Heparin, a sulfated polysaccharide belonging to the family of glycosaminoglycans, has numerous important biological activities, associated with its interaction with diverse proteins. Heparin is widely used as an anticoagulant drug based on its ability to accelerate the rate at which antithrombin inhibits serine proteases in the blood coagulation cascade. Heparin and the structurally related heparan sulfate are complex linear polymers comprised of a mixture of chains of different length, having variable sequences. Heparan sulfate is ubiquitously distributed on the surfaces of animal cells and in the extracellular matrix. It also mediates various physiologic and pathophysiologic processes. Difficulties in evaluating the role of heparin and heparan sulfate in vivo may be partly ascribed to ignorance of the detailed structure and sequence of these polysaccharides. In addition, the understanding of carbohydrate–protein interactions has lagged behind that of the more thoroughly studied protein–protein and protein–nucleic acid interactions. The recent extensive studies on the structural, kinetic, and thermodynamic aspects of the protein binding of heparin and heparan sulfate have led to an improved understanding of heparin–protein interactions. A high degree of specificity could be identified in many of these interactions. An understanding of these interactions at the molecular level is of fundamental importance in the design of new highly specific therapeutic agents. This review focuses on aspects of heparin structure and conformation, which are important for its interactions with proteins. It also describes the interaction of heparin and heparan sulfate with selected families of heparin-binding proteins.

Keywords: carbohydrates • coagulation • heparin • medicinal chemistry • molecular recognition

1. Introduction

1.1. Historical Perspective on Heparin

Heparin, an anticoagulant drug, is widely recognized to be a biologically important and chemically unique polysaccharide. Heparin was discovered in 1916 by Jay McLean, a second-year medical student, working under the direction of physiologist William Howell at Johns Hopkins University.[1] An understanding of heparin’s structure developed gradually. In 1928 Howell correctly identified one of the sugars in heparin to be a uronic acid,[2] and in 1935–1936 Jorpes and Bergstrom found glucosamine to be the second sugar component in heparin.[3] Jorpes and later Charles established that heparin contained a high level of covalently linked sulfate making it one of the strongest acids in nature.[4] Subsequent studies also identified the 1→4-linkage between C-1 of glucosamine and C-4 of the uronic acid and the location of the O-sulfo groups.[5] The uronic acid was confirmed to be L-iduronic acid by Perlin et al. in 1968 using NMR spectroscopy.[5] Thus, heparin finally emerged as a sulfated, linear polysaccharide with a repeating disaccharide unit containing iduronic acid and glucosamine.

By 1935, enough heparin of satisfactory purity had been prepared by Jorpes in Sweden, in collaboration with Charles and Scott in Canada, to allow clinical testing. The effectiveness of heparin treatment in the prevention of postoperative thrombosis was quickly established through the work of Crafoord and Best,[6] and this medical practice continues to this day. However, using heparin also leads to undesirable side effects, such as bleeding complications or heparin-induced thrombocytopenia (HIT). These complications, an improved understanding of the coagulation cascade, and the fractionation of heparin led to the development of low-molecular-weight (LMW) heparin fractions with better defined chemical and biological properties.[7] These agents have more predictable pharmacological actions, sustained activity, improved bioavailability and a better therapeutic index (ED50/LD50). Thus, at the beginning of the new millennium, LMW heparins have displaced heparin as the major clinical anticoagulant.[8]

In 1973, Rosenberg and Damus suggested that heparin binds to antithrombin, causing a conformational change within antithrombin III (AT III) leading to a greatly accel-
erated reaction with thrombin and the formation of an inactive complex of the two proteins.[9] Affinity fractionation helped in the identification of the minimal sequence in heparin required for binding AT III.[10] The interaction of heparin with antithrombin was the first reported case of a specific heparin–protein interaction of physiological significance. Over the past two decades a growing number of biological activities have been discovered to be regulated by the interaction of heparin and heparan sulfate with heparin-binding proteins.[11] These interactions play important roles in the normal physiological as well as pathological processes.

The connection of our laboratory to this field started with studies on the purification of the heparin lyases from a soil bacterium, Flavobacterium heparinum, and the characterization of their substrate specificity.[12, 13] To study the action of these enzymes we needed to develop new methods for the isolation, purification and structural characterization of heparin- and heparan-sulfate-derived oligosaccharides.[14] The preparation of these structurally defined pure oligosaccharides represented a breakthrough in understanding heparin’s interaction with proteins and in determining the precise structural requirements within heparin essential for each interaction.

2. Heparin

2.1. Structure of Heparin and Heparan Sulfate

Heparin is a linear polymer consisting of repeating units of 1→4-linked pyranosyluronic acid and 2-amino-2-deoxyglucopyranose (glucosamine) residues.[15] The uronic acid residues typically consist of 90% 1-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.[11] Indeed, the average heparin disaccharide contains 2.7 sulfo groups. The most common structure occurring in heparin is the trisulfated disaccharide (Scheme 1). However, a number of structural variations of this disaccharide exist, leading to the microheterogeneity of heparin. The amino group of the glucosamine residue may be substituted with an acetyl or sulfo group or unsubstituted. The 3- and 6-positions of the glucosamine residues can either be substituted with an O-sulfo group or unsubstituted. The uronic acid, which can either be l-iduronic or D-glucuronic acid, may also contain a 2-O-sulfo group. Glycosaminoglycan heparin has a molecular weight range of 5–40 kDa, with an average molecular weight of about 15 kDa and an average negative charge of approximately ~75. The molecular weight distribution ($M_w/M_n$) of heparin corresponds to its polydispersity. This structural variability makes heparin an extremely challenging molecule to characterize.

The structural complexity of heparin can be considered at several levels. At the proteoglycan (PG) level, different numbers of polysaccharide (or glycosaminoglycan) chains (possibly having different saccharide sequences) can be attached to the various serine residues present in heparin’s core protein. During their biosynthesis, heparin chains are

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attached to a unique core protein, serglycin, found only in mast cells and some hematopoietic cells. Tissue proteases act on this core protein to release peptidoglycan heparin, a small peptide to which a single long polysaccharide chain (100 kDa) is attached. This peptidoglycan is short-lived as it is immediately processed by a β-endoglucooluronidase to a number of smaller (ca. 15 kDa) polysaccharide chains called glycosaminoglycan (GAG) heparin. Most of the chemical and physical properties of heparin are related to GAG structure or sequence, conformation, chain flexibility, molecular weight, and charge density.

