td>Chondroitin 4-sulfotransferase⁹⁵</td>
<td>Sulfation of 4-OH position of GalNAc</td>
</tr>
<tr>
<td>Chondroitin 6-sulfotransferase⁹⁶</td>
<td>Sulfation of 6-OH position of GalNAc</td>
</tr>
<tr>
<td>GalNAc (4SO₄) 6-sulfotransferase⁹⁷</td>
<td>Sulfation of 6-OH position of GalNAc4S</td>
</tr>
<tr>
<td>Uronosyl 2-O-sulfotransferase⁹⁸</td>
<td>Sulfation of 2-OH of GlcA or IdoA</td>
</tr>
</tbody>
</table>
All HS sulfotransferases, with the exception of 2-OST, are present in multiple isoforms. These isoforms are highly homologous in amino acid sequences and expressed at distinct levels among different tissues, probably playing an important role in biosynthesizing tissue-specific HS. For example, NDST is present in four different isoforms, 6-OST is present in three isoforms, and 3-OST is present in seven isoforms. The unique substrate specificities of different 3-OST isoforms have been reported, demonstrating that different isoforms recognize the saccharide residues at the nonreducing end of the acceptor site (Figure 9). Very interestingly, the HS modified by different 3-OST isoforms exhibit distinct physiologic/pathophysiologic functions. HS modified by 3-OST-1 binds to antithrombin, carrying anticoagulant activity, whereas HS modified by 3-OST-3 binds to herpes simplex virus glycoprotein D and serves as an entry receptor for herpes simplex virus 1. HS modified by 3-OST-5 exhibit both the anticoagulant activity and serves as an entry receptor for herpes simplex virus 1 entry receptor. NDST has four different isoforms. It is believed that these isoforms are specialized in generating specific N-sulfoglucosamine clusters and control relative ratio of GlcNH2/GlcNAc/GlcNS. The substrate specificities among 6-OST isoforms are not experimentally distinguishable. The substrate subtleties among the isoforms allow the synthesis of unique sulfonated saccharide sequences.

4.1. Structure of HS sulfotransferases

There is considerable interest in understanding the mechanism used by the sulfotransferases to recognize their substrates. Furthermore, understanding this mechanism could aid the design of specific GAG sulfotransferase inhibitors to manipulate the biosynthesis of the unique subtypes of sulfonated saccharide structures. Among GAG sulfotransferases, only three crystal structures of HS sulfotransferases are available, including NST-1, 3-OST-1 and 3-OST-3. NST-1 is the N-sulfotransferase domain of NDST isoform -1, which transfers the sulfo group to the N-position of glucosamine unit. All three enzymes show a very similar overall folded structure, consisting of a five-stranded parallel β-sheet flanked on both sides by α-helices. The structural features of HS binding sites are distinct between NST-1 and 3-OST-1, supported by the fact NST-1 sulfonates the polysaccharide with low or no sulfation, whereas 3-OST-1 sulfonates the polysaccharide with high level of sulfation. The structures of the active sites of 3-OST-1 and 3-OST-3 are very similar. Based upon the crystal structures and the results of mutagenesis and computational analyses, the role of several amino acid residues in catalyzing the sulfotransferase activity were identified. A unique hydrogen bond network involved in key catalytic residues was discovered in 3-OST-1 and 3-OST-3, but not in NST-1, suggesting that this network contributes to the 3-O-sulfotransferase activity.
The best available example to understand how the sulfotransferases interact with their substrates is from the study of the structure of a ternary complex of 3-OST-3/PAP/tetrasaccharide (where PAP represents 3’-phosphoadenosine 5’-phosphate). From this structure, one can clearly observe the interaction between the amino acid residues that participate in the binding to the substrate. The 3-OH position of the glucosamine unit (acceptor site) is locked into a position that is about 2.8 Å to the catalytic base residue. At least six amino acid residues interact with the different functional groups of the saccharide units around the reducing end and the nonreducing end of the glucosamine acceptor (Figure 10).

![Figure 10. Model of the substrate recognition of 3-OST-3.

