HEPARIN, HEPARINOIDS AND HEPARIN OLIGOSACCHARIDES: STRUCTURE AND BIOLOGICAL ACTIVITIES

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Heparin is a polydisperse, highly sulfated polysaccharide which has been in widespread clinical use over the past half century. This chapter describes our current understanding of heparin's structure and its biosynthesis. In addition to heparin's usefulness as an anticoagulant, it has a wide range of additional activities. These include its antithrombotic activity, complement inhibitory activity, angiogenic activity, and other additional, recently discovered activities. The rationale for developing biomimetic polymers to replace the natural product, heparin, is discussed. Synthetic polymers, semi-synthetic sulfated polysaccharides, fully synthetic heparin oligosaccharides, fractionated heparins, low molecular weight heparins, and enzymatically prepared heparin oligosaccharides all have been used as heparin substitutes. This chapter examines heparin's structure-activity relationships with a focus on the potential utility of these biomimetic polymers as new therapeutic agents.

HEPARIN: ITS STRUCTURE AND SIGNIFICANCE

Heparin is a polydisperse, highly sulfated, linear polysaccharide comprised of repeating 1,4 linked uronic acid and glucosamine residues. Although heparin has been used clinically as an anticoagulant for the past 50 years, its precise structure remains unknown. The failure to understand completely heparin's structure is not the result of a lack of effort but rather is due to its extremely complex structure. Heparin is biosynthesized as a proteoglycan (PG, MW is approximately 1 million) consisting of a central core protein from which approximately eleven long linear polysaccharide chains extend (Figure 1). The drug heparin is recovered from porcine intestinal mucosa or bovine lung as a glycosaminoglycan (GAG) or simply a linear polysaccharide chain without any associated protein. Originally, it was believed that the harsh commercial processing of tissues from which the drug heparin was prepared resulted in the characteristic complexity of GAG heparin. This processing included treatment with alkali, proteases, bleaching agents. Proteases and β-glucuronidases present in the tissue itself are actually
The structural complexity of heparin can be considered at several levels. At the PG level the number, position and nature of the polysaccharide chains attached to the protein core can be examined. Once freed from their core protein by the action of tissue proteases, peptidoheparan is formed (i.e., a small peptide to which a single long polysaccharide chain, MW 100,000, is attached). This peptidoheparan is short-lived as it is immediately processed by a β-endoglucuronidase to a number of smaller polysaccharide chains (only one, corresponding to the original site of attachment to the core protein, would contain peptide material) called GAG heparin. At the level of GAG heparin some of the structural complexity results from its polydispersity. GAG heparin has a MW ranging from 5,000-46,000 (degree of polymerization (DP) 10-60) with an MW (average) of 11,000. Even the heparin chain corresponding to the most prevalent DP represents a mere 5 mole% of a typical GAG heparin preparation. GAG heparin has a second level of structural complexity associated with its primary structure or sequence. Unlike nucleic acids which have a four letter alphabet (A/G/C) heparin has at least ten different sugar units comprising its alphabet with many additional sugars possible but as yet unobserved.

The structural features of PG heparin have been primarily established by studying its biosynthesis. The structural features of GAG heparin have relied on biosynthetic, chemical, enzymatic, and spectroscopic techniques. Recent efforts in our laboratory as well as others have sought to use techniques originally developed to sequence nucleic acids and proteins to establish the sequence of heparin.

PG heparin is just one in a number of families of PG macromolecules (Figure 2). Heparin and heparan sulfate are structurally similar macromolecules with polysaccharide side chains comprised of alternating (1→4) linked sulfated and or acetylated glucosamine and sulfated or nonsulfated
iduronic or glucuronic acid residues.\textsuperscript{2} The core protein of PG heparin and PG heparan sulfate are different as is the core protein between heparan sulfate found in the extracellular matrix and that found in the mesenchyme.\textsuperscript{11,12} Although structurally similar, GAG heparin and GAG heparan sulfates can be distinguished from one another on close examination of their ratio of N-acetylation to O-sulfation.\textsuperscript{13} Chondroitin sulfates are another class of PGs which can be broken down into three classes: chondroitin sulfate A, \(\alpha\)) \(\beta\)-D-N-acetylgalactosamine-4 sulfate (\(1\rightarrow\)) \(\beta\)-D-galacturonic acid (\(1\rightarrow\)) chondroitin sulfate C, \(\beta\)) \(\beta\)-D-N-acetylgalactosamine-6 sulfate (\(1\rightarrow\)) \(\beta\)-D-glucuronic acid (\(1\rightarrow\)) and chondroitin sulfate D, \(\gamma\)) \(\beta\)-D-N-acetylgalactosamine-4 sulfate (\(1\rightarrow\)) \(\alpha\)-L-iduronic acid (\(1\rightarrow\)).

Heparan sulfate is yet another class of GAGs which has a major disaccharide repeating unit: \(\rightarrow\)) \(\beta\)-D-galactose(\(1\rightarrow\)) N-acetylgalactosamine-6 sulfate (\(1\rightarrow\)). A final class of glycosaminoglycan, hyaluronic acid contains no sulfate and is not found linked to a core protein.\textsuperscript{14} Its structure is \(\rightarrow\)) N-acetylgalactosamine (\(1\rightarrow\)) glucuronic acid (\(1\rightarrow\)).

Heparin's primary application is as an anticoagulant.\textsuperscript{13} It may be more appropriate to consider heparin as a polyelectrolyte drug having a multiplicity of biological activities.\textsuperscript{15,16} Heparin is the strongest acid present in the body and thus is present under physiologic conditions as a highly charged polyanion.\textsuperscript{16} Virtually any cationic protein (pI>7) is capable of binding to heparin under physiological conditions and the activity of such a protein is more often than not affected by heparin. Even anionic proteins are capable of interacting with heparin. In fact, ATIII, the most studied heparin binding protein, has an isoelectric point of 4.9-5.3.\textsuperscript{17} Within the past decade a growing number of biological activities\textsuperscript{18} have been demonstrated to be regulated by heparin ranging from
its effect on angiogenesis to the regulation of the immune response. The discovery of these new activities has brought about an urgency to understand heparin's structure and to elucidate its structure-activity relationship (SAR). Only limited success has been made along these lines primarily due to the efforts of two research groups, headed by Ulf Lindahl and Robert Rosenberg, to understand the SAR of heparin's anticoagulant activity. Their success has spawned additional research aimed at understanding heparin's other biological activities including the true physiological role of endogenous proteoglycan heparin.

A review of heparin would be incomplete if it did not discuss new trends in improving heparin's anticoagulant/antithrombotic activity. A new class of drugs, low molecular weight heparins and heparinoids, have been undergoing clinical trials as anticoagulant/antithrombotic agents for use in a wide variety of disease states ranging from deep vein thrombosis to non-hemorrhagic stroke. These agents include both natural products chemically or enzymatically derived from heparin as well as fully synthetic oligosaccharides and polysaccharides. These new drugs represent the first generation of biomimetic heparins. In addition to being used systemically as soluble agents, recent advances in heparin chemistry has permitted their immobilization onto supports. Stable, active, heparinized biomaterials can now be prepared. These may someday replace soluble heparins in devices such as the artificial kidney and heart-lung machine.

PROTEOGLYCAN HEPARIN

1. Biosynthesis

The biosynthesis of heparin has been well studied in a mast cell culture system (Figure 3). The core protein, which contains a high number of serine-glycine repeats, is first synthesized in the rough endoplasmic reticulum. To this core, a linkage region consisting of three neutral sugars is attached to serine through the action of a xylosyl transferase. Onto this neutral sugar linkage region, a repeating copolymer of 1,4-linked glucuronic acid and N-acetylgalactosamine is assembled through the stepwise addition of UDP-sugars. The linear polysaccharides chain is extended by approximately 300 sugar units before its synthesis terminates. The chain is then partially de-N-acetylated and sequentially N and O-sulfated. Finally a unique ATIII binding site is introduced by the action of a 3-O-sulfotransferase. The structural variability in the heparin polymer is primarily the result of the incomplete nature of these postpolymerization modifications. The sequence heterogeneity in the polysaccharides chains results in a family of PG heparin macromolecules.

2. Biological Functions

PG heparin is primarily found in the granules of mast cells. The precise function of these molecules is not yet understood but they may have a role in: (1) the packing of histamine within the mast cell; (2) the stabilization, inhibition and binding of proteases which are present at very high concentrations within mast cells; and (3) the regulation of complement activation following mast cell degranulation. When mast cells degranulate heparin is released, but as GAG heparin - the result of processing by proteases and endo-β-glucuronidases. Although the GAG
Figure 3. Proposed pathway for the biosynthesis of heparin. (A) Polymerization of UDP sugars; (B) N-deacetylase; (C) N-sulfotransferase; (D) Hexuronosyl C5-epimerase; (E) 2-O-sulfotransferase; (F) 6-O-sulfotransferase; (G) 3-O-sulfotransferase.

