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Synthetic and Natural Polysaccharides with Anticoagulant Properties
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1. INTRODUCTION
Anticoagulant polysaccharides have been of interest to the medical profession since discovery of heparin by McLean in 1916 [1]. Since that time considerable research has been directed at improving anticoagulant and antithrombotic properties of polysaccharides. Other compounds with structures similar to heparin have been studied as heparin substitutes. This chapter will discuss polysaccharides, both natural and synthetic, which have action on the hemostatic system.

A. Polysaccharides
Polysaccharides are important molecules that are often neglected in most reviews of bioactive biopolymers. Other biopolymers such as proteins, DNA, and RNA have been highly publicized in both the scientific literature and the lay press. Polysaccharides have received little such promotion even though they are widely distributed throughout nature and have highly organized structure. These are important molecules involved throughout the body in signal transduction and cell adhesion. Polysaccharides are also widely distributed as constituents of foods found in most of our diets. These biodegradable molecules are also often utilized as gellants, thickeners, film formers, fillers, and delivery systems in pharmaceutical and cosmetic applications. Moreover, they are often derived from renewable sources and therefore serve the above purposes in a relatively cost-effective fashion. Although often used as adjuncts in pharmaceutical applications, some polysaccharides are also used for their pharmacological action. The most notable pharmacologically active polysaccharide is heparin, which is used medicinally as an anticoagulant. Others polysaccharides with pharmacological activity are also exploited as therapeutic agents [2]. Such compounds along with heparin will be discussed within this chapter.

B. Anticoagulants and Antithrombotics
It is difficult to understand the action of anticoagulants and antithrombotics without a basic understanding of the hemostatic system, therefore a brief overview will be provided here. When blood vessels are damaged, bleeding occurs and the formation of a hemostatic plaque is initiated. Platelets adhere to the perivascular collagen through platelet surface glycoproteins and adhesion proteins [3]. Platelet aggregation is also important to the hemostatic system, but the physiology and biochemistry surrounding platelet function is beyond the scope of this chapter. Of most importance is thrombin formation in the blood coagulation system. Blood coagulation is based on an intrinsic homeostasis and represents a balance of procoagulant and anticoagulant factors [3]. Thrombin (factor IIa) formation is essential to coagulation as it catalyzes the formation of an insoluble fibrin clot, which occurs late in hemostasis [4]. Thrombin is formed through the sequential activation (Fig. 1) of coagulation factors normally circulating within plasma as zymogens (Table 1). Upon activation (i.e., factor II—factor IIa) a catalytic carboxy-terminal domain is exposed and the activated coagulation factor functions as a serine protease. Activation of coagulation occurs through two distinct pathways. The intrinsic pathway is initiated by collagen or contact with a charged surface. The extrinsic pathway begins with damage to vessel walls, causing the release of tissue factor [3]. These two pathways converge with the activation of factor X (factor X—factor Xa) and the cascade continues down a common pathway, resulting in thrombin (factor IIa) generation and eventually fibrin (clot) formation (Fig. 1) [4].
The current consensus is that coagulation is predominantly initiated through the extrinsic pathway [3]. Several anticoagulant systems regulate the procoagulant pathways described above. The first and most well known of these systems is the antithrombin III (ATIII) system. Antithrombin III is a circulating serine protease inhibitor (serpin) synthesized in the liver. Heparan sulfate, having a structure closely related to heparin, residing on the surface of endothelial cells, forms a complex with ATIII. This complex interacts with coagulation factors IIa, IXa, Xa, and Xla rendering them inactive [3]. Heparin cofactor II (HCII) is another serpin capable of inhibiting thrombin after binding heparan sulfate or the structurally related polysaccharide, dermatan sulfate. Protein C and thrombomodulin are the main players in another anticoagulation system. Activation of protein C occurs when it is presented with thrombin and thrombomodulin (endothelial cofactor) on the endothelial cell surfaces. Active protein C together with protein S, a cofactor, causes the degradation of factors V and VIII. This system is believed to be a major anticoagulation pathway, based on data from studies of acquired and inherited coagulation defects [3]. Another anticoagulation system employs a protein called tissue factor pathway inhibitor (TFPI) as its major player. This system is a major inhibitor of the extrinsic pathway and studies suggest that its disruption is not compatible with life. TFPI (a protein bound to the endothelium) is capable of forming quaternary complexes with factors VIIa and Xa in the presence of calcium and phospholipids [3]. This quaternary complexation renders these factors inactive.

New studies on hemostasis are reported daily and this brief review does not do justice to the immense complexity of this system. The human body requires consistent circulation of nutrients and oxygen to its tissues while maintaining a repair process capable of reversing the liquid

<table>
<thead>
<tr>
<th>Table 1 Coagulation Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathway</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td><strong>Intrinsic</strong></td>
</tr>
<tr>
<td>Factor XII</td>
</tr>
<tr>
<td>Factor XI</td>
</tr>
<tr>
<td>Factor IX</td>
</tr>
<tr>
<td>Factor VIII</td>
</tr>
<tr>
<td><strong>Extrinsic</strong></td>
</tr>
<tr>
<td>Tissue factor</td>
</tr>
<tr>
<td>Factor VII</td>
</tr>
<tr>
<td><strong>Common</strong></td>
</tr>
<tr>
<td>Factor X</td>
</tr>
<tr>
<td>Factor V</td>
</tr>
<tr>
<td>Prothrombin (factor II)</td>
</tr>
<tr>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Factor XII</td>
</tr>
</tbody>
</table>
nature of blood when needed. This feat is truly amazing
and to this day is unreplicated by man.

II. NATURAL POLYSACCHARIDES WITH
ANTICOAGULANT PROPERTIES

A. Heparin and Low Molecular Weight Heparins

1. Heparin and Heparan Sulfate

Background

Heparin is derived from animal tissues and has been
used widely as a clinical anticoagulant since 1935. It was
discovered in 1916 by Jay McLean, a second-year medical
student, working under the direction of physiologist
William Howell at Johns Hopkins University [5]. An
understanding of heparin's structure developed gradually.
In 1928, Howell correctly identified one of the sugars in
heparin to be a uronic acid [6]. Early researchers showed
that heparin also contained O-sulfated and N-sulfosubstituted
glucosamine residues. By 1970, iduronic acid
was demonstrated to be the major uronic acid component
and a generalized structure of heparin could be drawn [7].
It is only in the past 20 years that the molecular mech-
anism behind the anticoagulant/antithrombotic effects
were elucidated. With the discovery of the structure of the
ATIII pentasaccharide binding site, portions of hepa-
rin's fine structure have been elucidated, and an improved
understanding of its conformation [8–10] and interaction
with proteins established [11–20]. A most important de-
velopment in recent years has been the growing awareness
of the ubiquitous distribution, structural diversity, and
biological importance of heparin sulfate (HS). Formerly
an unwanted by-product of heparin manufacture, HS is
now recognized as a family of closely related yet distinct
polysaccharide species. In fact, heparin is now considered
by many as just another member of in the HS family [21].

Heparin is the most commonly used clinical anticoag-
ulant. Over 33 metric tons of heparin is manufactured
worldwide each year representing over 500 million doses
[22]. For industrial-scale production, heparin is prepared
by extraction from mammalian tissues that are rich in mast
cells (i.e., porcine intestine, bovine lung). The extraction of
1 kg starting material followed by complex formation,
fractionating precipitation, alkaline treatment, and bleaching
results in only 150 mg heparin [23]. Heparin obtained
from different tissues and different species differ structur-
ally (Table 2) [11]. In addition, individual manufac-
turers use different methods of isolation and purification.
A multitude of different commercial heparin products exists
with some variations in their chemical and physiological
properties [23]. Other species of mammals as well as birds,
fish, and even invertebrates such as lobster and clams,
which do not have a blood coagulation system, also contain
heparin [22].