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 a uronic acid 1→4-linked to glucosamine (Scheme 1).[16] While β-glucuronic acid predominates in heparan sulfate, it can contain substantial amounts of L-iduronic acid. Heparan sulfates generally contain only about one sulfo group per disaccharide, but individual heparan sulfates may have higher contents of this group. Heparan sulfate chains also often contain domains of extended sequences having low or high sulfation.[17] 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 of about 30 kDa ranging from 5 to 50 kDa.[18]

Heparan sulfate is biosynthesized, as a proteoglycan, through the same pathway as heparin, however, unlike heparin, the heparan sulfate GAG chain remains connected to its core protein. Heparan sulfate is ubiquitously distributed on cell surfaces and is also a common component of the extracellular matrix.[17, 19] Two types of core proteins, the syndecans (an integral membrane protein) and the glypicans (a GPI-anchored protein), commonly carry heparan sulfate GAG chains and correspond to the two major families of heparan sulfate proteoglycans (heparan sulfate PGs).[19, 20] The heparan sulfate chains on these heparan sulfate PGs bind a variety of proteins and mediate various physiologically important processes including, blood coagulation, cell adhesion, lipid metabolism, and growth factor regulation.[21] Although structurally similar, heparin and heparan sulfate GAGs can often be structurally distinguished through their different sensitivity towards a family of GAG-degrading, microbial enzymes, the heparin lyases.[22] In the following sections, most of the properties that we discuss with respect to heparin are also applicable, except where specified, to heparan sulfate.

### 2.2. Conformation of Heparin

Heparin is a linear, unbranched, highly sulfated polysaccharide that exists primarily as a helical structure (Figure 1).[23] Unlike proteins, heparin is not known to display or fold into any particular tertiary structure. The specificity of its interactions with a diverse range of biologically important

![Figure 1. Helical conformation of a heparin dodecasaccharide sequence having the major disaccharide repeating structure shown in Scheme 1 with sulfur atoms (yellow), oxygen atoms (red), nitrogen atoms (blue) and hydrogen atoms (cyan).](image)
proteins suggests that it displays its sulfo and carboxyl groups in defined patterns and orientations to promote specific protein interactions. The conformational flexibility of the 1-iduronic acid residue within heparin is believed to be responsible for the wide range of specific protein interactions exhibited by this family of GAGs.

Analysis of the conformation of individual sugars within heparin indicates that the α-glucosamine and α-glucuronic acid residues assume the preferred 1C₄ conformation (Scheme 2A). The conformation of the flexible 1-iduronic acid (IdoA) residue varies, depending on the substitution pattern and on its relative position in the chain. When the IdoA residue is at the reducing end of an oligosaccharide, NMR data suggest three possible conformers, the 4C₁, 1C₁, and 2S₀ conformers of IdoA. C) 2 conformations of IdoA. C) The predominant 4C₁ form is favored in terms of conformational stability, as it tends to minimize the unfavorable 1,3-diaxial nonbonded interactions that are expected in the 1C₄ form, where four of the substituents are axially oriented and only the carboxyl group is equatorial. However, the energy barrier to the 1C₄ form is not high, and so it is possible for the iduronate ring to change between the 2S₀ and 1C₄ forms so that in the protein-bound state it can make favorable electrostatic interactions with basic amino acids on the protein.

Indeed, while the spatial orientation of the 2-O-sulfo group in the IdoAp2S residues is altered during 1C₄–2S₀ interconversion, no significant conformational change in the backbone of the polysaccharide chain takes place. The crystal structure analysis of FGF-2 bound to a heparin hexasaccharide revealed that one internal IdoAp2S residue makes contact with the protein and resides in a 1C₄ conformation while the other makes contact in the 2S₀ conformation. Thus, while the iduronate rings are flexible and in equilibrium between the 2S₀ and 1C₄ conformation in the unbound state, they can be locked into either conformation when bound to a protein. The prevalence for one iduronate conformer is influenced by the substitution pattern of the α-glucosamine residue bound to its nonreducing end. For example, when the IdoAp2S residue has a N-acetylgalactosamine (α-GlcNAc) substituted at its 4-position, it resides almost exclusively in the 1C₄ chair conformer.

Despite the conformational flexibility of the 1-iduronate residues, heparin saccharides show relatively conserved angles φ and ψ in glycosidic linkages. NMR studies on a series of modified heparins with systematically altered substitution patterns indicate that all derivatives, regardless of sulfation pattern, exhibit similar glycosidic bond conformations. The helical parameters for heparin oligosaccharides bound to fibroblast growth factors have been determined by X-ray crystallography, and these values are comparable.

Homogenous, structurally defined heparin and heparan sulfate oligosaccharides are commonly enzymatically prepared from these GAGs using heparin lyases. These enzymes break down heparin into oligosaccharides that contain a nonreducing terminal 4-deoxy-α-L-threo-2-sulfopyranosyluronic acid (4UAp2S) residue. Understanding the conformation of these residues is important when using these oligosaccharides to study heparin–protein interactions and also to understand the unusual reactivity of these 4U-uridonates. Based on the conformation about the 4,5-double bond, the 4UAp2S residue can exist in either the 1H₂ or 1H₁ forms (Scheme 2C). The equilibrium between these two conformers is controlled by their substitution patterns. Crystallographic data also show that these two forms coexist within the same unit cell, suggesting that they are nearly equal in energy. However, the solution structures of heparin-derived oligosaccharides determined by NMR spectroscopy suggest that the terminal 4UAp2S residue is predominantly represented by the 1H₁ form with minor contribution from the 3H₁ form.
2.3. Biosynthesis of Heparin