Heparin released on mast cell degranulation demonstrates anticoagulant activity, the role of this activity is unclear. There is no direct evidence that endogenous mast cell heparin plays a role in maintaining blood flow through the vasculature, even though this is the primary application for exogenously administered GAG heparin.

The true biological function of heparin still remains hotly contested. The structurally related PC, heparan sulfate, has been isolated from the endothelial surface and has "heparin-like" sequences which permit it to bind ATIII, and thus inactivate plasma serine proteases responsible for blood coagulation. PC heparan sulfate, found in membranes of a wide variety of cells and in the extracellular matrix, appears to have an active role in cell-to-cell communication through the binding of protein growth factors, hormones, and various regulators. Although the biological roles of endogenous PC heparan sulfate and the related PG chondroitin sulfates are not completely understood, this has not precluded the use of GAGs derived from these natural products as well as GAG fractions, fragments and synthetic analogues, for a variety of medical applications.

GLYCOSAMINOGLYCAN HEPARIN

Glycosaminoglycan (GAG) heparin (commercial or the drug heparin) has been prepared and used as a clinical anticoagulant since 1939. Nearly all the studies on heparin structure and activity have been performed on this form of heparin.
1. Preparation

GAG heparin is prepared from animal tissues that are rich in mast cells, such as porcine intestinal mucosal and bovine lung. Other species of mammals, as well as birds, and even invertebrates, such as lobster and clams, which do not have a blood coagulation system, also contain heparin. The method of commercial processing of heparin varies between manufacturers and is generally regarded as trade secrets. The basic approach involves the collection of the appropriate tissue followed by proteolytic treatment, extraction and complexing with ion pairing reagents such as octylpyridinium chloride, followed by fractional precipitation. Treatment with base to remove residual protein by β-eliminative cleavage of xylose O-serine glycoside and/or bleaching with oxidizing agents is commonly used to prepare the pure white drug form of heparin. The major criteria for purity is a high specific activity expressed as USP units per milligram.

The extensive processing of commercial heparin preparations has often lead to speculation that its structure is extensively modified and might be in some ways different than GAG heparin stored in mast cell granules. Heparin has been prepared by researchers using very mild conditions at low temperatures, at neutral pH and without the use of oxidants and bleaches. Such mild preparation methods resulted in a heparin preparation indistinguishable from the commercial one. Studies on conditions required for desulfation of heparin have confirmed heparin's stability under conditions used in its processing. Recent work by our research group, using a new oligosaccharide mapping technique involving the enzymatic depolymerization of heparin followed by the analysis of the oligosaccharide products by either strong anion exchange high pressure liquid chromatography or gradient polyacrylamide gel electrophoresis, have demonstrated remarkable similarities between heparins prepared from the same tissue by different manufacturers and to heparins prepared under mild conditions (i.e., without bleaching) (Figure 4).

2. Properties

GAG heparin is a polydisperse preparation with a MW ranging from 5,000-40,000 having an MW (average) of 13,000 (20.55). The average molecular weight of porcine mucosal and bovine lung heparin preparations are similar. The primary repeating unit is β-D-glucosamine 2,6-disulfate (1→), α-L-iduronic acid 2-sulfate (1→). This repeating structure accounts for 85 wt% of porcine and 90 wt% of bovine heparin. The remaining 10-15 wt% of the heparin polysaccharide is comprised of a small number of rarer disaccharide sequences shown in Table 1. These minor disaccharide sequences are the result of incomplete biosynthesis and have a reduced degree of O-sulfation, N-sulfation (containing N-acetyl residues instead) and less idaronic acid (containing glucuronic instead). The disaccharide sequences in Table 1 do not account for all of heparin's mass. Other minor disaccharide sequences have been reported but their presence has not been independently confirmed and some may be artifacts formed in commercial processing or in depolymerization.

The disaccharide sequences are the building blocks of the heparin polymer and represent an alphabet through which information can be stored in the heparin polymer. We will see how this is the case for the antithrombin binding site, which is defined by a particularly rare saccharide sequence, later in this chapter. Until recently, there was little information on the sequence of the heparin polymer or, indeed, whether or not
there was even a sequence. Early researchers viewed heparin as a polymer having a random sequence where the only restrictions were the type of saccharide units present, the (1→4) linkage configuration, and the required alternating substituted uronic acid and glucosamine residues. Biosynthetic studies, combined with the frequent occurrence of a rare

Table 1. Structure and frequency of disaccharide sequences found in heparin.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Sequence$^a$</th>
<th>Mole%$^b$</th>
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<tbody>
<tr>
<td>a</td>
<td>→4)IDU2S(1→4)SN2S,6S(1→</td>
<td>7.1</td>
</tr>
<tr>
<td>b</td>
<td>→4)IDU2S(1→4)SN2S(1→</td>
<td>6.8</td>
</tr>
<tr>
<td>c</td>
<td>→4)TGU(1→4)GNAc,6S(1→</td>
<td>4.4</td>
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<tr>
<td>d</td>
<td>→4)GA(1→4)GN2S,6S(1→</td>
<td>9.3</td>
</tr>
<tr>
<td>e</td>
<td>→4)IDU(1→4)GN2S,6S(1→</td>
<td>1(7.57</td>
</tr>
<tr>
<td>f</td>
<td>→4)GA(1→4)GN2S,3S,6S(1→</td>
<td>4.4</td>
</tr>
</tbody>
</table>

(a) IDU, α-D-idopyranosyluronic acid; GA, β-D-glucopyranosyl-
uronic acid; GN, 2-deoxy-β-D-glucopyranose; GNAc, 2-deoxy-2-acetamido-
β-D-glucopyranose; S, sulfate.
(b) Mole% found in a typical commercial porcine mucosal
heparin (heparin R).$^{43}$
(c) Not determined.
sequence associated with the antithrombin binding site, has changed this perception.

One approach to understanding order (sequence or information storage) in the heparin polymer has used computer or mathematical simulation.\(^6\)\(^7\) For example, the major repeating unit in the heparin polymer, a trisulfated disaccharide (Table 1), could be: (1) clustered in one region of the heparin polymer; (2) distributed uniformly throughout the polymer; (3) distributed randomly throughout heparin; or (4) occur in unique, well defined positions within the polymer. By computer or mathematical simulation, number chains representing the heparin polymer are constructed following these site distribution patterns. After preparing an ensemble of number chains having the correct polydispersity, the computer simulates their breakdown by cutting the number chains through these sites. The size distribution of the smaller number chains formed are then calculated. These simulated distributions are matched against experimentally measured product distributions obtained using an enzyme, heparinase, which cuts heparin through these sites. The results of this study demonstrated that the major disaccharide repeating sequence was either randomly distributed through the heparin polymer or distributed in a unique sequence that was indistinguishable from the random solution.\(^6\)

A more sophisticated computer simulation examined whether a random distribution of the monosaccharide units, in the experimentally determined quantities found within the heparin polymer, could give rise to the oligosaccharides found in oligosaccharide mapping experiments.\(^7\)\(^9\) The results of this study demonstrated that the individual sugar units found in heparin could not be randomly arranged. Further studies along these lines look at the kinetics of heparinase depolymerization of heparin and the distributions of products formed throughout the reaction (not just

<table>
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<th>Table 2. Compositional and sequence analysis of transient oligosaccharides.</th>
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<td>Transient Oligosaccharide</td>
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<tr>
<td>---------------------------</td>
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<td>HEXASACCHARIDES</td>
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<td>octasulfated-1</td>
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<tr>
<td>octasulfated-2</td>
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<td>undecasulfated-1</td>
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</tr>
<tr>
<td>pentadecasulfated</td>
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(a) The structure of disaccharide units a, b, c, d, and f are given in Table 1.
(b) a' is α-L-DU2S(1→4)NU2S6 where α-L-DU2S is 4-deoxy-α-L-threo-Hex-4-enopyranosyluronic acid.
(c) nd, not determined.
the final product distribution). These simulation studies represent a novel way of understanding sequence of polydisperse polymers such as heparin.

Classical sequencing methods have also been applied to studying the primary structure of the heparin polymer. CAS heparin can be partially depolymerized (either enzymatically or chemically) to prepare transient oligosaccharides (i.e., oligosaccharides not found in the final products because they still contain cleavable sites). These oligosaccharides can then be purified to homogeneity. If a transient oligosaccharide is then exhaustively depolymerized to smaller, previously characterized, final oligosaccharides their composition can be determined. For introducing a tag, such as a radiolabel, at one end of the transient oligosaccharide a sequence can be determined. For more complex transient oligosaccharides, a series of partial depolymerizations can be used and the mixture of these run out on a gel. This represents the polysaccharide equivalent of Maxam-Gilbert sequencing of nucleic acids. The composition and sequence of a number of transient heparin-derived oligosaccharides, which have been determined in this way, are given in Table 2.