Structure

Heparin is a polydisperse, highly sulfated, linear poly-
saccharide consisting of repeating units of 1→4-linked
pyranosyluronic acid and 2-amino-2-deoxyglycopyranose
(glucosamine) residues [24,25]. The uronic acid typically
consists of 90% L-idopyranosyluronic acid (L-iduronic
acid) and 10% D-glucopyranosyluronic acid (D-glucuronic
acid). Heparin has the highest negative charge density of
any known biological macromolecule. This is the result of
its high content of negatively charged sulfo and carboxyl
groups. Indeed, the average heparin disaccharide contains
2.7 sulfo groups. The most common structure occurring in
heparin is the trisulfated disaccharide (Fig. 2). However,
a number of structural variations of this disaccharide exist,
leading to the microheterogeneity of heparin. The amino
group of the glucosamine residue can be substituted with
an acetyl or sulfo group or unsubstituted. The 3- and 6-
positions of the glucosamine residues can be either substi-
tuted with an O-sulfo group or unsubstituted. The uronic
acid, which can be either L-iduronic or D-glucuronic
acid, may also contain a 2-O-sulfo group. Glicosaminoglycan
(GAG) heparin has a molecular weight range of
5–40 kDa, with an average molecular weight of ~15 kDa
and an average negative charge of approx. ~75. This
structural variability makes heparin extremely challenging
molecule to characterize.

Heparan sulfate is structurally related to heparin but is
much less substituted with sulfo groups than heparin and
has a more varied structure (or sequence). Like heparin,
heparan sulfate is a repeating linear copolymer of uronic
acid 1→4-linked to glucosamine (Fig. 2) [26]. While D-

Table 2  Heparins from Different Species and Tissues

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Average number in one heparin chain*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N-acetylated AT binding site</td>
</tr>
<tr>
<td>Porcine</td>
<td>Intestine</td>
<td>0.5 (0.3–0.7)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Lung</td>
<td>0.3</td>
</tr>
<tr>
<td>Bovine</td>
<td>Intestine</td>
<td>0.3</td>
</tr>
<tr>
<td>Ovine</td>
<td>Intestine</td>
<td>0.7</td>
</tr>
<tr>
<td>Hen</td>
<td>Intestine</td>
<td>0.3</td>
</tr>
<tr>
<td>Clam</td>
<td>—</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*a The numbers shown in parentheses indicate a range of values typically observed.
glucuronic acid predominates in heparan sulfate, it can contain substantial amounts of L-iduronic acid. Heparan sulfates generally contain only $\sim 1$ sulfo group per disaccharide, but individual heparan sulfates may have higher content of O-sulfo groups. Heparan sulfate chains also often contain domains of extended sequences having low or high sulfation [27]. While heparan sulfate contains all of the structural variations found in heparin (and vice versa), the frequency of occurrence of the minor sequence variants is greater than in heparin, making heparan sulfate’s structure and sequence much more complex. Heparan sulfate chains are also polydisperse, but are generally longer than heparin chains, having average molecular weight (MW$_{avg}$) of $\sim 30$ kDa ranging from 5 to 50 kDa [28]. Heparan sulfate is biosynthesized, as a proteoglycan, through the same pathway as heparin; however, unlike heparin, the heparan sulfate GAG chain remains connected to core protein. The core protein of PG heparin and PG heparan sulfate are different. Heparan sulfate PG is ubiquitously distributed on cell surfaces and is a common component of the extracellular matrix, whereas heparin is only found intracellularly in certain granule-containing cells [29,30]. There are two types of HS core proteins, the syndecans (an integral membrane protein) and the glypicans (a GPI-anchored protein) [31,32]. Although structurally similar, heparin and heparan sulfate GAGs can often be structurally distinguished through their different sensitivity toward a family of GAG-degrading, microbial enzymes, the heparin lyases [33].

**Biosynthesis**

The biosynthesis of heparin and heparan sulfate and the regulatory mechanisms resulting in the placement of different saccharide sequences in their structure are only partly understood (Fig. 3) [20]. Heparin and heparan sulfate are synthesized by (1) formation of a region linking the HS chain to protein, (2) generation of the polysaccharide chain, and (3) enzymatic modification of the chain to yield the specific saccharide sequences and structural organization that are responsible for protein binding [32].

Studies on heparin biosynthesis were performed in a mastocytoma cell culture system with radiolabeled metabolic precursors of heparin [34]. The core protein, serglycin, contains a high number of serine and glycine repeats and is primarily synthesized in the rough endoplasmic reticulum. The biosynthesis of the GAG chain predominantly takes place in the Golgi apparatus. The first step in the pathway involves the attachment of a tetrasaccharide fragment to a serine residue in the core protein [35]. The sequence of this linkage-region tetrasaccharide is $\beta$-GlcAp-(1→3)-$\beta$-Galp-(1→3)-$\beta$-Galp-(1→4)-$\beta$-Xyp-(1→Ser. There are four different glycosyltransferases responsible for the synthesis of the linkage region [36]. Onto this neutral sugar linkage region the first GlcNpAc residue or N-acetylgalactosamine (GalNpAc, in the biosynthesis of chondroitin sulfates) is added. This addition decides whether the chain will be either a glucosaminoglycan (heparin and heparan sulfate) or a galactosaminoglycan (chondroitin sulfate/dermatan sulfate). It has been suggested that peptide sequence motifs close to the linkage-region substituted serine residues act as a signal for the addition of a GlcNpAc residue, thus initiating heparin/heparan sulfate formation [37,38].

After the first residue has been added, alternating transfer of GlcAp and GlcNpAc residues from their corresponding UDP-sugar nucleotides to the nonreducing termini of growing chains forms the rest of the GAG chain. One enzyme (present in humans in two isoforms) has both GlcAp transferase and GlcNpAc transferase activities [39]. Approximately 300 sugar residues are added to the linear polysaccharide chain before its synthesis terminates [34]. As the chain elongates it also undergoes other modification
Anticoagulant Properties

Figure 3 Pathway of heparin proteoglycan synthesis and its degradation to peptidoglycans and glycosaminoglycans. Protein synthesis takes place in the endoplasmic reticulum, linkage synthesis, chain elongation, and modification take place in the Golgi, and proteolysis and glucuronidase digestion take place in the mast cell granules. Heparan sulfate is biosynthesized through a similar pathway but with reduced modification of the GAG chain and little breakdown of the proteoglycan.

reactions [40]. Modification of the polymer is initiated by N-deacetylation and N-sulfonation of the GlcNpAc residues by an N-deacetylase/N-sulfotransferase enzyme. Subsequent steps occur sequentially and either on or adjacent to the N-sulfoglucosamine (GlcNpS)-containing residue. A C-5 epimerase then catalyzes transformation of some of the D-glucuronic acid residues to L-iduronic acid residues [41]. This is followed by O-sulfonation of the iduronic acid residues at the C-2 position by an iduronosyl 2-O-sulfotransferase [42]. Studies have also shown that a very active glucuronosyl 2-O-sulfotransferase in mouse mastocytoma microsomal fractions is responsible for the O-sulfonation of GlcAp residues at the C-2 position [43,44]. The 2-O-sulfonation of the uronic acid is followed by the action of glucosamine 6-O-sulfotransferase, which transfers an O-sulfo group to the C-6 position of GlcNpAc and GlcNpS [41]. Finally a 3-O-sulfotransferase acts upon the polymer and modifies certain GlcNpS6S residues [31]. The 3-O-sulfonation is required for the anticoagulant activity of heparin, and the pentasaccharide sequence formed by the 3-O-sulfotransferase is the minimum structure required for binding antithrombin III.