The biosynthesis of heparin and heparan sulfate and the regulatory mechanisms resulting in the placement of different saccharide sequences in their structures are only partly understood (Scheme 3). Studies on heparin biosynthesis were carried out in a mastocyte cell culture system with radiolabeled metabolic precursors of heparin. 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. The sequence of this linkage-region tetrasaccharide is β-GlcAp(1→3)-β-Galp(1→3)-β-Galp(1→4)-β-Xylp-1→Ser. There are four glycosyltransferases responsible for the synthesis of the linkage region. The glucuronyltransferase that attaches the terminal glucuronic acid (GlcAp) and the galactosyltransferase that attaches the second galactose (Galp) residue have been cloned and characterized. The neutral sugar linkage region of the first GlcNpAc residue or N-acetylgalactosamine (GalpNpAc, in the biosynthesis of chondroitin sulfates) is added. This addition decides whether the chain will either be a glucosaminoglycan (heparin and heparan sulfate) or a galactosaminoglycan (chondroitin sulfates). It has been suggested that peptide sequences close to the linkage-region substituted serine residue are added to the linear polysaccharide chain before its synthesis terminates. As the chain elongates it also undergoes other modification reactions. Modification of the polymer is initiated by N-deacetylation and O-sulfation of the GlcNpAc residues by an N-deacetylase/N-sulfotransferase enzyme. Subsequent steps occur sequentially and either on or adjacent to the N-sulfogalcosamine(GlcNpS)-containing residue. A C-5 epimerase then catalyzes transformation of some of the α-glucuronic acid residues to β-iduronic acid residues. This is followed by O-sulfation of the iduronic acid residues at the C-2 position by an iduronosyl 2-O-sulfotransferase. It has been shown that the IdoAp in an IdoAp→GlcNpS disaccharide can be sulfated by this enzyme provided the GlcNpS residue does not contain an O-sulfo group at the C-6 position. Studies have also shown that a very active glucuronosyl 2-O-sulfotransferase, in mouse mastocytoma microsomal fractions, is responsible for the O-sulfation of GlcAp residues at the C-2 position. There is also evidence suggesting that the glucuronosyl 2-O-sulfotransferase is the same enzyme as the iduronosyl 2-O-sulfotransferase. The 2-O-sulfation 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.

Finally a 3-O-sulfotransferase acts upon the polymer and modifies certain GlcNpS6S residues. The 3-O-sulfation 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. The 3-O-sulfotransferase has been cloned and purified and like many of the other biosynthetic enzymes, there are multiple isoforms having different specificities. All the sulfotransferase reactions described above require 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as a sulfate donor.

The apparently random and incomplete nature of the initial N-deacetylation is primarily responsible for the introduction of structural heterogeneity into the polymer at an early stage of biosynthesis. The specificity of this enzyme after its initial modification is also responsible for the block structures seen in heparan sulfate, where there are highly sulfated sequences separated by sequences of nonsulfated disaccharides. The overall structural variability of the heparin/heparan sulfate polymer is the result of the incomplete nature of modifications by the biosynthetic enzymes and it is still not clearly understood what additional factors serve as the regulating elements for these enzymes in the biosynthetic pathway.
2.4. Medical Importance of Heparin

Heparin and LMW heparins are the most commonly used clinical anticoagulants. Heparin is administered intravenously (LMW heparin 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. The use of these devices, requiring heparinization, can often lead to hemorrhagic complications. Systemic heparinization is also used in the treatment of deep vein thrombosis and in a variety of other surgical procedures. Heparin-induced thrombocytopenia (HIT), a complex process which results in loss of platelets, is currently recognized to be one of the most catastrophic complications of heparin treatment. [25]

3. Interaction of Heparin with Proteins

With the discovery of increasing numbers of heparin-binding proteins there was a need to characterize the molecular properties, within the proteins and heparin, responsible for specific recognition (Table 1). The first study to look at the general structural requirements for GAG–protein interactions was conducted by Cardin and Weintraub in 1989. [30] They compared the heparin-binding domains of four proteins, apolipoprotein B, apolipoprotein E, vitronectin, and platelet factor 4. This study demonstrated that, in addition to sequence similarity, these domains also had defined motifs and were classified into two consensus sequences, XBXXBX and XBBBXXBX (B is a basic and X a hydrophobic (neutral and hydrophobic) amino acid residue). These consensus sequences were also shown to occur in other proteins known to bind heparin. Molecular modeling studies performed on these consensus sequences showed that their presence in certain secondary structural conformations resulted in the oriented display of the basic residues facilitating their interaction with the negatively charged sulfo groups on heparin. Their results suggested that if the XBXXBX sequence were in a β-strand conformation, the basic amino acids would be aligned on one face of the β-strand while the hydrophobic amino acids would point back into the protein core. Similarly, if the XBBBXXBX sequence were folded into

Table 1. Characteristics of selected heparin-binding proteins.