New methods of sequencing heparin are now being proposed which involve sequencing the whole intact proteoglycan. These methods depend on the introduction of a reading frame from which the sequence can be determined. One such reading frame is the reducing terminus of the polysaccharide chain where it attaches to the core protein. If the polysaccharide chains can be removed and a label introduced into the same position where each was attached to the core, then a sequence might be read from this label, down the polysaccharide chain. This method is attractive as it might lead to the total sequence of heparin. A potential pitfall of this approach is that the sequence from the reducing terminus might be interrupted by a series of random sequences that might result in the loss of the reading frame.

3. Medical Applications of Heparin

Systemic Anticoagulation. Heparin is the most commonly used clinical anticoagulant. Over six metric tons of heparin are manufactured worldwide each year, representing over 50 million doses. Heparin is administered intravenously 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 by-pass procedures. Systemic heparinization is also widely used in treating deep vein thrombosis and a variety of other surgical procedures. Heparin is also given by the subcutaneous route and, despite its reduced bioavailability, a low level of heparinization can often be maintained this way for prolonged periods of time. Although it would be highly desirable to prepare an orally active heparin which could be administered outside a hospital setting, no such drug derivative or formulation is currently available. Heparin's major side-effect, hemorrhagic complications, is closely linked to its anticoagulant activity.

Thus, while heparin is widely used, it has been cited as the drug most responsible for death in otherwise healthy patients. After administering heparin, it is often necessary to neutralize its anticoagulant activity and this is usually done by administering a cationic polypeptide based drug called protamine. Protamine is not without its own set of side-effects and the resulting protamine heparin complex has been associated with an immune related loss of platelets.
Regional Heparinization. The heparin required to maintain flow in an extracorporeal circuit is needed only in the blood outside the body. Once the blood is returned to body and its vascular container it is naturally anticoagulated. In systemic heparinization, heparin is returned to the body along with the blood and this is where it demonstrates many of its side-effects.\textsuperscript{70} One approach directed at solving this problem is to infuse heparin as the blood leaves the body and enters the device, and then to remove the heparin from the blood as it leaves the device and re-enters the body.\textsuperscript{16} This approach makes use of the ability of immobilized microbial heparinase to destroy heparin's anticoagulant activity catalytically,\textsuperscript{56, 71} forming inactive and non-toxic\textsuperscript{60, 72} oligosaccharide products which can be cleared from the body without metabolism.\textsuperscript{73} Animal studies in dogs and sheep have demonstrated that regional heparinization is possible, but the need for human clinical trials remains.\textsuperscript{74}

Blood compatible Polymers. The preparation of blood compatible polymers has been undertaken to reduce the undesirable side-effects associated with systemic heparinization during extracorporeal therapy. One approach has been the design and preparation of heparin-coated and heparin-bonded surfaces.\textsuperscript{23, 27} The rationale behind preparing such surfaces is their similarity to the natural luminal surface of blood vessels which are lined with a heparin-like proteoglycan heparan sulfate. Recently, antithrombin binding sites common to heparin and responsible for its anticoagulant activity have been identified in endothelial heparan sulfate.\textsuperscript{24, 27} Early efforts at preparing heparinized surfaces have been aimed at the non-covalent attachment of heparin by entrapment,\textsuperscript{29} adsorption,\textsuperscript{23} or ionic interaction.\textsuperscript{23} These approaches had certain limitations, the most serious of which was the leaching of the weakly bonded heparin from the device's surface resulting in gradual loss in blood compatibility.\textsuperscript{25} In addition a number of different polymers (some porous, some flexible, some rigid, etc.) are commonly used in the construction of an extracorporeal device such as a membrane oxygenator or a hollow fiber kidney dialyzer. Not all of these material types are equally amenable to heparin bonding nor are they equally stable.\textsuperscript{25}

Recent advances in heparin chemistry have resulted in a better understanding in the covalent coupling of heparin to polymers.\textsuperscript{25} This stronger covalent coupling has resulted in several spectacular advances in fully heparinized extracorporeal devices.\textsuperscript{27} The simplest of these is the heparinized venous lines that are now becoming widely used in hospitals, as these involve only a single material type.\textsuperscript{25} More complicated devices including fully heparinized membrane oxygenators are just entering clinical trials following very successful animal studies.\textsuperscript{27, 28} In hind-leg profusions in dogs and sheep membrane oxygenators remained open to flow for up to 24 hours without the use of external pumps.\textsuperscript{27, 28} Progress in this area depends on understanding which type of binding is the best preventing both leaching and the stripping of surface heparin by the bodies naturally occurring heparinas.\textsuperscript{74} The limited effect of those surfaces on formed blood components such as platelets is also of primary importance.\textsuperscript{17, 28} Other questions which need to be addressed is what type of heparin should be immobilized and whether low molecular weight heparins or synthetic heparinoids would be better ligands for immobilization.

HEPARIN AND ITS ANTITHROMBOTIC ACTIVITY

1. Blood Coagulation

Hemostasis, "the spontaneous arrest of bleeding from ruptured blood
is a broad physiological process of which the blood coagulation system is just one part. In vivo hemostasis involves plasma coagulation factors, platelets, monocytes, and endothelial cells which line the blood vessels. The coagulation cascade consists of a sequence of reactions in which protease precursors (apoenzymes) are transformed from enzymatically inactive to enzymatically active forms. In the final stages of the coagulation cascade, fibrinogen is transformed by thrombin (factor IIa) into the spontaneously polymerizable fibrin monomer. Polymerization and subsequent crosslinking of fibrin monomers produces gelatinous fibers which ensnare platelets forming a primary hemostatic plug.

The coagulation cascade is divided into two pathways, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is activated by tissue damage which releases thromboplastin, calcium and phospholipid from the cell membrane. These factors activate factor VII, which transforms factor X into its proteolytically active form, factor Xa. The second pathway, the intrinsic pathway, is activated by blood contacting non-endothelial surfaces followed by the transformation of several circulating coagulation factors finally leading to the formation of factor Xa. The remaining steps, referred to as the final common pathway, results from factor Xa catalyzing the conversion of prothrombin to thrombin which cleaves fibrinogen to form a fibrin monomer, which produces a clot. The coagulation cascade appears to be autocatalytic and self-limiting and thrombin (factor IIa) plays a central role. The generation of active coagulation factors is explosive and is initiated by a local injury at a vessel wall, while inhibitors of these proteases are present throughout the entire vascular system. The active coagulation processes, therefore, only exist momentarily at the site of injury where their rate of formation is more rapid than their rate of inactivation.

2. Serine Protease Inhibitors

The blood coagulation enzymes are serine proteases with trypsin-like specificity for arginyl linkages. Unlike trypsin, they have a higher degree of specificity for the linkages they cleave and often require cofactors to accelerate their rate of reaction. The entire coagulation process is under the control of a group of glycoproteins serine protease inhibitors, the most important of which is antithrombin III.

Antithrombin III. ATIII is a single chain anionic (pI 4.9-5.3) glycoprotein of molecular weight 58,000. All the coagulation proteases (except for VIIa) are inhibited by ATIII which forms an equimolar covalent complex with these enzymes. ATIII has a high affinity for these enzymes (low K_M) but a slow turnover rate (low K_C). The reaction affords an intact protease covalently linked to the amino terminal portion of ATIII. The ATIII which is released is proteolytically modified at a single site near the carboxy terminus. Heparin binds to thrombin and ATIII in a ternary complex accelerating the rate of thrombin inhibition by ATIII by 2000-fold.

Heparin Cofactor II. Heparin cofactor II is structurally similar to ATIII with a molecular weight of 65,000 and a pI of 4.9-5.3, having a similar carboxy terminal sequence but a distinctly different amino terminal sequence. The physiological role of HCII might be as a reserve of thrombin inhibitor when the plasma concentration of ATIII becomes abnormally low. Unlike ATIII, HCII can inhibit thrombin but no other coagulation proteases. In addition to this unusual specificity, HCII can also be potentiated by GAGs other than heparin including dermatan sulfate and heparan sulfate both of which are found lining the luminal
3. Other Antithrombotic Actions of Heparin

Heparin is known to activate platelets, which represent an important component of thrombosis. Once a clot is formed, heparin can act to accelerate fibrinolysis or the dissolution of the clot. A new generation of fibrinolytic drugs including streptokinase, urokinase, and tissue plasminogen activator are particularly promising for the treatment of coronary clots formed in heart attacks. These agents have been tested in conjunction with heparin to accelerate clot dissolution and to prevent clot reformation. The most serious side-effect with such therapy is bleeding complications, but these might be eliminated by using new, low molecular weight heparins and heparinoids with reduced hemorrhagic side-effects.