Anticoagulant Activity and Its Mechanism

The anticoagulant activity of heparin is primarily mediated through its binding and regulation of a plasma serine proteinase inhibitor (serpin) antithrombin III (ATIII). ATIII functions as the principal plasma inhibitor of most blood coagulation proteinases [45]. This serpin forms tight, irreversible, equimolar complexes with its target enzymes by formation of an ester between an arginine residue of its active center and the serine residue of the active center of the enzyme. This slow, time-, temperature-, and pH-dependent process is accelerated 2000-fold by heparin [46]. An essential component of the mechanism of heparin’s effect is the binding of a lysine-rich region of ATIII to the highly specific ATIII-binding site in the heparin molecule (Fig. 4) [47]. This reversible, electrostatic interaction induces a conformational change in ATIII, which considerably reinforces its anticoagulant activity [23].

Another anticoagulant activity mechanism of heparin is mediated through heparin cofactor II (HCII), which is structurally similar to ATIII having a similar carboxyl terminal sequence but a distinctly different amino terminal sequence [48]. The physiological role of HCII might be as reserve of thrombin inhibitor when the plasma concentration of ATIII becomes abnormally low [49]. Unlike ATIII, HCII can inhibit thrombin but no other coagulation proteases [46]. In addition to this unusual specificity, HCII also can be potentiated by dermatan sulfate in addition to heparin and heparan sulfate [50,51]. The mechanism by which HCII inhibits thrombin is similar to that proposed for ATIII [46].
Other Biological Activities

The interaction of heparin with various proteins that play important roles in the regulation of normal physiological processes as well as disease states has led to an interest in using heparin in roles outside its normal application as an anticoagulant/antithrombotic agent. Randomized trials to study the effectiveness of low molecular weight heparin (LMWH, MW~6000) as compared with unfractionated heparin (see Sec. II.A.2) (MW~12,000) in treating venous thromboembolism in cancer patients led to a surprising observation: that treatment with heparin may affect survival of patients with malignancy [52,53]. Cancer patients who had been treated with LMWH for their thrombosis had a slightly improved 3-month survival as compared to cancer patients receiving unfractionated heparin. Heparin can potentially exert its activity at various stages in cancer progression and malignancy-related processes. It can affect cell proliferation, interfere with the adherence of cancer cells to vascular endothelium, regulate the immune system, and have both inhibitory and stimulatory effects on angiogenesis [54]. There is recent evidence showing that heparin treatment reduces tumor metastasis in mice by inhibiting P-selectin-mediated interactions of platelets with carcinoma cell-surface mucin ligands [55].

Problems in the Medical Applications of Heparin

In clinical applications, heparin is administered intravenously (LMWH can be administered either intravenously or subcutaneously, improving its scope of therapeutic applications) during most extracorporeal procedures (where blood is removed from the body and passed through a device), such as kidney dialysis and membrane oxygenation, used in heart bypass procedures [56]. The use of these devices requires heparinization and can often lead to hemorrhagic complications. Systemic heparinization is also used in treatment of deep vein thrombosis and in the variety of other surgical procedures [57]. Heparin-induced thrombocytopenia (HIT), a complex process that results in loss of platelets, is currently recognized as one of the most catastrophic complications of heparin treatment [58,59].

Anticoagulant Activity of Fractionated Heparins

Heparin can be fractionated, on the basis of size, using gel permeation chromatography (GPC) [60]. Both low-pressure and high-pressure GPC has been used to obtain heparin fractions of MW~5000 and 40,000. Anticoagulant activities of fractionated heparin with different chain size are shown in Table 3 [22]. Being a sulfated polysaccharide, heparin can be fractionated on the basis of its charge using anion exchange chromatography. Strong anion exchange (SAX) chromatography of heparin typically involves the use of a salt gradient [61]. Heparin fractions can be prepared with from two to three sulfogroups per disaccharide repeating unit [62]. Anticoagulant activities of fractionated heparin vary with degree of sulfonation (Table 3) [22] making this a useful technique to separate or enrich specific heparin fraction based on activities. Chemical sulfonation or desulfonation can also be used to prepare oversulfated or undersulfated heparins with various activities (see Sec. III.A)

2. Low Molecular Weight Heparin

Background

Low molecular weight heparins (also referred to as low molecular mass heparins (LMMHs)) are a group of hepa-

<p>| Table 3 Heparin Activities as a Function of Polymer Chain Length and Degree of Sulfonation or Charge |
|--------------------------------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Anti IIa activity (U/mg)</th>
<th>ATIII</th>
<th>HCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Based on size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrasaccharides (MW 1200)</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Hexasaccharides (MW 1900)</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Octasaccharides (MW 2400)</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Decasaccharides (MW 2900)</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Dodecasaccharides (MW 3500)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Tetradecasaccharides (MW 4100)</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Hexadecasaccharides (MW 4700)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Octadecasaccharides (MW 5300)</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>Eicosasaccharides (MW 5900)</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Heparin fraction (MW ~4000)</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Heparin fraction (MW ~5700)</td>
<td>26</td>
<td>44</td>
</tr>
<tr>
<td>Heparin fraction (MW ~14,500)</td>
<td>196</td>
<td>271</td>
</tr>
<tr>
<td>Heparin fraction (MW ~16,900)</td>
<td>130</td>
<td>331</td>
</tr>
<tr>
<td>Heparin fraction (MW ~25,300)</td>
<td>92</td>
<td>356</td>
</tr>
<tr>
<td>Unfractionated heparin (MW ~14,000)</td>
<td>171</td>
<td>225</td>
</tr>
<tr>
<td>Based on charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin fraction (very low charge)</td>
<td>17</td>
<td>90</td>
</tr>
<tr>
<td>Heparin fraction (low charge)</td>
<td>108</td>
<td>261</td>
</tr>
<tr>
<td>Heparin fraction (intermediate charge)</td>
<td>125</td>
<td>336</td>
</tr>
<tr>
<td>Heparin fraction (high charge)</td>
<td>227</td>
<td>468</td>
</tr>
<tr>
<td>Heparin fraction (very high charge)</td>
<td>540</td>
<td>448</td>
</tr>
</tbody>
</table>
rin-derived anticoagulant/antithrombotic agents. The introduction of LMWHs primarily resulted from an improved understanding of the molecular basis of the biochemistry associated with the coagulation cascade [63]. The isolation of the serine protease inhibitor, antithrombin III (ATIII), and the characterization of coagulation factors (serine proteases), such as thrombin (factor IIa) and factor Xa (inhibited by ATIII), were critical in driving the development of LMWHs [64,65]. Heparin accelerates the inhibition of these coagulation factors by ATIII, preventing the generation of a fibrin clot. In the coagulation cascade (Fig. 1), one factor activates the next until prothrombin (factor II) is converted to thrombin (factor IIa) by factor Xa. It is thrombin that acts on fibrinogen to form a fibrin clot. The very nature of this cascade suggested a therapeutic opportunity, to develop an agent that was more specific than heparin (which acts at many points in the cascade) that might provide more subtle regulation of coagulation, reducing the major hemorrhagic side effects associated with heparin. LMWHs were originally developed based on this rationale.