This model is based on the crystal structure of the ternary complex (3-OST-3/PAP/tetrasaccharide) as described above. The acceptor site (OH) is depicted in red. The catalytic base (Glu184) does not contribute to the substrate specificity, rather increase the acidity of the proton of the –OH acceptor. R represents proton (-H) or a sulfate (-SO₃); RE represents reducing end; NRE represents non-reducing end. The three saccharide residues at the NRE of the tetrasaccharide are shown.](image)

In addition, the iduronic and uronic acid units of the tetrasaccharide substrate are present in a skew-boat conformation when the tetrasaccharide binds to 3-OST-3, whereas, these two units are present in a chair conformation when this tetrasaccharide binds to fibroblast growth factor 2. This observation suggests that the conformations of the saccharide units might contribute to the binding recognition between enzymes and substrates. Using site-directed mutagenesis approach, two amino acid residues (Q255 and K368) of 3-OST-3 were identified to participate in binding the substrate during the catalysis. Mutation of Q255 and K368 led to the significant loss of 3-OST-3 activity,
whereas mutations of similar amino acid residues of 3-OST-1 have no effect on the enzymatic activity. It was also reported that 3-OST-1 undergoes a conformational change when it binds to the substrate, suggesting that protein flexibility or potential allosteric effects on the substrate recognition.\textsuperscript{125} The contribution of the conformational changes of 3-OST-1 to the substrate specificity requires further investigation.

### 4.2 Synthesis of HS using HS biosynthetic enzymes

Chemical synthesis has been the major route to obtain structurally defined heparin and HS oligosaccharides.\textsuperscript{126} The most important example involves the structure of AT-binding pentasaccharide (Arixtra).\textsuperscript{127} As described above, Arixtra is a specific factor Xa inhibitor. Unfortunately, the total synthesis of heparin and HS oligosaccharides, larger than pentasaccharides, is extremely difficult. HS analogs with 14 or 16 saccharide units inhibit the activity of thrombin, but these compounds are simplified hybrid molecules of HS oligosaccharides and highly sulfonated glucose units and are not the naturally occurring structures.\textsuperscript{54} While a number of groups continue to pursue the synthesis of heparin,\textsuperscript{128} it has become clear that chemical synthesis alone will be incapable of generating most larger oligosaccharide structures. The application of HS biosynthetic enzymes for generating large heparin and HS oligosaccharides with desired biological activities offers a promising alternative approach.

The enzymatic synthesis includes one-step enzymatic modification or multi-step modification as described below. One step enzymatic modification to synthesize biologically active oligosaccharides or polysaccharide saccharides is straightforward. For example, 2-OST was used to modify HS oligosaccharide libraries to synthesize FGF and FGF receptor binding domains of HS.\textsuperscript{115, 129} Likewise, a herpes simplex virus glycoprotein D-binding domain was isolated from 3-OST-3-modified HS oligosaccharide library.\textsuperscript{130} However, this approach is limited by the availability of the oligosaccharide library. Furthermore, the subsequent purification is often difficult.

The multi-step enzymatic synthesis of the antithrombin-binding pentasaccharide from E. coli capsular polysaccharide was reported by Kuberan and colleagues (Figure 11).\textsuperscript{60} This approach demonstrated for the first time the feasibility of enzymatic synthesis of a HS oligosaccharide with defined structure and anticoagulant polysaccharides. Unfortunately, only microgram amounts of products were generated, making a general approach for extensive biological studies impossible. Recently, Lindahl and colleagues reported an alternative chemoenzymatic approach for the synthesis of anticoagulant heparin from heparosan, the E. coli K5 capsular polysaccharide.\textsuperscript{131} This method utilized the C5 epimerase to convert D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), followed by the chemical persulfonation and finally selective desulfonation.
While this approach afforded gram quantities of a heparin-like polysaccharide with anticoagulant activity, unnatural saccharide units, such as 3-O-sulfo-D-glucuronic acid, were present in their product, suggesting a limitation in the selectivity of chemical sulfonation/desulfonation in HS synthesis.