HEPARIN'S OTHER BIOLOGICAL ACTIVITIES

1. Antiatherosclerotic Activity

Effect on Lipoprotein Lipase. When heparin is administered intravenously, it causes the release of lipoprotein lipase (LPL) from the endothelium. This may result in increased triglyceride lipolysis occurring in the blood stream, thereby lowering the concentration of cholesterol-rich remnant particles in contact with the arterial wall. The effect of heparin on the release and activation of LPL has been studied in a number of different animal models including humans. Two major problems stand in the way of the application of heparin as an antiatherosclerotic agent. These are its primary activity as an anticoagulant and heparin's low bioavailability when administered orally. To circumvent these problems, low molecular weight heparins, heparin-oligosaccharides, and heparinoids are being studied.

Effect on Smooth Muscle Proliferation. The proliferation of smooth muscle cells following damage to the endothelium is an important part of atherogenesis. Both anticoagulant and non-anticoagulant heparins have demonstrated the ability to inhibit the proliferation of smooth muscle cells. A heparin-like AGG, present on the luminal surface of the endothelium, probably plays a physiological role in the regulation of smooth muscle proliferation during atherogenesis. A synthetic pentasaccharide containing an ATIII binding site demonstrates anti-proliferative activity comparable to heparin's, as demonstrated in rat aortic smooth muscle cell culture. A similar pentasaccharide, missing the 3-O-sulfate required for ATIII binding, showed a markedly reduced activity, as did disaccharide and tetrasaccharide samples.

Heparins and low molecular weight heparins, with low affinity for ATIII, also demonstrated antiproliferative activity comparable to commercial heparins thus indicating, at least for higher oligosaccharides, that the presence of an ATIII binding sequence is unnecessary. It is likely, therefore, that the anticoagulant and antiproliferative activities are separable. Smooth muscle proliferative activity is highly source dependent as heparins from different commercial suppliers show 2 to 3-fold differences in activity. Full structure-activity studies will be required to exploit heparin as an antiproliferative agent.
effect on smooth muscle proliferation continues to be an active area of research interest.\textsuperscript{109-114}

2. Ability to Inhibit Complement Activation

Heparin's principle location in man is the granules of tissue mast cells and basophils. Because heparin's primary location is so closely linked to the immune response, its ability to regulate complement has become an active area of interest. Heparin acts at multiple sites in both the classical\textsuperscript{113-118} and the alternative amplification pathways\textsuperscript{10,67,119,123} of complement. Heparin, heparin-Oligosaccharides and other polyanions can inhibit the cell-bound alternative amplification pathway C3 convertase, C1b,B, and C3b,Bb,P\textsuperscript{113} as well as fluid-phase consumption of B by D in the presence of C1b, suggesting a direct action on C1b.\textsuperscript{119} Heparin's anticoagulant activity is primarily associated with a specific pentasaccharide sequence at which ATIII binds.\textsuperscript{120,121} Heparin's structure-activity relationship on the complement system, however, is still poorly understood. Although studies indicate the importance of O-sulfation\textsuperscript{17,116} and a minimum molecular size,\textsuperscript{20} a specific binding site for a complement factor such as C1b (similar to the ATIII binding site) has not yet been implicated in heparin's ability to inhibit complement activity.\textsuperscript{41}

3. Angiogenic and Antiangiogenic Activities

Angiogenesis or neovascularization is defined as the formation of new blood vessels. This process, originally identified with vascularization in the placenta,\textsuperscript{110} has been extended to other capillary growth including tumor angiogenesis.\textsuperscript{122} After a tumor takes hold it grows slowly, remaining quite small (1-2 mm\textsuperscript{3}) until new capillaries come to within 150-200 mm (the distance which oxygen can diffuse).\textsuperscript{124} Once vascularized, the tumor cells multiply at a rapid rate resulting in serious damage to the tissue surrounding the growing tumor. The process of angiogenesis, or the ingrowth of blood vessels, requires the enzymatic degradation of the extracellular matrix underlying tissue (basement membrane), the movement of endothelial cells into this space and their replication.\textsuperscript{123} Heparin may play a variety of roles in angiogenesis. Immediately before capillary ingrowth, mast cells, containing heparin, congregate. The heparin from these mast cells can stimulate endothelial cell migration and this activity can be blocked by the addition of protamine.\textsuperscript{124}

Heparin can increase the activity, stability or binding of growth factors which stimulate angiogenesis, such as fibroblast growth factor (FGF), endothelial cell growth factor (EGF),\textsuperscript{125-127} Heparin and heparin Oligosaccharides can inhibit angiogenesis\textsuperscript{139} in the presence of angiotensic steroids, such as the naturally occurring metabolites of cortisone, which were previously thought inactive.\textsuperscript{139,128} Specific sequences in the heparin polymer are capable of very tight chelation of copper,\textsuperscript{129} long implicated as an angiogenesis modulator.\textsuperscript{136} Folkman and Klagsbrun proposed that heparin-like glycosaminoglycans, lining the endothelium, in the presence of other soluble factors, such as steroids, restrain capillary growth.\textsuperscript{38} This quiescent microvasculature\textsuperscript{131} can rapidly respond to heparin modulated growth factors produced during ovulation, by wounds,\textsuperscript{132} or inflammation (as occurs in stroke where the damaged blood brain barrier requires repair),\textsuperscript{132} in immune reactions and tumors.\textsuperscript{134}

To understand fully the role of heparin in the process of angiogene-
siss. specific saccharide sequences within the heparin polymer which bind growth factors and growth modulators such as copper need to be elucidated. Once the necessary sequences have been determined heparin-oligosaccharides might be prepared either enzymatically or synthetically. These biomimetic oligomers would potentially represent an important new class of drugs capable of regulating angiogenesis.

4. Additional Activities

Interaction of Heparin with Enzymes. Heparin has been found to bind to, inhibit or activate a large number of enzymes (Table 3). Heparin inhibits leukocyte elastase while having no effect on the related porcine pancreatic elastase or Pseudomonas aeruginosa elastases. The inhibition was established to be tight-binding (K_i 40nM-100 μM depending on chain length) and hyperbolic non-competitive. Over-sulfation of the heparin chain enhances its activity. The therapeutic application of this activity might be in treating disease states where the role of leukocyte elastase has been well established, such as emphysema or rheumatoid arthritis. Heparin can activate protein kinases found in both liver and skeletal muscle. Heparin inhibits the activity of acetylcholinesterase. Heparin's ability to inhibit topoisomerase I, important in cell replication, has been demonstrated to be independent of its anticoagulant activity and primarily the result of its highly charged nature. Heparin accelerates the antiprotease inhibition of protease nexin, a protease found predominantly in the brain and one whose activity is associated with Alzheimer's disease. Heparin effects the proteases involved in fertilization of the egg by the sperm in mammals. Heparin is a potent inhibitor of phospholipase A2 which is found elevated in uremia.

Interaction of Heparin and Proteins. Heparin has been shown to bind, to stimulate, or to protect a number of different growth factors including platelet, epithelial, epidermal, fibroblast, insulin-like, and other growth factors.

<table>
<thead>
<tr>
<th>Table 3. Enzymes which interact with heparin.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BINDING</strong></td>
</tr>
<tr>
<td>RNA polymerase, THF synthetase, Streptokinase, Tryptase, Adenylate cyclase, Complement Convertases, Thrombin</td>
</tr>
<tr>
<td><strong>INHIBITION</strong></td>
</tr>
<tr>
<td>Neutrophil elastase, Topoisomerase I, Topoisomerase II, Esterase, Lipase, Kinase, Glycogen synthase Kinase, Diamine oxidase, Casein Kinase II, Lysyl oxidase, Sialyl transferase, Protein C</td>
</tr>
<tr>
<td><strong>ACTIVATION</strong></td>
</tr>
<tr>
<td>Kinases, Phosphatases, RNA-dependent protein kinase, Lipoprotein lipase and Hepatic triglyceride lipase</td>
</tr>
</tbody>
</table>
Heparin interacts with blood components, blood proteins, and extracellular matrix proteins such as fibronectin, laminin, and vitronectin. Heparin also binds to low density lipoproteins (LDLs).

**Heparin's Other Biological Activities.** An endogenous heparin-like anticoagulant has been found in the blood of an AIDS patient and has been associated with coagulation abnormalities. Exogenous heparin is being clinically tested in the treatment of AIDS and has shown itself effective (as has dextran sulfate) in blocking human immunodeficiency virus (HIV) replication in vivo.

Heparin and other proteoglycans have a role in neoplasia. Heparin binding sites have been found on B16 melanoma cells. Heparin's effect on angiogenesis has been exploited to inhibit tumor growth. Heparin-like polysaccharides have recently been found in the neuritic plaque formed in Alzheimer's disease. Low concentrations of heparin inhibit the binding of inositol phosphate to its receptor thus effecting calcium release from cells.