**Preparation of LMWH**

LMWHs are generally prepared through the controlled, partial, chemical, or enzymatic depolymerization commercial heparin (Fig. 5 and Table 4) [22]. These depolymerization methods were selected to give a product with (1) suitable average molecular weight and low polydispersity, (2) antifactor Xa/antifactor IIa activity > 1, (3) structurally similar to a LMWH prepared through fractionation and with few structural artifacts resulting from the depolymerization method used, (4) no residual toxic reagents, and (5) a cost-effective reproducible and scalable process having a minimum number of process steps, little if any required purification, neither labor, reagent, nor capital intensive, and high yielding. While no current manufacturing process meets all of these goals, the currently used processes have afforded a first generation of clinically useful LMWHs [63].

Heparin can be oxidatively broken down using a variety of oxygen-containing reagents including hydrogen peroxide, hypochlorous acid, Cu\(^{2+}/O_2\), Fe\(^{2+}/O_2\), or ionizing \(\gamma\)-irradiation (Fig. 5) [66]. Each of these methods relies on the generation of oxygen radicals that are believed to act by oxidizing sensitive saccharide residues within the heparin polymer. Nonreducing sugars are essentially inert to aqueous hydrogen peroxide except in the presence of alkali or in the presence of a metal catalyst. Both of these conditions lead to the generation of the hydroxyl radical, which will react with sugar residues and degrade them to 1-, 2-, and 3-carbon fragments without modifying the residues on either side of the point of attack. The most susceptible residues appear to be those that are unsubstituted at positions numbered 2 and 3 in the sugar ring. Studies of

![Figure 5 Depolymerization of heparin to prepare LMWHs. The heparin chain in the center can undergo depolymerization by each of the four processes shown. Heparin chain size is reduced (\(n > m\)), affording a LMWH.](image-url)
Table 4 Properties of commercially available LMWHs

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Manufacturer</th>
<th>Preparation</th>
<th>$M_W^{\text{av}}$</th>
<th>Potency$^{\text{a}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natroparin calcium</td>
<td>Sanofi/Choay</td>
<td>EtOH fractionation</td>
<td>5,500</td>
<td>95</td>
</tr>
<tr>
<td>Fraxiparine</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fraxiparin</td>
<td></td>
<td></td>
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<tr>
<td>Fragmin</td>
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<td></td>
</tr>
<tr>
<td>Dalteparin sodium</td>
<td></td>
<td>Nitrous acid degradation</td>
<td>3,400</td>
<td>60</td>
</tr>
<tr>
<td>Tediparin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low liquemine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Certiparin sodium</td>
<td>Sandoz</td>
<td>Isoamyl nitrite degradation</td>
<td>6,300</td>
<td>83</td>
</tr>
<tr>
<td>Sandiparin</td>
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<td></td>
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<tr>
<td>Parnaparin sodium</td>
<td>Opocrin</td>
<td>Peroxidative degradation</td>
<td>6,500</td>
<td>83</td>
</tr>
<tr>
<td>Minidaltion</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ardeparin sodium</td>
<td>Hepar/Wyeth-Ayerst</td>
<td>Peroxidative degradation</td>
<td>6,200</td>
<td>83</td>
</tr>
<tr>
<td>Sandiparin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Enoxaparin sodium</td>
<td>Rhone Poulenc/Aventis</td>
<td>Benzylaion/$\beta$-elimination</td>
<td>3,800</td>
<td>96</td>
</tr>
<tr>
<td>Cleparin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lovenox</td>
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<tr>
<td>Kleparin</td>
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<tr>
<td>Tinzaparine sodium</td>
<td>Novo/Leo/Dupont</td>
<td>Enzymatic $\beta$-elimination</td>
<td>4,900</td>
<td>87</td>
</tr>
<tr>
<td>Logiparin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regeparin sodium</td>
<td>Knoll AG</td>
<td>Nitrous acid degradation</td>
<td>4,000</td>
<td>83</td>
</tr>
<tr>
<td>Clivarin</td>
<td></td>
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</tr>
<tr>
<td>Clivaeparin</td>
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<td></td>
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</tr>
<tr>
<td>Std LMWH 85/600</td>
<td>NIBSC</td>
<td>Nitrous acid/GPC</td>
<td>14,000</td>
<td>100</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ By GPC.
$^{b}$ Relative anti-Xa potency against NIBSC standard LMWH.

drivate. Partial deaminative cleavage is possible by controlling the process conditions (i.e., temperature, pH, time) or by limiting the amount of nitrosation reagent [69,70]. The LMWH product formed using these controlled conditions is obtained in high yield and has the appropriate chemical and biological properties. Several LMWHs prepared through deaminative cleavage are currently used clinically (Table 4).

Enzymatic $\beta$-eliminative methods are used to commercially prepare LMWHs. Heparin lyases eliminatively depolymerize heparin affording unsaturated oligosaccharide products. Three major polysaccharide lyases (heparin lyases I, II, and III), isolated from Flavobacterium heparinum, are capable of cleaving linkages present in heparin [71]. The extent of this reaction can be conveniently monitored by measuring the change in absorbance associated with the unsaturated uronate residue formed in each product molecule. Cleavage with heparin lyase I takes place only at the 2-O-sulfoiduronic acid residue [72]. The depolymerization is stopped by removing or inactivating the enzyme. This method is used to prepare the clinically used LMWH product, tinzaparin sodium (Table 2).

Chemical $\beta$-elimination can involve the direct treatment of heparin or its quaternary ammonium salt with
base [73,74]. Alternatively, the benzyl ester can be prepared by treatment of the benzethonium salt of heparin with benzyl chloride and base with heating [75]. Under these conditions, chemical β-elimination takes place, affording LMWH containing an unsaturated uronate residue in the nonreducing end. Cleavage occurs specifically at iduronic acid without preference for the presence or absence of a 2-O-sulfo group. Proper control of the process conditions affords the clinically used LMWH, enoxaparin sodium (Table 4).

Medical Applications of LMWHs

Based on the method of the preparation, different structural modifications are induced with the consequence that LMWHs may differ considerably in their chemical and physical as well as in their biological and pharmacological properties (Table 4). Although each LMWH is different, they all have several features in common such as being polydisperse with MW_{avg} ~ 5000 and MW ranging from 2000 to 8000. In comparison with unfractionated heparin, LMWHs have better defined chemical and biological properties giving predictable pharmacological actions, sustained activity, improved bioavailability, and a better therapeutic index. These can be administered subcutaneously once a day due to their high bioavailability and longer half-life [23]. Thus, LMWHs have been successfully introduced as new effective and improved anticoagulant/antithrombotic agents.

B. Nonheparin Glycosaminoglycans

Heparin has been the drug of choice in clinical, presurgical and postsurgical prophylaxis of thrombotic events. However, because of its side effects, such as bleeding and other disadvantages, developing alternatives to heparin is an important research goal. Other polysaccharides and modified polysaccharides have been examined as potential heparin analogs in drug development [76].

1. Animal Nonheparin Glycosaminoglycans

Nonheparin GAGs, originally side products of heparin manufacture, have raised great interest as anticoagulant/antithrombotic drugs in the past several years, especially after the demonstration that the interaction with ATIII is not essential to produce antithrombotic effects [77].