<table>
<thead>
<tr>
<th>Heparin-binding protein</th>
<th>Physiological/Pathological role</th>
<th>Characteristics of heparin binding</th>
<th>Oligosaccharide size</th>
<th>Sequence features[d]</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>proteases/estrases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT III [a]</td>
<td>coagulation cascade serpin</td>
<td>ca. 20 nm</td>
<td>5-mer</td>
<td>GlcNS6S3S</td>
<td>enhances</td>
<td>[83, 104]</td>
</tr>
<tr>
<td>SLPI</td>
<td>inhibits elastase and cathepsin G</td>
<td>ca. 6 nm</td>
<td>12-mer to 14-mer</td>
<td>IS</td>
<td>enhances</td>
<td>[76]</td>
</tr>
<tr>
<td>C1 INH</td>
<td>inhibits C1 esterase</td>
<td>ca. 100 nm</td>
<td>–</td>
<td>HS</td>
<td>enhances</td>
<td>[87]</td>
</tr>
<tr>
<td>VCP</td>
<td>protects host cell from complement</td>
<td>nm</td>
<td>–</td>
<td>HS</td>
<td>unclear</td>
<td>[114, 115]</td>
</tr>
<tr>
<td>growth factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-1 [c]</td>
<td>cell proliferation, differentiation, morphogenesis, and angiogenesis</td>
<td>nm</td>
<td>4-mer to 6-mer</td>
<td>IdoA2S-GlcNS6S</td>
<td>activates signal transduction</td>
<td>[34, 93]</td>
</tr>
<tr>
<td>FGF-2 [b]</td>
<td>same as FGF-1</td>
<td>nm</td>
<td>4-mer to 6-mer</td>
<td>IdoA2S-GlcNS</td>
<td>(same as FGF-1)</td>
<td>[31, 88]</td>
</tr>
<tr>
<td>chemokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-4 [a]</td>
<td>inflammation and wound healing</td>
<td>nm</td>
<td>12-mer</td>
<td>HS/LS/HS</td>
<td>inactivates heparin</td>
<td>[136, 137]</td>
</tr>
<tr>
<td>IL-8</td>
<td>pro-inflammatory cytokine</td>
<td>ca. 6 μm</td>
<td>18-mer to 20-mer</td>
<td>HS/LS/HS</td>
<td>promotes</td>
<td>[139]</td>
</tr>
<tr>
<td>SDF-1 [a]</td>
<td>pro-inflammatory mediator</td>
<td>ca. 20 nm</td>
<td>12-mer to 14-mer</td>
<td>HS</td>
<td>localizes</td>
<td>[142, 143]</td>
</tr>
<tr>
<td>lipid-binding proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin II</td>
<td>receptor for TPA and plasminogen, CMV and tenasin C</td>
<td>ca. 30 nm</td>
<td>4-mer to 5-mer</td>
<td>HS</td>
<td>unclear</td>
<td>[151]</td>
</tr>
<tr>
<td>Annexin V [a]</td>
<td>anticoagulant activity; influenza and hepatitis B viral entry</td>
<td>ca. 20 nm</td>
<td>8-mer</td>
<td>HS</td>
<td>assembles</td>
<td>[85, 157]</td>
</tr>
<tr>
<td>Apo[a]</td>
<td>lipid transport; AD risk factor</td>
<td>ca. 100 nm</td>
<td>8-mer</td>
<td>HS</td>
<td>localizes</td>
<td>[166]</td>
</tr>
<tr>
<td>pathogen proteins</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>HIV-1 gp120</td>
<td>viral entry</td>
<td>0.3 μm</td>
<td>10-mer</td>
<td>HS</td>
<td>inhibits</td>
<td>[173, 175]</td>
</tr>
<tr>
<td>Tat</td>
<td>viral localization and entry</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>inhibits</td>
<td>[177]</td>
</tr>
<tr>
<td></td>
<td>transactivating factor, primes cells for HIV infection</td>
<td>ca. 70 nm</td>
<td>6-mer</td>
<td>HS</td>
<td>antagonizes</td>
<td>[181]</td>
</tr>
<tr>
<td>HSV gB and gC</td>
<td>viral entry into cell</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>inhibits</td>
<td>[184, 186]</td>
</tr>
<tr>
<td>HSV gD</td>
<td>viral entry and fusion</td>
<td>–</td>
<td>–</td>
<td>GlcNH23S</td>
<td>inhibits</td>
<td>[70]</td>
</tr>
<tr>
<td>Dengue virus envelope protein</td>
<td>viral localization</td>
<td>ca. 15 nm</td>
<td>10-mer</td>
<td>HS</td>
<td>inhibits</td>
<td>[187]</td>
</tr>
<tr>
<td>Malaria CS protein</td>
<td>sporozoite attachment to hepatocytes</td>
<td>ca. 40 nm</td>
<td>10-mer</td>
<td>HS</td>
<td>inhibits</td>
<td>[193–195]</td>
</tr>
<tr>
<td>adhesion proteins</td>
<td>selectins</td>
<td>adhesion, inflammation, and metastasis</td>
<td>μm</td>
<td>≥ 4-mer</td>
<td>HS with GlcNH2</td>
<td>blocks</td>
</tr>
<tr>
<td></td>
<td>vitronectin</td>
<td>cell adhesion and migration</td>
<td>nm</td>
<td>–</td>
<td>–</td>
<td>removes</td>
</tr>
<tr>
<td></td>
<td>fibronectin</td>
<td>adhesion and traction</td>
<td>μm</td>
<td>8-mer to 14-mer</td>
<td>HS with GlcNS</td>
<td>reorganizes</td>
</tr>
<tr>
<td></td>
<td>HB-GAM [a]</td>
<td>neurite outgrowth in development</td>
<td>ca. 10 nm</td>
<td>16-mer to 18-mer</td>
<td>HS</td>
<td>mediates</td>
</tr>
<tr>
<td></td>
<td>AP</td>
<td>in amyloid plaque</td>
<td>μm</td>
<td>4-mer</td>
<td>HS</td>
<td>assembles</td>
</tr>
</tbody>
</table>

an α-helix, then the basic amino acids would be displayed on one side of the helix (Figure 2) with the hydrophobic residues pointing back into the protein core. Using this model, Sobel and coworkers proposed a third consensus sequence, XBBBXXBBXBBX, in a heparin-binding protein, the von Willebrand factor.[60] These motifs serve as initial sequence probes to determine whether a protein can possibly bind heparin. However, with the discovery of more heparin-binding proteins, it was observed that the motif hypothesis did not always hold. Basic residues that are distant in sequence could be closer in the folded state. Thus, spatial orientation of basic residues, rather than sequence proximity, is an important factor in determining heparin binding ability. Margalit and coworkers also used molecular modeling to examine linearly contiguous heparin-binding sites that were shown by others to interact with heparin. In established heparin-binding sites, with known secondary structure, they observed that a distance of approximately 20 Å between basic amino acids is important for interaction, irrespective of whether the heparin-binding site exists as an α-helix or a β-strand.[61] As most of the basic residues were aligned on opposite sides of the secondary structural element, the authors proposed that heparin coils around its binding domain to interact with these positively charged residues. They also suggested that this coiling can induce conformational changes in the heparin-binding protein.

Heparin-binding sites are commonly observed on the external surface of proteins and correspond to shallow pockets of positive charge. Thus, the topology of the heparin-binding site is also an important factor in heparin-binding consensus sequences. Structural analysis of the heparin-binding sites in acidic fibroblast growth factor (FGF-1), basic FGF (FGF-2), and transforming growth factor β-1 (TGF β-1) implicated a TXXBXXTBXXTBB motif (Figure 3), where T defines a turn, B a basic arginine or lysine residue (or occasionally a hydrogen-bonding glutamine) and X a hydrophobic residue.[62] The importance of the shape of this binding site in the FGF family was further demonstrated by affinity studies with synthetic linear and cyclic peptides based on a sequence analogous to the heparin-binding site in FGF.[63] The cyclic peptide, designed to resemble the structure of the heparin-binding site in FGF, bound more tightly to heparan sulfate than the acyclic peptide of the same sequence or a cyclic peptide containing a D-proline turn. Not all heparin-binding proteins contain linearly contiguous heparin-binding sites. Basic amino acids that are distant in sequence can be brought spatially close through the folding of the protein. This is observed for antithrombin III, where both a linearly contiguous heparin-binding domain and sequence-remote basic amino acids are appropriately positioned to bind heparin.[64]