**RATIONALE FOR DEVELOPING BIOMIMETIC POLYMERS TO REPLACE HEPARIN**

1. Structural Requirements for Active Biomimetic Polymers

Heparin's biological activities are primarily mediated through its binding to proteins and its regulation of their activities. To substitute for heparin, biomimetic polymers must bind to these proteins and regulate these same activities. Heparin's binding is primarily through electrostatic interactions and depends on its high charge density. Most of these biomimetic polymers are highly sulfated polymers, prepared enzymatically or chemically, and can substitute for heparin by the positioning of these charged groups. Ideally these heparin substitutes should exhibit tighter binding to these proteins in order to have higher potency. Only the heparin ATIII binding site has been sufficiently studied to develop a well defined structure activity relationship (SAR). The synthetic heparin pentasaccharide binding site and heparin have comparable ATIII binding avidity (heparin-ATIII, \(K_{\text{iso}} = 10^{-7}\), synthetic pentasaccharide-ATIII, \(K_{\text{iso}} = 10^{-7}\)) as well as comparable activity towards the inhibition of ATIII. Structure modification studies are currently underway in an effort to prepare analogs with even higher binding affinities. Unfortunately, heparin’s interaction with ATIII is the only heparin-protein interaction that has been demonstrated to be restricted to a specific saccharide sequence. Even in this thoroughly studied system, it is uncertain which amino acids within ATIII are interacting with the required sulfate groups in heparin. To systematically develop heparin biomimetic polymers, an increased understanding of heparin's interaction with the proteins it binds to and regulates will be required.

2. Combating Heparin's Side-Effects

Although heparin's major activity as an anticoagulant is widely exploited, it is also associated with its most common side-effect. Bleeding complications can range from mild mucosal ooze to intracranial hemorrhage. Low molecular weight (LMW) heparins represent a new class of therapeutic agents called antithrombotics. These LMW heparins offer several advantages over the anticoagulant heparin. These include a reduc-
ed effect on platelets and increased specificity of action. LMW heparins
catalyze the ATIII mediated inhibition of Factor Xa to a greater extent
than the inhibition of Factor IIa. This greater specificity towards an
early step in the coagulation cascade may be partially responsible for
the reduced hemorrhagic side-effects of LMW heparin. Heparin's hemor-
rhagic side-effect have been reduced by modifying its molecular weight.
In the future more subtle structural modifications may be used to prepare
antithrombotic heparins which are free from hemorrhagic side-effects.

Heparin-induced osteoporosis (decalcification of the bones) can be
reduced by either increasing heparin's anticoagulant potency, thereby
reducing the dose of the agent required, or by decreasing the length of
time a patient is heparinized. Many of heparin's other secondary side-
effects can also be reduced by this approach.

3. Exploiting Heparin's Side-Effects

Not all of heparin's side-effects are undesirable. Some of these
side-effects could be exploited and new pharmacologically active agents
prepared if only these activities could be enhanced. For example, heparin
releases and activates lipoprotein lipase (LPL), but does so only at
concentrations which fully anticoagulate. If a heparin could be pre-
pared that was devoid of anticoagulant activity with high LPL releasing
activity, it might represent a useful agent in the treatment of athero-
sclerosis.105 Heparin inhibits complement activation, but only at con-
centrations ten-times those required for full anticoagulation.41 Recent
results in our laboratory have demonstrated that it is possible to pre-
pare heparin-oligosaccharides equipotent with heparin (on a weight basis)
towards complement, but without anticoagulant activity.41 Such a drug, or
one which contained equal potency as an anticoagulant and as a complement
activation inhibitor, might be useful in preventing both coagulation and
complement activation in extracorporeal therapy. There are scores of
other heparin side-effects which might be usefully exploited resulting in
the preparation of new classes of drugs.

4. Controlling Pharmacokinetics

One major problem with heparin is its poor bioavailability when ad-
ministered by certain routes.104 Heparin is primarily administered intra-
venously, and acts systemically as an anticoagulant.10 It has also has
been administered by the subcutaneous route.102,103 By this route, how-
ever, only a fraction of heparin ever makes it to the circulation, and
then only after a long delay.102,103 This delayed release of low levels of
heparin into the circulation is often desirable.102,103 Low-dose heparini-
zation has been effectively used in the treatment of deep vein throm-
bosis.10,103 Both intravenous and subcutaneous routes require hospitali-
ization of the patient being treated. If an orally active heparin could be
prepared which was safe, it might permit the treatment of out-
patients.104,105 This type of therapy might be useful for disorders which
do not generally call for hospitalization, such as the reduction of VLDLs
and LDLs in the prevention of atherosclerosis.105

A major second problem associated with the use of heparin has been
the lack of understanding of its metabolism and its erratic and sometimes
unpredictable rate of clearance.105 Many of these problems are due to the
absence of a sufficiently sensitive assay for the measurement of chemical
heparin in the plasma.112 Currently the only methods having the required

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sensitivity are biosassays, which are also affected by subtle changes in the levels of coagulation proteins in the plasma and are subject to numerous additional interferences. The preparation of homogeneous heparin, which are pure single entities, would go a long way towards solving these problems in facilitating the development of sensitive chemical assays.

5. Preparing Stable Blood Compatible Surfaces

Part of the problem, associated with the preparation of blood compatible surfaces, is our lack of a complete understanding of coagulation and thrombosis. Ideally, one would like to mimic blood's natural container, the vessel lined with endothelial cells, as closely as possible. In addition, the flow characteristics of the device being designed must also closely resemble those observed in the vasculature. Lastly, the surface must be stable and survive enzymatic and chemical attack from the components present in the circulation. It is beyond the scope of this review to consider the first two requirements except for saying that the natural endothelium is lined with heparin-like molecules. The last requirement, the production of a stable antithrombotic surface, is not as simple as it might appear. Simple adsorption of heparin onto a polymer produces a blood compatible surface which only lasts a short period of time until the heparin leaches from the surface.

Covalent immobilization offers an alternative in that the linkage is chemically stable, but these surfaces also have a short lifetime, possibly due to the enzymatic stripping of heparin from the surface. Enzymes which act on heparin are present in the circulation such as exo and endo-glycosidases. The precise nature of the surface-heparin linkage as well as control on the enzyme accessible regions of the immobilized heparin chain are required to form a stable heparinized surface. Our

Figure 5. Immobilization of heparin to surfaces. (A) heparin coupled through carboxyl group; (B) heparin coupled through amino group; (C) heparin coupled through its reducing end; (D) heparin coupled through its non-reducing end (R = linkage arm).
Figure 6. Structure of heparinoid natural products and their derivatives. (A) Xylan sulfate; (B) Ribose sulfate; (C) Pentosan sulfate; (D) Dextran sulfate \( X = O \) or \( NH \); and (E) Fucoidan \( m = \) degree of polymerization.

Laboratory is currently examining the question of how the orientation of a heparin chain immobilized to a surface (i.e., coupled through either its reducing-end or its non-reducing end) effects its linkage stability when exposed to enzymes in the circulation (Figure 5). Work along these lines is required to design blood compatible surfaces intelligently.

SYNTHETIC SULFATED POLYSACCHARIDES

Polymers that are structurally related to heparin and which possess certain of its biological properties, such as its anticoagulant activity, are commonly called heparinoids. These heparinoids have been prepared by modification of naturally occurring polysaccharides (Figure 6), by the total synthesis of heparin-like polymers, and most recently by the synthesis of small sulfated heparin-like oligomers.

1. Sulfation of Natural Polysaccharides

Chitin, a polymer of 2-acetamido-2-deoxy-D-glucose, is the major organic component of the exoskeleton found in insects, crabs, etc.\(^{14}\) Chitin can be de-N-acetylated to prepare chitosan, which on chemical sulfation and/or carboxymethylation results in a polymer having certain structural similarities to heparin.\(^{21a}\) These chitosan derivatives show anticoagulant activity related to their degree of sulfation. Sulfated, carboxymethylated chitosan inhibits thrombin activity through ATIII to almost the same degree as heparin.

Pentosan, a (1→4)-\( \beta \)-linked xylopyranose with a single laterally
positioned 4-O-methyl-α-D-glucuronic acid, is extracted from the bark of the beech tree, Fagus sylvatica. When fully sulfated by chemical methods, it has been demonstrated to be an anticoagulant with one-tenth of heparin's activity on a weight basis. Its primary anti-Ⅱa activity has been proposed to be HCII mediated. This heparinoid also has a demonstrated antihemipar activity in that it can compete with heparin for antithrombin III binding, reducing the rate of heparin catalyzed ATⅢ mediated inhibition of thrombin.

Dextran, a (1→6)-D-glucan (1→3)-α-D-branched polymer, can be chemically sulfated to prepare dextran sulfate. Both dextran (a plasma extender) and dextran sulfate have been used as pharmaceuticals. Dextran sulfate has low anticoagulant activity with high LPL releasing activity. This has permitted the exploitation of this agent as an antiatherogenic in Japan. Dextran sulfate has been used as a heparin replacement in anticoagulation and has recently been immobilized on plastic tubes to prepare non-thrombogenic surfaces. A clinical study on the use of dextran sulfate as an HIV inhibitor in the treatment of AIDS has recently begun.