Dermatan Sulfate

Dermatan sulfate (DS, chondroitin sulfate B) is found in a variety of tissues in virtually all animals, and is a (1→3,4-linked) copolymer of N-acetyl-d-galactosamine, l-iduronic acid, and d-glucuronic acid with O-sulfo groups commonly found on the 4-position of the N-acetyl-d-galactosamine residues (Fig. 6). DS has been used clinically as an antithrombotic agent [23]. Similar to HS, DS is a relatively weak anticoagulant in vitro (70 times less than heparin) and, apart from the inhibition of the thrombin-induced platelet aggregation, does not interact with platelets [78,79]. DS has neither ATIII-mediated anti-Xa nor anti-IIa activity. Its anticoagulant effect is mainly based on enhancement of HCII activity [80]. DS is also able to inhibit thrombin generation and thrombin-mediated amplification reactions, but less effectively than heparin [81,82]. Several DS preparations have been developed for prophylaxis of venous thromboembolism [23]. Compared to heparin, native DS is a less active but safer antithrombin drug by intravenous administration due to its low bleeding potential, even at very high doses (up to 16 mg/kg). Low molecular weight DS has been developed (e.g., Desmin 370) to improve bioavailability. Chemical modifications such as oversulfation and succinyl derivatization were examined to improve anticoagulant/antithrombotic activity and safety [83,84]. Dermatan disulfate (Intimatan), prepared by chemical 6-O-sulfonation of dermatan sulfate, shows substantial antithrombotic activity [85].

Chondroitin Sulfates

Chondroitin sulfates are families of structurally complex, sulfated, linear polysaccharides with repeating (1→3,4-linked) disaccharide units of D-glucuronic acid and N-acetyl-d-galactosamine, with the O-sulfo groups at either the 4-position (chondroitin sulfate A) or 6-position (chondroitin sulfate C) of the N-acetyl-d-galactosamine [86] (Fig. 6). This polysaccharide is part of the proteoglycans found localized on cell surfaces and in the extracellular matrix and is important in cell-cell communication [87]. While chondroitin sulfates appear to be involved in maintaining hemostasis, chondroitin sulfates lack clinically relevant levels of anticoagulant activity, presumably due to the lack of iduronic acid and their low level of sulfation [88]. Oversulfated chondroitin sulfates with two to three sulfate groups per disaccharide unit have been shown to exhibit enhanced antithrombotic activity [89]. These chemically prepared oversulfated chondroitin sulfates still contain glucuronic acid residues, making them both structurally and conformationally different from the iduronic acid residues found in heparin [90,91].

Acharan Sulfate

Acharan sulfate is composed of a simple disaccharide repeating unit of 1→4-linked N-acetyl-d-glucosamine and 2-O-sulfo-l-iduronic acid (Fig. 6) and is isolated from the giant African snail, Achatina fulica [92]. The natural function of this molecule in snail, while still unclear, may be as an antidesiccant, a metal chelator, an anti-infective, or a locomotive (slime) agent. Its structure is related to heparin and heparan sulfate but is distinctly different from all known members of these classes of glycosaminoglycans. Because of its structural similarities to heparin, chemically modified acharan sulfate was studied to understand the chemical structure effecting its anticoagulant activity [93]. After de-N-acetylation, acharan sulfate was N-sulfonated using either chlorosulfonic acid–pyridine or sulfur trioxide–trimethylamine complex. The level of sulfonation in these products ranged from 22% to 24% (w/w), significantly less than that of heparin at 36%. The MW_{avg} of both N-sulfoacharan sulfates were comparable with that of heparin. In vitro anticoagulant activity assays showed that N-sulfoacharan sulfate derivatives were moderately active.
Figure 6  The structures of the major disaccharide units of chondroitin sulfate, dermatan sulfate, dermatan disulfate (Intimatan), acharan sulfate, keratan sulfate and per-O-sulfonated hyaluronic acid (Ac is acetate and n is the degree of polymerization).

A corneal cell culture system has been developed in which long-term KS biosynthesis is maintained. Progress has been made toward identification of the glycosyl- and sulfotransferases responsible for KS biosynthesis. Evidence has also been presented supporting functional roles of KS in cellular recognition of protein ligands, axonal guidance, cell motility, and in embryo implantation. These findings have served to expand the concept of what KS is and the potential roles it may play in the cellular biology of diverse tissues [94]. No report has been found so far on KS as an anticoagulant agent.

2. Nonheparin Glycosaminoglycans from Plant Sources

Carrageenans

Besides animal GAGs, important natural sources for sulfated polysaccharides are the marine flora and fauna,
Anticoagulant Properties

i.e., algae and organisms like mussels and corals [23]. Carrageenans are obtained commercially by aqueous extraction of certain species of red seaweeds in the Rhodophyceae class. They consist of sulfate esters of 1→3,4-linked D-galactose and 3,6-anhydro-D-galactose copolymers [95]. Carrageenans provide structural support for the spatial arrangement of plants and are widely used in food, pharmaceutical, and industrial applications. They have been well known for a long time and are used therapeutically as antieptic and immunosuppressive agents. Their anticoagulant activity, which was first demonstrated by Elsner, is considered to be a side-effect [23]. In vitro, the carrageenans prolong a PTT and the PT, show only a small effect in the Heptest®, and inhibit the amidolytic thrombin but not FXa activity [96–98]. Recently, the antiviral and anticoagulant properties of the insoluble polysaccharide fraction from carrageenans extracted from cystocarpic and tetrasporic Stenogramme interrupa were analyzed [98]. These analyses showed the polysaccharides exhibit no or very slight anticoagulant activity at the concentrations assayed for antiviral activity, indicating a lack of correlation between antiviral and anticoagulant properties of the polysaccharides studied in their work.

Sulfated Fucans

Since the description of high amounts of sulfated fucans in marine brown algae over 50 years ago, these polysaccharides have been widely tested for biological activities in different mammalian systems [99,100]. These fucans comprise a wide, continuous spectrum ranging from high-uronic-acid-, low-sulfate-containing polymers, which are glucuronofucoglycans and fucogluconurons, to the relatively pure fucans, the so-called homofucans or fucoidans [23]. Algal-sulfated fucans have anticoagulant activity as measured in several different assays and have also venous antithrombotic activity [101,102]. Apart from the anticoagulant and antithrombotic activity, these polysaccharides are inhibitors of native and recombinant human immunodeficiency virus reverse transcriptase activity in vitro [103]. Furthermore, because of their interference with molecular mechanisms of cell-to-cell recognition, algal-sulfated fucans are potent blockers of a wide range of biological processes. Thus, algal-sulfated fucans are inhibitors of cell invasion by retroviruses such as human immunodeficiency virus, herpes, cytomegalovirus, and African swine fever virus [104–106].

The mechanisms by which the algal fucans exert their anticoagulant action remain controversial. Mechanisms related to both antithrombin and heparin cofactor II-mediated action have been described for algal-sulfated fucans from different species [100,102]. Direct anticoagulant activity by an antithrombin-independent pathway has also been reported for some preparations of algal fucans [107]. In addition, some fucans have antifactor Xa activity, whereas others do not. The structures of sulfated fucans vary from species to species and must give rise to variation in the detailed mechanisms of anticoagulant action [99].

Pereira and coworkers [99] compared the anticoagulant activity of the regular and repetitive fucans from echinoderms with that of the more heterogeneous fucans from three species of brown algae. The results indicate that different structural features determine not only the anticoagulant potency of the sulfated fucans but also the mechanism by which they exert this activity. Thus, the branched fucans from brown algae are direct inhibitors of thrombin, whereas the linear fucans from echinoderms require the presence of antithrombin or heparin cofactor II for inhibition of thrombin, as reported for mammalian glycosaminoglycans. The linear sulfated fucans from echinoderms have an anticoagulant action resembling that of mammalian dermatan sulfate and a modest action through antithrombin. A single difference of one sulfo per tetrasaccharide repeating unit modifies the anticoagulant activity of the polysaccharide markedly. Possibly, the spatial arrangements of sulfo groups in the repeating tetrasaccharide unit of the echinoderm fucan mimic the site in dermatan sulfate with high affinity for heparin cofactor II.

To improve the activities of genuine sulfated fucans, chemical modifications of a fucoidan from Fucus vesiculosus were conducted, indicating that oversulfation as well as the introduction of alkylamino residues increase in vitro and in vivo anticoagulant/antithrombotic activity [23].