Studies performed in our laboratory looked at the common amino acids in heparin-binding domains in proteins and the importance of spacing of basic amino acids in heparin binding. Using peptide libraries we assessed the affinity of randomly synthesized heptamers for both heparin and heparan sulfate. Peptides enriched in arginine and lysine, but not histidine, bound with greatest affinity. Peptides with high affinity for heparin and heparan sulfate were also enriched in other polar amino acids including serine.[65] It was also observed that known heparin-binding domains contain amino acids (asparagine and glutamine) capable of hydrogen bonding. The relative strengths of heparin binding by basic amino acid residues was compared and arginine was shown to bind 2.5 times more tightly than lysine. The tight interaction observed for arginine appears to result from strong hydrogen bonding between the guanidinio group of arginine and a sulfhydrol group in heparin.[66]
The effect of the pattern and spacing of the basic amino acids in heparin-binding sites was also studied using a series of synthetic peptides.[67] The relative binding affinities of two peptide series, -RRG<sub>n</sub>RR- and -RRRG<sub>n</sub>R- (R is arginine and G glycine) was examined. Heparin interacted most tightly with peptides containing a complementary binding site of high positive charge density while less sulfated heparan sulfate interacted most tightly with a complementary site on a peptide that had more widely spaced basic residues. Thus, the results of this study are entirely consistent with our understanding of heparin and heparan sulfate structures.

3.1. Protein-Binding Motifs within Heparin and Heparan Sulfate

Specific recognition between heparin and proteins also requires defined sequences within the heparin chain. Heparin and heparan sulfate predominantly exhibit linear helical secondary structures with sulfo and carboxyl groups displayed at defined intervals and in defined orientations along the polysaccharide backbone. A heparin-binding domain on a protein would, therefore, require a minimum number of saccharide residues within the heparin chain to appropriately display these charged groups to facilitate a tight and specific interaction.

The first example of a defined protein-binding motif within heparin was the discovery of a discrete pentasaccharide sequence required for binding antithrombin III.[10] This pentasaccharide sequence is rare, occurring in only about one-third of the chains in heparin. Its most distinguishing feature is the unusual 3-O-sulfo group on an internal GlcNpS6S residue (Scheme 4), which is absolutely essential for its high affinity to antithrombin III.[89] The 3-O-sulfation reaction is the final step in heparin and heparan sulfate biosynthesis and there are multiple 3-O-sulfotransferase isoforms having different substrate specificities. The treatment of a biochip-immobilized heparan sulfate, that has a low affinity for AT III, with one of these isoforms affords a structurally modified high affinity chain.[90] A heparan sulfate modified at specific glucosamine residues by a second of these 3-O-sulfotransferase isoforms offers sites for the binding of the viral envelope glycoprotein of herpes simplex virus type 1 (HSV-1).[79] This binding is postulated to be important in the initiation of HSV-1 entry into cells. The 3-O-sulfo group in heparan sulfate is also believed to be important for the specific interaction with fibroblast growth factor 7 (FGF-7).[71] Another study suggests that the antithrombin-binding motif within heparan sulfate is required for the formation of a functional complex with the FGF receptor kinase ectodomain.[77]

The proteins of the FGF family are probably the most extensively studied heparin-binding proteins. X-ray crystallography data on FGF-1[34] and FGF-2[33] complexes with heparin oligosaccharides have been useful in defining the precise groups within heparin that are important for these biologically significant interactions. Both structural and experimental data suggest that the 6-O-sulfo groups within heparin, while not directly important in the binding of FGF-2 to heparin,[73] are apparently required for the mitogenic activity of FGF-2.[74] In contrast, the interaction of FGF-1 with heparin is directly mediated through contacts with the 6-O-sulfo groups on heparin. These differences suggest a specificity of interaction for various members of the same family of growth factors.[75] While the interaction between proteins and heparin is primarily ionic and based on the presence and appropriate positioning of sulfo and carboxyl groups, this may not always be the case. For example, it has been observed that the interaction between secretory leukocyte protease inhibitor (SLPI) and heparin, undersulfated heparin oligosaccharide sequences are required for specific high affinity interaction, suggesting the importance of hydrogen-bonding interactions through the hydroxyl groups present in heparin.[76]

3.2. Types of Interactions

Clearly the most prominent type of interaction between heparin and a protein is ionic. Clusters of positively charged basic amino acids on proteins form ion pairs with spatially defined negatively charged sulfo or carboxyl groups on the heparin chain. However, in some cases there is a significant contribution to the binding by nonionic interactions such as hydrogen bonding. Isothermal titration calorimetry (ITC) studies of the interaction of brain natriuretic peptide (BNP) with heparin revealed that only a small portion of the free energy of this interaction resulted from ionic contributions. The major contribution was from hydrogen bonding between the polar amino acids on BNP and heparin.[77] Hydrophobic forces may also play a minor role in heparin–protein interactions. Based on NMR data, Bae and coworkers suggested the importance of a tyrosine residue in a synthetic antithrombin III peptide in an apparently specific, hydrophobic interaction with the N-acetyl group in the AT-III-binding pentasaccharide sequence in porcine mucosal heparin.[79]

3.3. Kinetics, Thermodynamics, and Measurement of Heparin–Protein Interactions

Heparin resembles DNA as both are highly charged linear polymers that behave as polyelectrolytes. The high repulsive energy of multiple negatively charged groups in these polyelectrolytes promotes cation (i.e., Na<sup>+</sup>) binding to minimize these forces.[79] The binding of Na<sup>+</sup> ions by a polyelectrolyte is entropically unfavorable. When a polyelectrolyte such as
Heparin binds a protein, the positively charged amino acid residues on the protein interact at anionic sites resulting in the entropically favorable release of Na\(^+\) ions. Thus, much of the free energy of interaction of heparin with proteins is derived from the entropically favorable release of Na\(^+\) ions, and this is called the “polyelectrolyte effect.” The free energy of interaction, which is related to the observed dissociation constant \((K_d)\), has contributions from the polyelectrolyte effect, hydrogen bonding, and hydrophobic interactions. Specifically, \(K_d\) is related to both ionic and nonionic contributions according to Equation (1) where \(K_{d_{\text{ionic}}}\) is the dissociation constant in the absence of the polyelectrolyte effect, \(m\) is the number of Na\(^+\) ions released on heparin–protein binding, and \(f\) is a small fraction of anionic charge on the polyelectrolyte that is not charge-neutralized by Na\(^+\) ions.

\[
\lg K_d = \lg K_{d_{\text{ionic}}} + m(1-f)\lg [Na^+] 
\]

The kinetics of interaction for heparin binding to antithrombin III have been extensively studied. Since AT III undergoes a conformational change on binding heparin, and this process can be monitored by a change in AT III fluorescence, it represents a good system to study by stopped-flow fluorimetry. For most other heparin-binding proteins, where there is no such observable change following the binding event, it is very difficult to study the kinetics of the interaction. However, the development of surface plasmon resonance (SPR), which allows binding events to be measured in real time, has made it possible to study the kinetics of heparin binding to proteins.