2. Fully Synthetic Sulfated Polymers

Synthetic polymers such as poly(vinyl sulfate) and poly(anethole sulfonate) are highly charged heparin-like polymers (Figure 7) which exhibit anticoagulant activity. This activity has only been exploited in vitro in preventing coagulation of collected blood or plasma samples during storage for assay. The use of these agents in vivo has largely been precluded by their high toxicity. These synthetic polymers are resistant to metabolism and thus remain in the body for extended periods during which they can display severe side-effects.

3. Small Sulfated Oligomers

Homogeneous, structurally defined, heparin oligosaccharides have been prepared by both enzymatic and synthetic means. These possess both anticoagulant activity as well as other heparin associated activities. Often these heparin-oligosaccharides are specific displaying only a single activity.

Figure 7. Structure of synthetic heparinooids. (A) Poly(vinyl sulfate) and (B) Poly(anethole sulfonate) where n = degree of polymerization.
The synthetic 3-O-sulfated pentasaccharide, representing heparin's ATIII binding site was first prepared by Choay et al., in a multi-step synthesis in less than 5% yield. This synthesis has been optimized by Choay et al. (Figure 8) and repeated by other groups. Activity studies demonstrate that this agent is a potent accelerator of ATIII mediated inhibition of Factor Xa. Clinical studies on this pentasaccharide as an antithrombotic agent demonstrated that it was not as effective as heparin itself. The cost of synthesis may also preclude its use as a therapeutic agent.

Heparin-oligosaccharides of defined structure have been prepared from heparin in our laboratory using enzymatic methods. These heparin oligosaccharides possess a number of important biological activities,
including their capacity to inhibit complement activation in vitro, with nearly equipotency to heparin on a weight basis. Further studies will be required to demonstrate this activity in vivo as well as to develop large-scale inexpensive methods to prepare these heparin oligosaccharides.

Synthetic, highly sulfated, lactobionic acids have recently been demonstrated to have high activity in the potentiation of HII inhibition of thrombin. The regulation of the coagulation cascade at this step resembles dornatan sulfate's activity and might represent an interesting new class of antithrombotic agents.

Synthetic analogs of heparin have a great advantage in that they can permit access to unusual structures which do not occur in nature and thus are not found in natural products. On the other hand, carbohydrate synthesis is extremely complex and tedious, involving many blocking and de-blocking steps required to protect sensitive functionality. Thus, it is difficult to predict when structurally complex pentasaccharide to decasaccharide sized structures will be preparable in a cost-effective manner by the synthetic chemist. A natural product, such as heparin, provides a relatively inexpensive source of the desired oligosaccharide sequence, thus its enzynatic recovery from the polymer probably represents the best method for its preparation. Ultimately, the biotechnologist might be able to replace both the synthetic and the natural product routes if the desired sequence can be prepared using recombinant genetics. This option is still far off as glycosaminoglycan biosynthesis requires many enzymes to act in concert in a highly compartmentalized cell. Prokaryotes, in which most recombinant genetics is currently carried out, probably are not sufficiently compartmentalized to conduct such synthesis. Thus the preparation of glycosaminoglycans by recombinant genetics awaits the development of improved eukaryotic vectors.

FRACTIONATED FEPARINS

1. On the Basis of Size or Charge

Heparin can be fractionated on the basis of size using gel permeation chromatography (GPC). Both low pressure and high pressure GPC has been used to obtain heparin fractions of average molecular weight between 5,000 and 40,000. Detection methods include continuous measurement of absorbance at 206 nm, colorimetric assay of fractions using metachromatic dyes such as azure A, or uronic acid assay using carbazole. Because of its high charge density, heparin exists as an extended helical rod and thus has a greater molecular size than does a globular protein of similar molecular mass as estimated by GPC. For example, a heparin of MW (average) 13,000 has a 

Being a sulfated polysaccharide (with an average of 2.7 sulfate groups per repeating disaccharide unit), heparin can be fractionated on the basis of its charge using anion exchange chromatography. Both low and high pressure strong anion exchange (SAX) chromatography of heparin has been reported. Typically, heparin is bound to an anion exchanger at low ionic strength and released by either a stepwise or gradient solution with solute of increasing ionic strength. Low pressure supports such

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### Table 4. Heparin activities as a function of polymer chain length.

<table>
<thead>
<tr>
<th>Approximate MW</th>
<th>Anti IIa activity (^{(a)}) ((U/mg))</th>
<th>Relative complement activation inhibition (^{(b)})</th>
<th>Relative lipase releasing activity (^{(c)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrasaccharides (^{(a)})</td>
<td>1200 &lt;1 &lt;1</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Hexasaccharides</td>
<td>1500 &lt;1 &lt;1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Octasaccharides</td>
<td>2400 &lt;1 &lt;1</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Decasaccharides</td>
<td>2500 &lt;1 &lt;1</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Dodecasaccharides</td>
<td>3500</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Tetradecasaccharides</td>
<td>4100</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Hexadecasaccharides</td>
<td>4700</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Octadecasaccharides</td>
<td>5300</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>Bicossaccharides</td>
<td>5900</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Heparin fraction 1</td>
<td>6,000</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>Heparin fraction 2</td>
<td>5,700</td>
<td>25</td>
<td>44</td>
</tr>
<tr>
<td>Heparin fraction 3</td>
<td>18,700</td>
<td>33</td>
<td>115</td>
</tr>
<tr>
<td>Heparin fraction 4</td>
<td>14,500</td>
<td>195</td>
<td>271</td>
</tr>
<tr>
<td>Heparin fraction 5</td>
<td>16,700</td>
<td>130</td>
<td>131</td>
</tr>
<tr>
<td>Heparin fraction 6</td>
<td>25,300</td>
<td>92</td>
<td>356</td>
</tr>
<tr>
<td>Unfractionated heparin</td>
<td>14,000</td>
<td>171</td>
<td>225</td>
</tr>
</tbody>
</table>

\(^{(a)}\) All heparin and heparin oligosaccharides are from porcine intestinal mucosa.

\(^{(b)}\) Oligosaccharides prepared using heparin lyase are mixtures of those shown in Table 2.

\(^{(c)}\) Not determined.

As DEAE or QAE cellulose, Sephadex or Sepharose bind heparin at ionic strengths of <0.2 M and release heparin at 0.5–1.5 M concentrations of salts such as sodium chloride. Heparin fractions can be prepared with a degree of sulfation ranging from 2 to 3 sulfate groups per disaccharide repeating unit. Some of heparin's biological activities vary with degree of sulfation making this a useful technique to separate or enrich specific heparin based activities (Table 5).

#### 2. Affinity Fractionation

A major breakthrough in heparin research came with the observation that there were two populations of heparin chains, having different affinities to ATIII, which could be separated by affinity chromatography. The different ATIII binding affinities of these heparin fractions translated into major differences in ATIII-mediated activities. For example, unfractionated heparin having an ATIII-mediated anti-thrombin activity of 150 U/mg can be fractionated into 33 wt% high-affinity heparin (500 U/mg) and 66 wt% low-affinity heparin (50 U/mg). The affinity separation is dependent on tight-binding of the high affinity fraction \((K_{on} = 10^{7})\) and weak-binding (through non-specific interactions) of the low-affinity fraction \((K_{on} = 10^{-4})\).
Table 5. Heparin activity as a function of degree of sulfation or charge.

<table>
<thead>
<tr>
<th>Heparin fraction</th>
<th>Anti IIa Activity (U/mg) mediated by $^{3}^{14}$HATIII</th>
<th>HCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>108</td>
<td>261</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>136</td>
</tr>
<tr>
<td>4</td>
<td>227</td>
<td>468</td>
</tr>
<tr>
<td>5</td>
<td>540</td>
<td>448</td>
</tr>
<tr>
<td>Unfractionated heparin</td>
<td>171</td>
<td>229</td>
</tr>
</tbody>
</table>

(a) Heparin fractionated on the basis of charge using DEAE Sephadex with salt gradient elution. The degree of sulfation of heparins 1 to 5 ranged from 2.73 - 2.83 sulfates/disaccharide repeating unit.

The minimum binding affinity of $K_{d} = 10^{-5}$ to $10^{-6}$ is required for efficient separations by affinity chromatography. Heparin has been fractionated by its affinity towards other proteins (Table 6), and although many of these demonstrate enhanced biological activities, none have resulted in the level of enrichment seen in ATIII affinity fractionation, nor have any resulted in the isolation and characterization of specific sequences through which binding and activity is expressed.