Sulfated Galactan

Farias and coworkers [108] reported an algal-sulfated D-galactan with repeating structure [→4-D-Galp-(1→3)-D-Galp-(1→)], but with a variable sulfation pattern (one-third of the total saccharide units are 2,3-di-O-sulfonated and another one-third contain only 2-O-sulfo groups), has a potent anticoagulant activity (similar potency as unfractionated heparin) due to enhanced inhibition of thrombin and factor Xa by ATIII and/or HCII. They also extended the experiments to several sulfated polysaccharides from marine invertebrates with simple structures composed of a single, repeating structure. A 2-O- or 3-O-sulfonylated L-galactan has a weak anticoagulant action when compared with the potent action of the algal-sulfated D-galactan. Possibly, the addition of two sulfo esters to a single galactose residue has an “amplifying effect” on the anticoagulant action, which cannot be totally ascribed to the increased charge density of the polymer. These results indicate that the wide diversity of polysaccharides from marine alga and invertebrates is a useful tool to elucidate structure/anticoagulant activity relationships [108].

3. Nonheparin Glycosaminoglycans from Microbial Sources

Sulfated polysaccharides are rarely found as microbial products. This may be due to the absence of a highly compartmentalized cellular structure found in eucaryotes that is required for the biosynthesis of sulfated polysaccharides [109]. Heparin-like compounds have been prepared by chemical modification of capsular polysaccharide from Escherichia coli K5. Capsular polysaccharide from E. coli K5, with the basic structure [GlcAα1→4GlcNAcα1→4], was chemically modified through N-deacetylation, N-sulfonation, and O-sulfonation [110]. Depending on the reaction conditions, the products showed different proportions of components with high affinity for ATIII.
Derivatives with high affinity for ATIII and high anti-Xa activity contained the trisulfated amino sugar GlcNSO$_3$-3, 6-SO$_3$. The 3-0-sulfo GlcN unit has been identified as a marker component of the ATIII-binding pentasaccharide sequence in heparin (Fig. 2) suggesting that the modified bacterial polysaccharide interacts with ATIII and promotes its anticoagulant action in a manner similar to that of heparin [110]. Thus, homogeneous heparin-like substances with special anticoagulant properties might be produced by biotechnological methods [23].

Hyaluronic acid (HA) is a very high molecular weight GAG having no sulfo groups [22]. Bacterial HA is identical to HA from animals and can substitute for animal HA previously used in eye or joint operations to replace the material lost during surgical manipulations [111]. Chemical O-sulfonation of this polysaccharide (Fig. 6) affords a polysaccharide displaying anticoagulant activity [112]. Magnani and coworkers [113] synthesized seven differently sulfonated HA derivatives (having a general formula Hya1Sx, where x can be 1, 2, 2.5, 3, 3.5, 3.8, 4). Coagulation tests (whole-blood clotting time and thrombin time) showed that significant prolongations were observed from Hya1S2.5 up to Hya1S4, suggesting that the anticoagulant activity increases by increasing the sulfonation degree of HA.

### III. MODIFIED AND SYNTHETIC POLYSACCHARIDES WITH ANTICOAGULANT PROPERTIES

#### A. Synthetic Chemistry: Sulfonation

As researchers began to look for analogs to build the pharmacological anticoagulant arsenal, chemical sulfonation became an area of intense interest. The starting materials for such studies were nonsulfated polysaccharides. It was believed that molecules comparable in structure to heparin upon sulfonation would produce compounds with the ability to perform as anticoagulants within the body. Many polysaccharides were targeted for sulfonation including agarose, amylose, arabinogalactan, cellulose, chitin, curdlan, dextran, guaran, hyaluronic acid, inulin, leintin, laminarin, pectin, xanthan, xylan, and others [23]. It was hypothesized sulfonation of such materials could produce improved anticoagulants for either systemic administration or coating of implantable objects [114]. Data indicate that specific desulfonation of heparin and selective sulfonation of heparinoid molecules may decrease the thrombogenesis of implanted objects. This effect is believed to be due to these molecules’ inability to affect platelet function, therefore decreasing platelet adhesion and aggregation [115]. These data are interesting because the molecules, which cause the least effect on platelets, often provide little anticoagulation. Further studies on molecular modifications and their effect on platelet function, along with continued study of the anticoagulant effects, may provide additional insights into the discovery of new and more effective antithrombotic coatings.

Scientists have been sulfonating polysaccharides since the middle of the 19th century but only recently have adequate methods been described [116]. Currently, several hundred methods for sulfation of polysaccharides have been described in the literature. However, most sulfonation reactions induce excessive destruction of the polysaccharide or produce scarce, uneven sulfonation of the final product [117]. Furthermore, such reactions result in polysaccharide sulfate mixtures, which must be separated [23]. Under these conditions, structure–activity relationships (SAR), used to yield improved therapeutics, are very difficult to study.

Years of investing time, money, and other resources into the sulfonation of polysaccharides with the intent to produce the perfect anticoagulant have yielded mixed results [118–122]. This demonstrates the immense complexity of the body’s ability to recognize and metabolize such compounds. Sulfonation of defined oligosaccharides such as oligomers might offer better antithrombotic agents for the study of structure–activity relationships [123].

The quest for the ultimate anticoagulant has associated with it yet another level of difficulty. Heparin-induced thrombocytopenia, a major complication of heparin therapy in specific patients, is mediated through an immune reaction. With the elucidation of the HIT mechanism at hand, the ultimate “modern” anticoagulant must not demonstrate cross-reactivity with heparin antibodies in HIT patients [124].

#### B. Dextran Sulfate

Dextrans have a historical mainstay in medicine in which they were used as plasma extenders in postsurgical and medical thromboembolic prophylaxis. Dextran is of bacterial origin and therefore is unsulfated in nature. It is a (→4)β-D-Glc (1→3)-α-D-Glc (1→ branched polymer [125]. The natural polysaccharide has a large MW$_\text{avg}$ (~1000 kDa) but for medicinal purposes the MW$_\text{avg}$ is reduced by partial degradation (10–100 kDa). Dextran’s effects are purely physical; the unsulfated polysaccharide has no direct effect on the coagulation system [23]. Therefore, unsulfated dextran has low efficacy as an anticoagulant and also exhibits multiple side effects rendering its use less than ideal.

Upon sulfonation, dextran is converted to dextran sulfate (Fig. 7), which was one of the first polysaccharides to be chemically sulfated. Dextran sulfate exhibits heparin-like activity but has a very narrow therapeutic index—an undesirable characteristic for a therapeutic agent [121]. There is direct relationship between MW$_\text{avg}$ and toxicity, with molecules less than 10 kDa showing little or no toxicity. This is a problem because dextran requires a relatively high degree of sulfonation for maximum activity (~1.3) [120]. Although the relative anticoagulant activity of dextran sulfate is low the molecule does exhibit lipoprotein-lipase-releasing activity, a characteristic exploited as an agent for the treatment of atherosclerosis [126]. Currently, the use of dextran sulfate as an anticoagulant and antilipemic has lost favor due to its narrow therapeutic
index and the marketing of more effective alternatives. It continues to be used within the laboratory in some diagnostic assays [127] and has regained some popularity due to its ability to inhibit HIV binding to CD4+ T cells [128].