Various methods are utilized in studying the binding affinity of heparin with proteins. In affinity chromatography, the most commonly used technique, heparin or the heparin-binding protein is immobilized on a solid matrix and its binding partner is applied to the column. The binding partner is then released with salt and the amount required for elution is a quantitative measure of the ionic component of the binding affinity. This approach fails to measure the hydrophobic and hydrogen-bonding contributions to binding. Affinity chromatography is difficult to use in studying interactions dependent on divalent cations and requires the immobilization of one of the binding partners. ITC provides information on the thermodynamics of heparin–protein interactions. In this technique, one of the interacting species is placed in solution in a thermostatted cell and the other species is injected into this cell. The heat released following each injection is measured, affording a sigmoidal titration curve. By fitting this curve, values for the enthalpy \((\Delta H)\), the association constant \((K_a)\), and the binding stoichiometry \((n)\) are obtained. ITC typically requires milligram amounts of each interacting species, is useful only in measuring association constants in the range \(10^4\) to \(10^6\), and involves relatively high concentrations of interacting species often leading to precipitation.

NMR spectroscopy can afford extremely valuable information about heparin–protein interactions. NMR analysis provides primarily structural and conformational data that are useful in identifying the precise contact points between interacting molecules. While NMR experiments give a relatively detailed picture of the interaction between heparin and protein, the insensitivity of this method requires milligram quantities of interacting species at high concentrations, raising solubility problems and making it difficult to accurately determine the association constant \((K_a)\).

Fluorescence spectroscopy is a very sensitive method that can provide a wealth of information about heparin–protein interactions on very small amounts of sample. Conformational changes in the protein, which often occur on heparin binding, can be measured through changes in the environment of intrinsic fluorophores such as tyrosine and tryptophan. The association constant can be obtained by monitoring the change in fluorescence as one interacting species is titrated into a solution of the other. This technique is limited to protein–heparin binding that results in a change in fluorescence. Fluorescence resonance energy transfer (FRET) between an intrinsic fluorophore in the protein and an extrinsic fluorophore in heparin (fluorescently labeled heparin) can afford both \(K_a\) and information on the distance between the two fluorophores of up to 80 Å. One concern associated with this approach is that derivatizing heparin (or particularly a small oligosaccharide) with an extrinsic fluorophore can perturb both its conformation and its interaction with the protein.

Surface plasmon resonance (SPR) is a powerful technique for studying interactions. Here one of the interacting species is immobilized on the surface of a chip and the binding partner is flowed over this chip. The resulting interaction changes the refractive index of the chip, which is measured as a change in

![Figure 4. Data obtained from an ITC experiment with heparin and BNP. A) Raw data showing the heat released on binding in \(\mu\text{s}^{-1}\) \((Q_t)\) with each injection. B) Fitted heats of interaction, giving a sigmoidal binding curve. The heat released \((Q)\) is plotted against the number of injections \((n)\).](image-url)
the intensity and angle of light reflected from the chip’s surface. The magnitude of this change is directly proportional to the mass of the binding partner being bound, affording a real-time measurement of association and dissociation rates from which the dissociation constant can be calculated (Figure 5). Signals are easily obtained from sub-microgram quantities of material. While this technique can provide important information on binding kinetics, it suffers from potential experimental artifacts that arise from ligand immobilization.

Figure 5. SPR sensorgram data obtained for the interaction of the apoE4 N-terminal domain with heparin. Solutions with varying concentrations (0.11 ± 2.30 μM) of the apoE4 domain were flowed over heparin immobilized on a chip. The change in surface response (R) over time (t) is plotted. RU = response unit.

Other techniques that can be used to study heparin–protein interactions include affinity coelectrophoresis (ACE), equilibrium dialysis, competitive binding techniques, analytical centrifugation, and circular dichroism. X-ray crystallography of heparin oligosaccharides in complexes with proteins provides high-resolution structural information but requires large quantities of homogeneous interacting species.

3.4. Modeling Studies on Heparin–Protein Interactions

Molecular modeling can provide useful information on molecular interactions but its predictive power still needs to be substantiated. The value of modeling is that intricate dynamic details of molecular level events can be visualized with a relatively small investment of time and cost. One of the problems of molecular mechanics and dynamics is the limited availability of reliable potential energy functions, particularly for molecules containing functionality such as N- and O-sulfo groups. Modeling protocols and reliable potential energy functions for studying proteins and DNA are well established and many empirical forcefields such as AMBER, CHARMM, MM2, and TRIPOS are widely used.

Modeling of heparin initially required the derivation of potential energy functions, using ab initio calculations, of the N- and O-sulfo groups attached to 2-, 3-, and 6-positions of the pyranosyl units. The development of efficient modeling protocols for understanding the steric and spatial considerations of oligosaccharides is also important in studying heparin–protein interactions. The NMR structure of a heparin dodecasaccharide (Figure 1) and the high-resolution X-ray crystallographic structures of heparin oligosaccharides provided an important starting point for modeling heparin–protein interactions. Docking studies with heparin oligosaccharides and their protein-binding partners can afford additional structural information on the interaction. However, such studies present a challenge because of the weak surface complementarity, the high charge density of heparin and the heparin-binding site, and the highly flexible nature of the heparin chain.

Docking studies have been carried out between the heparin-binding peptide sequences of antithrombin III and the AT-III-binding pentasaccharide sequence to study the structure–activity relationship (SAR) of this interaction. In a recent study, several docking protocols for the prediction of heparin-binding sites on AT III, FGF-1, and FGF-2 were tested and compared to crystallographic data available for these proteins in complexes with their oligosaccharide binding partners. A global search for likely binding sites was initially performed using sulfated monosaccharides and disaccharides, followed by a local docking of a hexasaccharide. After correctly predicting the binding site for the test molecules, these protocols were used to predict the heparin-binding site on interleukin-8 (IL-8), a chemokine with a central role in immune response. This strategy significantly reduces the required computational time compared to a global docking of the hexasaccharide.