Immunoadfinity chromatography has been successfully used to fractionate a wide variety of proteins. The application of this technique to fractionating heparin, based on the presence of specific saccharide sequences which could be recognized as individual epitopes, would represent an important advance. The problem with this approach is that heparin is remarkably non-immunogenic. No true (IgE-mediated) allergic reactions to heparin have ever been demonstrated in patients, (only one unconfirmed report of a human antibody to heparin is reported in the scientific literature).

Table 6. Proteins which have been used to affinity fractionate heparin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III</td>
<td>239</td>
</tr>
<tr>
<td>Heparin Cofactor II</td>
<td>236</td>
</tr>
<tr>
<td>Thrombin</td>
<td>240</td>
</tr>
<tr>
<td>Poly(Lysine)</td>
<td>241</td>
</tr>
<tr>
<td>Platelet Factor IV</td>
<td>242</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>(a)</td>
</tr>
<tr>
<td>C3b</td>
<td>(a)</td>
</tr>
<tr>
<td>Lipoprotein Lipase</td>
<td>105</td>
</tr>
<tr>
<td>Concanaavillin A</td>
<td>243</td>
</tr>
<tr>
<td>Protamine</td>
<td>244</td>
</tr>
</tbody>
</table>

(a) Unpublished data from our laboratory.
to unmodified heparin chains in animals using a variety of approaches including adjuvants, immobilized heparin, a variety of heparin (hapten) carriers, etc., have failed. Recently, our group has prepared polyclonal antibodies to heparin-oligosaccharides having a modified unsaturated uronic acid residue at the chains nonreducing terminus. A recent report of antibodies has also appeared which again uses heparin-oligosaccharides modified at the reducing terminus, containing a ring contracted anhydro-
mannose produced by deaminative cleavage.58

LOW MOLECULAR WEIGHT HEPARINS

1. Preparation

Low molecular weight (LMW) heparins are generally prepared through the controlled, partial, chemical or enzymatic depolymerization of commercial GAG heparin. Early attempts at acid or base catalyzed hydrolysis of heparin failed primarily due to the resistance of the glycosidic link-
ages in uronic acid to hydrolysis, coupled with the sensitivity of other functional groups to the strong hydrolysis conditions required.59 A con-
venient method to cleave heparin involves its treatment under relatively mild conditions with nitrous acid.444 Heparin is cleaved at glucosamine residues containing an N-sulfate group.445 Not all N-sulfated glucos-
amines are equally susceptible to deaminative cleavage, the resistance of 3,6-0-sulfated glucosamine N-sulfate (found in the center of the ATIII binding site) was a fortunate accident which lead to the isolation of ATIII binding oligosaccharides following deaminative cleavage of he-
parin.121 This method of preparing LMW-heparins introduces an artifact into the oligosaccharide’s reducing end, a ring-contracted anhydromannose residue.446-448 There is currently no easy way of removing this residue from LMW-heparins prepared by this method. Because of the selectivity of nitrous acid for residues outside the ATIII binding site, an enrichment in this site is obtained in these preparations. Thus depolymerization by nitrous acid treatment is seldom run to completion to preserve these resistant sulfated glucosamine residues and to retain ATIII binding activity.118,121

Peroxidative cleavage of sugars by Smith degradation permits the cleavage of heparin. Heparin contains unsulfated glucuronic acid residues which have vicinal diol functionality particularly susceptible to certain oxidants. The cleavage of the glucuronic results in an acyclic residue which is sensitive to hydrolysis under relatively mild conditions. The ATIII binding site contains an unsulfated uronic acid and thus can also be cleaved by this method. Again controlled, partial oxidation followed by hydrolysis is required to retain activity. It is possible that some acyclic, oxidized sugar residues may remain in LMW-heparin prepared using this method, however further studies will be required to establish this point.449,452 Heparin can be eliminatively cleaved at its iduronic acid residue (this residue contains the appropriate anti-relationship between the 5-position proton and the 4-position glycoside anion. This elimina-
tive cleavage can be further facilitated by first forming the benzyl ester of uronic acid before treatment with base. Only the glucuronic acid residues survive such eliminative cleavage and these can then be debenzy-
lated.447 The resulting oligosaccharides have an unsaturated uronic acid residue at their non-reducing end. This residue may prolong the in vivo half-life of these oligosaccharides by blocking their biotransformation by exoglucuronidases.79 This unsaturated sugar, however, can be easily re-
moved by treatment with ozone followed by acidic work-up. More selective eliminative depolymerization of heparin can be accomplished enzymatically
using heparin and heparan sulfate lyases (Figure 9). 249

Heparin lyase cleaves heparin's most common linkage, 2,6-disulfated-glucosamine-(1->4)-2-sulfated iduronic acid. 249 This enzyme will cleave this linkage even if the 6-sulfate is missing or in the presence of a 3-O-sulfate in the glucosamine residue, found in heparin's ATIII binding site. 249 In fact, it now appears that heparin lyase preferentially cleaves heparin's ATIII binding site possibly because of its high degree of sulfation. This makes heparin lyase a more useful tool in preparing oligosaccharides with low ATIII mediated anticoagulant activity (for applications such as inhibition of complement activation). 41 Heparan sulfate lyase can also be used to depolymerize heparin and, although it cuts at fewer linkages, one of these is contained within the ATIII binding site as well. 245 Thus, both lyases must be used in controlled partial depolymerizations to prepare LMW heparins with anticoagulant activity. 250, 251 As with the LMW-heparin prepared by chemical eliminative cleavage, the unsaturated sugar can either be left on each chain to block biotransformation, or can be removed by ozonolysis. 251

2. Chemical and Physical Properties

Although each LMW heparin is different (i.e., each possesses artifacts generated in their preparation) they all have several features in common. 252 These LMW heparins are still polydisperse with average molecular weights of approximately 5000 (Table 7), and having molecular weights ranging from 2000-8000. 250, 253 These LMW heparins have been mapped using SAX-hplc and gradient PAGE techniques (Figures 10 and 11). As with the standard commercial heparins which have been mapped, LMW heparins are primarily comprised of six oligosaccharides (Figures 10 and 11). 252 In addition to these major oligosaccharides these maps show uncharacterized oligosaccharide components which are probably introduced in the depolymerization step. 251, 255, 256 More study of the structure of these LMW heparin preparations will be required to understand and to rationalize the differences in their biological activities.

3. Biological Activities

The anticoagulant activities of several common LMW heparins are presented in Table 7. A ratio of ATIII mediated anti-Xa to ATIII mediated anti-IIa activities of 5-25 is observed in LMW heparins resulting in their inhibition of coagulation early in the coagulation cascade, 238 presumably reducing their hemorrhagic side-effects. 21 These LMW heparins also exhibit a decreased effect on platelets reducing both their activation and aggregation. 21 Increases in bioavailability following subcuta-
Table 7. Properties of commercial low molecular weight heparins.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Source</th>
<th>Origin</th>
<th>Preparation*</th>
<th>Wt Avg Mw</th>
<th>Anti Xa Potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV216</td>
<td>Choay(FR)</td>
<td>PM</td>
<td>EtOH FRC, NA</td>
<td>5566</td>
<td>95</td>
</tr>
<tr>
<td>(Fraxiparin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV222</td>
<td>Choay(FR)</td>
<td>PM</td>
<td>Prolonged NA</td>
<td>5410</td>
<td>60</td>
</tr>
<tr>
<td>Kabi 2165</td>
<td>Kabi(SW)</td>
<td>PM</td>
<td>NA, FPC</td>
<td>6370</td>
<td>142</td>
</tr>
<tr>
<td>(Fragnin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMWH/DHE</td>
<td>Sandoz(SZ)</td>
<td>PM</td>
<td>AN</td>
<td>6122</td>
<td>-</td>
</tr>
<tr>
<td>RD118385</td>
<td>Hepar/Wyeth(US)</td>
<td>PM</td>
<td>OX</td>
<td>6221</td>
<td>62</td>
</tr>
<tr>
<td>OP2123</td>
<td>Opocrin(ITAL)</td>
<td>RM</td>
<td>OX</td>
<td>6511</td>
<td>63</td>
</tr>
<tr>
<td>FK10159</td>
<td>Pharmaka(FR)</td>
<td>PM</td>
<td>Benzylation/β-E</td>
<td>3789</td>
<td>96</td>
</tr>
<tr>
<td>(Enoxaparin/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lovinox)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LWM-1</td>
<td>Novo(DN)</td>
<td>PM</td>
<td>Enzymatic 3-E</td>
<td>4850</td>
<td>87</td>
</tr>
<tr>
<td>(Logipraim)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StdLMWH 85/600</td>
<td>NEBSC(UK)</td>
<td>PM</td>
<td>NA, FRC</td>
<td>14,000</td>
<td>150</td>
</tr>
<tr>
<td>Heparin</td>
<td>Hepar(US)</td>
<td>PM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) NA, nitrous acid; GPC, gel permeation chromatography; AN, isoamyl nitrate; OX, peroxidative; β-E, β-elimination; FRC, fractionation.
(b) By GPC.238
(c) Relative Anti-Xa potency against NIBSC standard LMWH.254

Venous administration and increased half-life of LMW heparin preparations have also been reported.211 These improved biological activities have paved the way for clinical trials of these new drugs.