C. Pentosan

Pentosan is a compound extracted from beech tree bark and sulfonated to form a clinically useful anticoagulant called pentosan polysulfate (PPS), often referred to as SP 54. It is a 1→4-linked xylopyranose with one lateral 4-O-methyl-α-D-glucuronic acid branch (Fig. 7) [20]. PPS has performed as an anticoagulant in Europe for well over 30 years and is often given subcutaneously twice daily [129]. It is thought that PPS functions as an anticoagulant by various mechanisms, independent of ATIII [130]. First, PPS exhibits behavior consistent with inactivation of factor VIII [131]; second, PPS appears to enhance fibrinolytic activity via activation of factor XII and kallikrein [132]. PPS also exhibits weak antifactor Xa properties believed to be the result of hepatic triglyceride lipase release [133]. Interestingly, PPS also has antiangiogenic activity due to its ability to effectively block heparin-binding growth factor (HBGF) both in vitro and in vivo [134]. Angiogenesis is important in rapid tissue growth and development as observed in tumor progression and metastasis [135]. This activity alone has led to considerable recent study of pentosan polysulfate as an antitumor agent [136].

Another interesting characteristic of PPS is its ability to be absorbed orally; although the bioavailability is low, significant reduction in thrombus formation is observed [137]. A derivative of PPS called Bego 0391 was prepared by Bene-Arzneimittel GmbH. This derivative is significantly more bioavailable relative to PPS, although its use clinically has been limited [138]. Both oral and subcutaneous administration of PPS yield increased plasmogen activator activity within the plasma [139,140]. Multiple studies suggest this increased fibrinolytic activity is due to endothelial tissue plasmogen activator (t-PA) release, not the factor XII pathway mentioned earlier [140–142].

There are multiple reports that PPS induces thrombocytopenia when it is used therapeutically [143–148]. Interestingly, PPS not only has the ability to induce thrombocytopenia, but exposure to PPS seems to sensitize patients to heparin. Subsequent exposure to heparin (and to a lesser extent LMWHs) thrombocytopenia should be expected [147]. It has also become evident this adverse reaction is mediated through an immune complex mechanism where the critical element for reactivity is a high negative charge rather than specific polymer molecular structure [148]. This would indicate this reaction could occur with other sulfated polysaccharides, making the search for polysaccharide anticoagulants devoid of properties capable of inducing HIT even more difficult.

D. Chitosan Sulfate

Chitin, a 2-acetamido-2-deoxy-α-D-glucopyranose polymer (Fig. 7), is the major component of the exoskeleton of insects, crabs, and shrimp, and can also be found in cell walls of bacteria and fungi. Like heparin, chitin and chitosan, the products of N-deacetylation of chitin, have 1→4 linkages making them suitable targets for anticoagulant construction through chemical sulfonation. Furthermore, their linear structure and inherent presence of acetamido and amino groups are even more attractive because such characteristics are challenging to introduce chemically [118]. Likewise, the sulfonation of both of these compounds yields products with anticoagulant properties. Since chitin is highly crystalline its modification to chitosan sulfate requires two-phase reactions [149]. The highest activity is observed with O-sulfated N-acetylated chitosan (>300 U/mg), followed by N,O-sulfonated chitosan (>200 U/mg), both showing more activity than heparin [150]. N-Sulfo chitosan is devoid of activity, but interestingly, the addition of a carboxyl group at the 6 position affords a product with one-fourth the activity of heparin [151]. This activity is potentiated by further O-sulfonation [149]. Position 6 [152] or amino group [118,153] carboxymethylation potentiates the anticoagulant activity of either sulfonated chitin or chitosan and if the degree of sulfonation is high these molecules function as well as heparin. A 6-O-sulfo group represents the main active site, although sulfonation at multiple positions is beneficial. The sulfamido group is not essential when a high degree of sulfonation is achieved [154]. In addition to the degree of sulfonation, molecular weight also influences anticoagulant activity [118,150,153], with reasonably higher molecular weight molecules exhibiting greater activity [150].

Recently, chitosan has been extensively studied as a component of drug delivery. More specifically, chitosan's biocompatible properties [154] along with its ability to
complex biomolecules [155–157] and its effects on transmucosal transit [158] led to its exploitation as a drug absorption enhancer. Chitosan polymers could potentially be used for delivery of a broad spectrum of therapeutics; DNA complexes with chitosan to form structures that have been shown to increase transfection rates in vitro as well as in vivo [155], whereas peptide and protein absorption also increases using chitosan carriers [156,157]. Chitosan’s ability to enhance absorption is believed to occur through interaction with cellular membrane components and tight junctions. This interaction increases paracellular permeation of hydrophilic compounds [158]. These properties show significant enhancements in absorption of LMWH.

A 3% (w/v) low-viscosity mono-N-carboxymethyl chitosan (LMCC) significantly increased the absorption of LMWH across the intestinal membrane. Area under the curve (AUC), a measure of total absorption of a drug, increased by 7 times, and the $C_{\text{max}}$ a measure of the maximum concentration achieved in the plasma, increased by 5.4 times. Moreover, anti-Xa concentrations rose to a level at or slightly above the therapeutic level [159]. Even though chitosan sulfate may not be the perfect anticoagulant its use with LMWH may provide significant improvements in oral administration over products that are currently available.

E. Curdlan

Curdlan is a natural polysaccharide with a linear β-1,3-glucan structure. It is produced by _Alcaligenes faecalis_ var. _myxogenes_, a bacterium normally found in the soil [160]. This naturally occurring carbohydrate is produced in large amounts and used in the food industry; chemical sulfonation results in β-1,3-glucans, which have been studied because of their biological properties. Curdlan possesses immunostimulating properties in its natural form, and chemically modified (sulfonation mainly) derivatives have been shown to possess antiviral, anticoagulant, and antitumor properties [161–164]. Some sulfonation products also exhibit the ability to interact with growth factors [165]. The chemical sulfonation of curdlan and other polysaccharides with similar structure and the products of their chemical sulfonation have been extensively studied due to the discrepancies between their in vitro and in vivo activity.

Data from the study of curdlan sulfates in coagulation assays indicate the anticoagulant effect of these compounds is dependent upon MW [166] and degree of sulfonation [167]. In addition to MW and degree of sulfonation, the substitution pattern of sulfo groups on the glucose monomer is also important in anticoagulant activity. Uniform sulfo group distribution around glucose monomers and, as a consequence, more secondary hydroxyl-linked sulfo groups improves the anticoagulant activity of β-1,3-sulfo glucans [168]. This indicates primary hydroxyl-linked sulfo groups at position 6 are not required for anticoagulant effects [168]. Furthermore, this provides valuable information about the need for primary hydroxyl-linked sulfo groups as a qualification for anticoagulant effects. In fact the above conditions plus the glycosidic branching play a role in the activity of β-1,3-sulfoglucans not only in vitro but also in vivo.

Although β-1,3-sulfoglucans seem to be very comparable to heparin as antithrombetics in vivo the data from coagulation assays indicate that these compounds have lower activity [169,170]. This suggests the antithrombotic activity observed is the result of additional mechanisms beyond anticoagulation activity. One study suggests new anticoagulants, such as β-1,3-sulfoglucans, whose effects are not adequately measured using current anticoagulant assays, and comparison of these agents to the gold standard (heparin) using PT and aPTT does not adequately show the agents’ antithrombotic potential [170].

Curdlan sulfate (CurS) serves as an anticoagulant through three dissimilar mechanisms: (1) the ability to directly inhibit the thrombin-fibrinogen interaction; (2) activation of plasma inhibitors (predominately HCII), which results in thrombin inhibition; and (3) complexation with fibrinogen [167]. CurS also activates the contact system as indicated by its ability to stimulate kaikirein generation [169]. This property of CurS is insignificant in regard to anticoagulation in vivo but rather functions to break down fibrin. Exploitation of CurS as a profibrinolytic needs further investigation.