Two obstacles remain in improving the scale the enzymatic synthesis of HS: the availability of large amount of HS sulfotransferases and the inhibition effect of 5′-phosphoadenosine 3′-phosphate (PAP), the desulfated product of PAPS. Liu and Linhardt Labs have recently developed an approach to enable the scale of the enzyme-catalyzed O-sulfations using completely desulfated and N-sulfated heparin (9) as a starting material (Figure 12).132

First, highly active HS O-sulfotransferases were expressed in E. coli, allowing the access of large amount of sulfotransferases. Second, the sulfotransfer reactions were coupled with the PAPS regeneration system that was developed by the Wong group,133 eliminating the inhibition effect of PAP. The PAPS regeneration system utilizes arylsulfotransferase IV, which can converts PAP to PAPS as illustrated in Figure 12B. In addition, the PAPS regeneration system permits the use of the p-nitrophenol sulfate PNPS as the sulfo donor and requires only catalytic amounts of PAP, significantly reducing the cost of synthesis.133 The immobilized enzymes used in this approach are capable of reuse and the HS sulfotransferases show improved thermal stability, suggesting that this approach can be easily expanded for a large scale synthesis. This method generates the milligram scale of sulfated polysaccharide with the desired biological activities, including the anticoagulant activity as measured by the anti-Xa and anti-IIa activities (compound 13), the activity in triggering FGF/FGF receptor signaling (compounds 10, 11, 12a and 12b) and the binding to herpes simplex virus glycoprotein D (compound 14).

5. CONCLUSIONS

Recent advances in carbohydrate chemistry have made GAG oligosaccharides more accessible while still posing a significant synthetic challenge. The enzymes involved in GAG biosynthesis and metabolism play crucial role in understanding the structures of these heterogeneous polysaccharides as well as preparing structurally defined GAGs. The use of three GAG modifying enzymes, lyases, synthases and sulfotransferases, have been applied to the preparation of biologically potent, structurally defined GAG oligosaccharides and polysaccharides.

Polysaccharide lyases can be combined with separation methods such as chromatography and electrophoresis for the preparation of glycosaminoglycan oligosaccharides for biological evaluations as well as for disaccharide analysis, oligosaccharide mapping and polysaccharide sequencing.
Figure 11. Synthesis of antithrombin-binding pentasaccharide from E. coli K5 capsular polysaccharide.
Figure 12. Schematic synthesis of sulfonated polysaccharides and PAPS regeneration system.

Panel A shows the stepwise enzymatic synthesis of sulfonated polysaccharides using HS sulfotransferases. The description of intermediate polysaccharides is shown in the text. Compound 12a and 12b were prepared by inverting the order of sulfonation steps. 12a was prepared by incubating compound 9 with 2-OST followed by 6-OST, while 12b was prepared by incubating compound 9 with 6-OST followed by 2-OST. Panel B shows the reaction catalyzed by arylsulfotransferase IV (AST-IV) to generate PAPS. R = -H or -SO₃.
Versatile, malleable Pasteurella synthases have been harnessed as useful catalysts for the creation of a variety of defined GAG or GAG-like polymers ranging in size from small oligosaccharides to huge polysaccharides. These materials should be useful for a wide spectrum of potential biomedical products for use in the areas of cancer, coagulation, infection, tissue engineering, drug delivery, surgery, and viscoelastic supplementation.

GAG sulfotransferases play critical roles in biosynthesizing the GAGs with diversified biological functions. The availability of the GAG sulfotransferases not only leads to an improved understanding of the structure-function relationship of the sulfated polysaccharides but also opens new routes to synthesize these molecules. Although the current attention has been focused on HS and heparin synthesis, it is conceivable that a similar enzymatic approach could be used for the synthesis of other GAGs, including chondroitin sulfates and keratin sulfates. A further simplified synthetic approach could lead to a general method to prepare “recombinant” glycosaminoglycans, in which will be possible to use glycomics approach to study the effects in different biological systems.

The use of these three enzyme families in combination should greatly enhance the success of target GAG synthesis. The lyase prepared oligosaccharides could be used as templates to be elongated with UDP-monosaccharide donors using synthases then the sulfotransferases could introduce the sulfo groups into the specific positions leading to the synthesis of desired GAG structures. In summary, expansion of the enzymatic synthesis, in combination with chemical synthesis, will lead to novel therapeutic reagents in physiological and pathophysiological events.

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