4. Clinical Studies

Studies using LMW heparins performed in healthy volunteers (Phase 1) have demonstrated their safety at doses that are generally considered effective.4,4 Phase 2 clinical trials have begun to study both the safety and efficacy of LMW heparins in the treatment of a variety of disease

Table 8. Clinical studies on low molecular weight heparins.

<table>
<thead>
<tr>
<th>Study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long term Prophylaxis</td>
<td>257</td>
</tr>
<tr>
<td>of thromboembolism</td>
<td></td>
</tr>
<tr>
<td>Non-hemorrhagic stroke</td>
<td>24</td>
</tr>
<tr>
<td>Deep Vein Thrombosis</td>
<td>258</td>
</tr>
<tr>
<td>Membrane Oxygenation</td>
<td>259</td>
</tr>
<tr>
<td>Kidney Dialysis</td>
<td>260</td>
</tr>
<tr>
<td>With Fibrinolytics</td>
<td>101</td>
</tr>
</tbody>
</table>
Figure 10. Strong anion exchange-hplc oligosaccharide map of low molecular weight heparins. These LMW heparins (see Table 7) were prepared oxidatively (Ox) by nitrous acid depolymerization (NA), by chemical β-elimination (β-E) and using heparin lyase. Absorbance at 232 nm is plotted against elution volume and chromatography is performed using conditions given in ref. 41. The position at which standard oligosaccharides elute are marked using vertical lines and abbreviations of their chemical structure are described in Tables 1 and 2.
states (Table 3). The results of these studies are promising, suggesting that they are safer than heparin, generally resulting in less hemorrhagic complications, and have greater bioavailability and longer half-lives resulting in better dose control. One potential problem of LMW heparins is the failure of protamine sulfate to reverse their action effectively (as it does for heparin), particularly when the longer half-life of LMW-heparin is considered. This may stimulate research into new methods of LMW-heparin reversal and the preparation of protamine sulfate replacements.

HEPARIN OLIGOSACCHARIDES

Chemically defined, homogeneous heparin oligosaccharides have several advantages over the polydisperse microheterogeneous preparations (including LMW heparins) currently in use. First, it should be possible to prepare extremely selective agents, i.e., one which regulates complement activation but not coagulation or one which targets a specific step in the coagulation cascade. Second, the concentration of homogeneous drug entities are easier to monitor in vivo and hence better dose control may be possible, reducing the side-effects common in heparinization. Third, homogeneous heparins make metabolism studies easier to perform as well as studies of heparin S-AK, potentially resulting in the faster development of second and third generation heparin-like drugs with a variety of pharmacological activities.

1. Chemical Synthesis

The first total synthesis of a heparin-oligosaccharide was accomplished in 1984 resulting in the preparation of a pentasaccharide ATIII binding site. This synthesis both confirmed the reported structure and activity described for the natural product and also permitted the unequivocal elucidation of the functionality required for the expression of ATIII mediated activity. The initial synthetic scheme affording ATIII binding pentasaccharide required multiple steps resulting in a less than 5% overall yield. Subsequent synthesis reported by a number of groups have reduced the required steps and increased the yield. Chemical synthesis of the ATIII binding pentasaccharide possesses two major advantages over its preparation from the natural product, heparin. The first is that it results in independent confirmation of structure and eliminates any doubts that the identified structure (as opposed to a minor contaminant) is responsible for biological activity. The second advantage of chemical synthesis is that, if the synthetic strategy is designed well, a large number of derivatives can be prepared from the natural product, can be synthesized and examined for biological activity.

2. Enzymatic Preparation

Heparin oligosaccharides can be prepared from the natural product in a single enzymatic step followed by fractionation and purification. The simplicity of this approach and the low cost of the heparin starting material certainly makes it competitive with classical synthesis. One major failure, however, is that often the desired sequence is either not present in the starting heparin or it cannot be enzymatically removed from the polymer. One approach to these problems is to first use enzymes such as heparin lyase and heparan sulfate lyase to prepare a collection

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of small oligosaccharide building blocks. These building blocks might then be combined either chemically or enzymatically in a number of proportions or sequences to prepare a wide variety of heparin sequences.

Initial studies on the use of heparin lyase to synthesize large oligosaccharides from smaller ones by reverse enzymic catalysis have been unsuccessful. An approach currently under study in our laboratory involves the treatment of a partial ATIII binding site a'cf (see Tables 1 and 2 for chemical structure) with heparin lyase in the presence of ATIII as a trap for a completely intact ATIII binding site arising from reverse catalysis. Further research along these lines will be necessary.
3. Chemical and Physical Properties

The chemical and physical properties of structurally defined oligosaccharides are essentially the same as those of polydisperse preparations. Structurally defined, homogeneous oligosaccharides, however, offer certain distinct advantages. For example, recent studies in our laboratory using \(^{13}C\) NMR over a range of pH have for the first time permitted the measurement of the pKa of the various types of carboxyl groups found in the heparin polymer.\(^{283}\) Such a measurement would not be possible by examining polydisperse mixtures. A second advantage of using homogeneous oligosaccharides of defined structure is that complete \(^{13}C\) and \(^{1}H\) NMR spectral assignments are often possible. Definitive and complete assignment is a prerequisite for analyzing the secondary structure of these oligosaccharides using advanced NMR techniques including 2D COSY and 2D NOESY.\(^{284}\) These permit distance, which can be used to deduce the average conformation of these molecules. Since these measurements are performed in aqueous solutions under near physiological conditions, the data collected may be more physiologically relevant than what might be obtained from X-ray crystal structure (this approach is also only possible using homogeneous preparations). Even a simple property such as molecular weight is complicated by polydispersity, particularly when the MW distribution of species is not symmetrical. Such distributions result in number average, weight average, and Z average molecular weights which can be quite different.

USING HEPARIN OLIGOSACCHARIDES TO UNDERSTAND HEPARIN SAR

1. Mapping the ATIII Binding Site

The story behind the determination of heparin’s ATIII binding site provides a useful lesson in understanding the structures behind heparin’s other biological activities. The first step is the demonstration of high affinity binding to a protein and that not all heparin molecules are capable of such binding (selectivity). The second step is a reductionist approach resulting in the isolation of the smallest oligosaccharide capable of high affinity binding. The third step is preparation of that oligosaccharide in its pure form from the natural product and determination of its structure. The fourth step is the independent synthesis of this high affinity oligosaccharide and the demonstration that the synthetic product has activity equivalent to that produced from the natural product. Fifth, the determination of the molecule’s SAR by chemically or enzymatically altering specific functional groups or by the de novo synthesis of a variety of structurally related derivatives.

2. Other Activities

Screening a library of structurally defined oligosaccharides with a battery of bioassays might result in separation of two biological activities, as it has for complement regulatory and anticoagulant activities.\(^{281}\) Oligosaccharide samples of defined structure have been sent by our laboratory around the world for screening by groups for a variety of biological activities including anti-gout, immunomodulatory, topoisomerase, antifertility,\(^{283}\) and osteoporosis.\(^{46}\) The use of pure, structurally characterized oligosaccharides can avoid misleading and often unrepeatable results frequently obtained when mixtures are examined.
3. Metabolism

Here the use of defined homogeneous oligosaccharides is self evident. Without such samples it is not possible to study the biotransformation and clearance of these drugs. The absence of such defined oligosaccharides in the past has made heparin the drug with one of the most poorly understood pharmacokinetics. The knowledge of heparin pharmacokinetics will undoubtedly improve with the introduction of these defined oligosaccharides. This may also lead to a better understanding of a variety of genetic diseases that result in the incomplete metabolism of GAGs resulting in their accumulation and ultimately causing mental retardation and death.

HEPARIN OLIGOSACCHARIDES POTENTIAL AS NEW THERAPEUTIC AGENTS

Whether or not structurally defined, homogeneous heparin-oligosaccharides will ever be used as therapeutic agents depends on several factors. The first would be improved anticoagulant properties including better bioavailability, pharmacokinetics, dose control and dose monitoring, reduced side-effects, easier reversal and higher specificity. The second would be the therapeutic exploitation of other activities such as complement regulatory activity, angiogenesis regulatory activity, smooth muscle proliferative regulation, lipoprotein lipase release and activation activities, antiviral activity, and heparin's many additional observed biological activities. Before these activities can be exploited they must be separated and the absence of side-effects as well as high potency (a high therapeutic index) must be demonstrated. The third potential is one based on understanding how heparin acts and what its natural physiological roles are. Once known, this may result in previously unforeseen applications for heparin oligosaccharides. Finally, by understanding heparins SAR and physiological roles, analogues with greatly improved properties and potency, the "next generation" of heparin development, might be possible.

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