F. Xylans

Aside from pentosan, the original xylan to gain status as a sulfonated polysaccharide with anticoagulant properties, others have also been found to have such properties. Xylans isolated from corn cobs, oatspels, and larchwood have been sulfated, resulting in potent anticoagulants more active than pentosan polysulfate [171]. Larchwood xylan sulfate is the most active of these derivatives with an activity comparable to heparin [172]. This xylan seems to function in a similar fashion as heparin, accelerating the interaction between ATIII and factor IIa. Furthermore, larchwood xylan sulfate seems to interact with thrombin, directly inhibiting its effects on fibrinogen [171].

G. Total Synthesis of Polysaccharide Sulfates

Only recently has complete synthesis of sulfated polysaccharides become an area of intense interest in anticoagulant development. The first fully synthetic heparin oligosaccharide was produced in 1984 [173]. The synthesized molecule consisted of a high-affinity ATIII-binding pentasaccharide found naturally in heparin. It was quickly realized that this molecule had significant advantages over the therapeutic agents available at that time. It also became evident to researchers that for the first time it may be possible to produce heparin derivatives with very specific activities [125]. Furthermore, use of homogeneous agents in humans would be far easier to monitor, thereby reducing adverse effects and improving therapy [174]. Aside from the above, this synthesis provided extensive SAR data to the scientific community. It confirmed the reported structure and activity described for the natural product, provided
complete explanation of ATIII SAR [175], and has allowed the production of unnatural derivatives [176].

The first synthetic scheme resulting in an ATIII-binding pentasaccharide was complicated with many steps and resulted in low yield of the product [173]. Subsequent schemes reported by multiple researchers decreased the number of steps, thereby increasing the yield [177–179]. The synthesis of this pentasaccharide ATIII-binding sequence resulted in the development of methods for the synthesis of larger sulfated polysaccharides modifying the function of the molecule.

The extended synthetic targets were chosen based upon the structure, activity, and side effects of heparin. Mechanistic data from heparin studies suggest oligosaccharides with the ability to mimic heparin fully need two distinct activities: the ability to bind to ATIII and thrombin [180]. With the full elucidation of ATIII binding at hand, further synthetic oligosaccharides were derived from extensions of the known ATIII-binding pentasaccharide (Fig. 2). Thrombin binding is based on electrostatic interactions between the oligosaccharide and the protein [181]. Therefore, introduction of the thrombin-binding domain significantly increases the anionic charge of the oligosaccharide and decreases the specificity of protein binding [182] (e.g., more PF4 binding). This was the downfall of the initial oligosaccharides that were generated [183]. Further refinement of the synthetic scheme was based on four considerations: (1) a specific pentasaccharide sequence is required for ATIII binding [184,185]; (2) a thrombin-binding domain must consist of two to three disaccharides [186]; (3) a 17-saccharide chain is required for significant thrombin inhibition [183]; and (4) the six to eight central saccharides are not critical to either ATIII or thrombin interactions, therefore the charge of these residues can be altered without significant loss in anticoagulant properties [183]. These findings lead to the synthesis of a hexadecasaccharide containing an ATIII-binding domain and a thrombin-binding domain separated by a neutral hexasaccharide sequence possessing the ability of binding ATIII and thrombin and devoid of PF4 binding (Fig. 8) [183,187]. Although these modifications to the original pentasaccharide have yielded products with improved properties and activities the utility of these derivatives in humans is still under investigation.

IV. CONCLUSIONS AND PERSPECTIVE

Heparin has been the most commonly used clinical anticoagulant for more than 60 years. However, there are some side effects related to it, e.g., bleeding and heparin-induced thrombocytopenia. Low molecular weight heparins were developed as a new class of therapeutic agents called antithrombotics and offer several advantages over anticoagulant heparin. These advantages include a decreased effect on platelets (reducing both their activation and aggregation) and increased specificity of action (presumably reducing their hemorrhagic side effects). The results of clinical trials are promising suggesting that LMWHs are safer than heparin, generally resulting in less hemorrhagic complication, and have greater bioavailability and longer half-lives resulting in better dose control. In the future, more subtle structural modifications may be used to prepare heparin analogs that are entirely free from hemorrhagic side-effects.

Over the last few decades heparin and heparan sulfate have been shown to interact with a number of biologically important proteins [20], thereby playing an essential role in the regulation of various physiological processes. Our understanding of these interactions at the molecular level is important for the design of new, highly specific therapeutic agents. In addition, an understanding of the specificity of heparin and heparan sulfate will be necessary to understand normal physiological and pathophysiological processes (Fig. 9). These processes are particularly important wherever cell–cell interaction plays an important role, such as in developmental biology, cancer, wound healing, infectious diseases, inflammatory processes, and neurite outgrowth.

Substantial research effort has focused on the preparation of improved heparins and heparin analogs that might exhibit higher specificity and decreased side-effects. These heparin analogs or heparinoids include polysaccharides from animals, plants, microorganisms, and invertebrates, synthetic derivatives of polysaccharides, and acidic oligosaccharides and their small synthetic analogs. Many mammalian nonheparin glycosaminoglycans also exhibit anticoagulant properties and present considerable therapeutic potential. However, like heparin, they are heterogeneous mixtures and not much is known about their mechanism of action. Several GAG mixtures are now clinically used as antithrombotic drugs; for example, danaparoid has been use as the usual alternative for anticoagulant treatment of HIT patients. Despite some successes in developing new heparinoid agents, substantial additional efforts are needed to expand both the types of molecules used as heparinoids and their different therapeutic applications.

There is now more interest in therapeutics prepared from nonmammalian sources to avoid the risk of contamination with pathogenic agents such as prions. Sulfated polysaccharides are widespread in the marine organisms, such as the carrageenans in red algae or the fucoidans in brown algae, which show anticoagulant activity. Substantial research effort has been put in this large field of potential heparinoids, but so far none of these has been clinically tested. Heparin-like compounds with anticoagulant activity were prepared by chemical modification of bacteria polysaccharides. Thus, industrial-scale, homogeneous heparin-like substances with special anticoagulant properties might be produced by biotechnological methods.

Many other nonsulfated polysaccharides were used as starting materials in an attempt to find alternatives to natural anticoagulants. These unsulfated polysaccharides often had similar structure to heparin. Upon sulfonation, it was found that many of these compounds were anticoagulants exhibiting considerable antithrombotic activity.
Nonetheless, only two such semisynthetic anticoagulants are currently in use: pentosan polysulfate and dextran sulfate. Although this search has not yielded considerably better anticoagulants, it has yielded information about structure and activity, which may ultimately play an important role in the development of further clinically useful compounds. One concern about semisynthetic molecules is that they contain unnatural structures that may be resistant to metabolism, preventing clearance and resulting in long half-lives and toxicity.

Dependence on natural sources for pharmaceutical products has its downside. The desire to be free from the variability of nature has led to the development of synthetic heparin oligosaccharides with considerable anticoagulant activity. These compounds lack the polydispersity found in the unfractioned heparin, low molecular weight heparin, and the semisynthetic compounds developed to date. These fully synthetic products have considerable consistency, a characteristic the Food and Drug Administration and other regulatory bodies admire. The most notable, fully synthetic heparin mimic is the ATIII-binding pentasaccharide found in heparin. The pentasaccharide was initially synthesized via a complicated and expensive scheme that may not be suitable for wide-scale, commercial exploitation. Far less complex synthetic procedures are under development of larger oligosaccharides with additional activities. These studies have also yielded considerable data about the cause of platelet activation in HIT patients, possibly facilitating the synthesis of oligosaccharides devoid of such activity.

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