4. Regulation of Proteases and Esterases

The interaction of heparin with antithrombin III is the first well-studied example of a heparin–protein interaction. Heparin is believed to function as an anticoagulant primarily through its interaction with AT III by enhancing AT-III-mediated inhibition of blood coagulation factors, including thrombin and factor Xa. These coagulation factors are serine proteases with trypsin-like specificity for arginyl linkages. AT III is a member of the serpin (serine protease inhibitor) superfamily of proteins that includes more than 40 proteins. The serpins react with serine proteases to form inactive complexes, which are then cleared from the circulation. All the coagulation factors (with the exception of factor VIIa) are inhibited by AT III, forming a covalent 1:1 complex with these enzymes. The inactivation of these proteases by AT III is greatly accelerated by the binding of heparin. Heparin binds to AT III and thrombin in a ternary complex, increasing the

Heparin

bimolecular rate constant for the inhibition of thrombin by a factor of 2000.\[9\] The interaction of heparin with AT III and serine proteases proceeds through several stages. Initial heparin binding to AT III is a low-affinity interaction mediated by a well-defined unique pentasaccharide sequence within heparin. This binding causes a conformational change\[10\] in the structure of AT III, which promotes additional interactions between AT III and heparin, resulting in a stronger binding. The conformational change also leads to the expulsion of a loop with a protease-reactive site in AT III that facilitates the reaction of the target protease with AT III yielding a cleaved and conformationally altered AT III covalently bound to the active site of the serine protease as an acyl–enzyme intermediate. As a result of the cleavage, the affinity of AT III for heparin is markedly diminished,\[10\] causing heparin to dissociate in an unaltered form, free to catalyze further reactions between AT III and its target serine proteases. The acyl–enzyme intermediate then dissociates very slowly (t_{1/2} \sim 5\text{ days}) to yield the unaltered serine protease and the cleaved antithrombin, which is no longer an active serpin and has lost its heparin binding affinity.\[10\]

Heparin chains at least 16 saccharides in length are required to accelerate the reaction of antithrombin with thrombin, even though only the pentasaccharide sequence is necessary to bind AT III.\[10\] In contrast, heparin chains as small as the AT-III-binding pentasaccharide are able to accelerate the inactivation of the other target coagulation enzymes, such as Factor Xa.\[10\] Heparin binding to AT III induces a conformational change, resulting in a 40% enhancement of AT III fluorescence, thus facilitating kinetic studies that rely on stopped-flow fluorimetry.\[10\] The X-ray crystal structure of an AT-III–pentasaccharide complex was used to identify residues in AT III involved in this interaction as well as important functional groups within the pentasaccharide (Figure 6).\[10\] The residues important in the interaction have been altered through site-directed mutagenesis and stopped-flow kinetic studies were carried out to assess the relative contributions of these residues to binding.\[10\] The role of individual saccharide residues of the heparin pentasaccharide in the allosteric activation of AT III has also been determined by studying the effect of truncating pentasaccharide residues, at either its reducing or nonreducing end, on oligosaccharide binding and the induction of a conformational change in AT III.\[10\] These studies established that the three saccharide residues on the nonreducing end of the pentasaccharide sequence are capable of fully activating AT III. While the reducing-end residues are not essential for this activation, they stabilize the activated conformation. Detailed characterization of the determinants involved in this physiologically relevant interaction is important, as it would enable the development of more specific heparin analogues that might retain anticoagulant activity without the undesirable side effects observed with heparin.

Heparin is principally located in the granules of tissue mast cells that are closely associated with the immune response. Thus, the ability of heparin to regulate the major activities of the complement cascade is an area of active interest.\[10\] The complement system consists of about 20 plasma proteins that interact in two related sets of reactions: the antibody-dependent classical pathway and the antibody-independent alternative pathway. Both pathways consist of the sequential activation of a series of serine esterases, much like in the blood coagulation pathway. Studies indicate the importance of O-sulfo groups in heparin and a minimum oligosaccharide size for complement regulation.\[10\] The classical pathway is initiated when complement protein C1 esterase, also called the recognition unit, specifically binds to a cell surface antigen–antibody aggregate. C1 esterase is regulated by a natural inhibitor present in serum, called C1 esterase inhibitor (C1INH).\[10\] Heparin and other glycosaminoglycans augment the activity of C1INH on C1 components in vitro.\[10\] The ability of heparin and heparan sulfate to bind C1INH and enhance its inhibitory activity has been studied using surface plasmon resonance and in vitro assays.\[10] Heparin immobilized on a biosensor chip interacted with C1INH (K_{d} \approx 100\text{ nm}), while no binding between heparan sulfate and C1INH was observed. In vitro assays indicated that heparin, and to a slightly greater extent LMW heparin, augmented the activity of C1INH on cell-bound C1 and the order of the reaction suggested the involvement of a ternary complex in which cell-bound C1 interacts with both heparin and C1INH. Heparin is also believed to directly bind to and inhibit C1 activity. There is evidence to suggest that C1 recognizes the fraction of heparin with high antithrombin III affinity.\[10\] Heparin also has a profound effect on the complement cascade through its interaction with a number of other complement proteins.\[10\]

Chemically modified, N-acetylated heparin inhibits cobra venom factor activation of the complement cascade in vivo.\[10\] Vaccinia virus complement control protein (VCP) is secreted from virally infected cells and is believed to protect
the host cell from complement attack prior to the release of viral progeny.[113] VCP inhibits both pathways of complement activation by binding the third and fourth complement components. VCP, a heparin-binding protein, may bind heparan sulfate PGs on the surface of human endothelial cells.[114] A recent crystal structure analysis of VCP revealed a putative heparin-binding site at its C-terminus and experiments indicate that VCP can bind heparin and control complement activation simultaneously.[115]

5. Interaction of Heparin with Extracellular Signaling Molecules

5.1. Interaction of Heparin with Growth Factors

Fibroblast growth factors (FGFs) are members of a large family of proteins that are involved in developmental and physiological processes including cell proliferation, differentiation, morphogenesis, and angiogenesis (currently 21 members).[116] FGFs are heparin-binding proteins that have a high affinity for cell surface heparan sulfate proteoglycans. With the possible exception of antithrombin III, no heparin-binding proteins have been more thoroughly studied. Vertebrate FGFs have an internal core region of 28 highly conserved and six invariant amino acids and range in molecular weight from 17 to 34 kDa.[117]

Acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2) were the first members of the family to be discovered, and the thermodynamics and kinetics of their interaction with heparin have been extensively studied.[118, 119] High-resolution X-ray crystallographic data on these proteins in complexes with heparin oligosaccharides have been published.[120, 121] These growth factors exert their biological effects by binding to different, specific cell surface receptors called fibroblast growth factor receptors (FGFR-1 – FGFR-4). The FGFRs are transmembrane tyrosine kinase receptors expressed as multiple splice variants with different affinities for the different FGFs.[79] The FGFRs are also heparin-binding proteins.[118] Thus, the three compounds FGF, FGFR, and heparan sulfate must interact simultaneously to initiate signal transduction.[119] FGF-stimulated signal transduction is similar to other receptor-mediated pathways in that it involves the dimerization of the FGFRs. Cell membrane heparan sulfate binds multiple FGF molecules promoting FGF dimerization and signal transduction. High-resolution X-ray crystal structures of complexes of FGF, FGFR, and a heparin oligosaccharide provide an insight into the stoichiometry and structural aspects of this physiologically relevant interaction (Figure 7).[120, 121]

The first study reports the crystal structure of a dimeric ternary complex of FGF-2, FGFR-1, and a heparin decasaccharide (2:2:2) at a resolution of 3 Å.[120] In each FGF – FGFR 1:1 complex, heparin makes numerous contacts with both FGF-2 and FGFR-1 stabilizing FGF – FGFR binding. Heparin also makes contacts with the FGFR-1 of the adjacent FGF – FGFR complex, thus seeming to promote FGF dimerization. The heparin binding mode in this structure is not in accordance with previous findings on the minimal heparin chain length sufficient to promote dimerization,[122] requiring additional biochemical studies to support these observations. Based on biochemical and crystallographic data, a model can be built in which heparin interacts through its nonreducing end with both FGF-2 and FGFR-1 resulting in the formation of a stable 1:1:1 complex of FGF, FGFR, and heparin. This complex can combine with another 1:1:1 complex through direct FGFR – FGFR contacts, secondary interactions between FGF-2 in one ternary complex and FGFR-1 in the adjacent ternary complex, and indirect heparin-mediated FGFR – FGFR contacts.

The second study reports the crystal structure of a 2:2:1 complex of FGF-1, FGFR-2, and a heparin decasaccharide at a resolution of 2.8 Å.[121] In this case the complex is assembled around a central heparin molecule that links two FGF-1 ligands into a dimer, similar to a previously proposed model.[34] There are significant differences in these models with respect to the relative orientation of FGF-1 and heparin. The 2:1 FGF-1 – heparin complex acts as a bridge between the two FGFR-2 molecules. The asymmetric heparin chain in this structure contacts only one receptor molecule. The role of heparan sulfate in this type of receptor dimerization also appears critical, as there are very few protein – protein contacts between the two halves of the pentameric complex.

While both these studies provide important information on the organization of these complexes, there are significant differences in the two structures possibly indicating that different members of the FGF family of ligands and their respective receptors may interact differently with heparan sulfate chains. These differences could result from differences in the distribution and structure of heparan sulfate PGs and FGF receptors on cell surfaces in different tissues.

The vascular endothelial growth factor (VEGF) is an angiogenic factor consisting of four alternatively spliced forms, three of which interact with heparin.[123] The hepatocyte growth factor (HGF) binds heparan sulfate to act as a tumor suppressor, morphogen, and angiogenic factor.[124] Heparin can also modulate the biological activity of the transforming growth factor-β1 (TGF-β1). This factor plays an important role in cell migration and proliferation, extracellular matrix synthesis, and is also involved in immune

Figure 7. Structures of the recently solved FGF – FGFR – heparin complexes. The FGFR is shown as a gold and the FGF as a green ribbon. The heparin oligosaccharides are shown as space-filling models with sulfur (yellow), oxygen (red) and nitrogen atoms (blue).
processes. Other heparin-binding growth factors include the platelet derived growth factor (PDGF) and the heparin-binding epidermal growth factor (HB-EGF). In summary, heparan sulfate PGs localize growth factors at the cell surface or in the extracellular matrix and promote their biological activities.

5.2. Interaction of Heparin with Chemokines

Chemokines are a group of small, cytokinelike proteins with a variety of biological functions including selective recruitment and activation of cells during inflammation. Chemokines also stimulate leukocyte degranulation and promote angiogenesis or angiostasis. The first member of the chemokine family to be discovered was the platelet factor 4 (PF-4, now called CXC chemokine ligand 4 or CXCL4). Chemokines were not recognized as chemotactic molecules until the characterization of interleukin 8 (IL-8, now called CXCL8). The more than 40 currently identified chemokines can be classified according to the distribution of cysteine residues near the NH₂-terminus into four major families: CXC, CC, C, and CX₃C. The interaction of chemokines with specific cell populations is mediated by G-protein-coupled receptors having seven transmembrane domains. Chemokine receptors are also believed to play an important role in breast cancer metastasis and in the entry of HIV-1 into cells. Chemokines can also bind heparin and cell surface GAGs at the vascular endothelium or in the extracellular matrix. This interaction has been suggested to play a role in the formation of haptotactic gradients on the surface of endothelial cells and to enhance the local concentration of chemokines in the vicinity of G-protein-coupled receptors.

PF-4 is released from platelets and is believed to have a number of properties associated with inflammation and wound healing that are thought to be due to its ability to neutralize the activities of heparin and heparan sulfate proteoglycans. When therapeutically administered heparin binds to PF-4, it can lead to a dangerous immunologically induced loss of platelets causing a condition called heparin-induced thrombocytopenia (HIT). PF-4 exists mainly as a tetramer under physiological conditions and binds to heparin and heparan sulfate with a high affinity in a 1:1 ratio. NMR studies on the interaction of a heparin dodecasaccharide chain with PF-4 suggest that residues in the Arg20–Arg22 loop are involved in heparin binding. These studies also indicate that, for certain PF-4:heparin ratios, the heparin chain induces PF-4 to undergo a conformational transition to a partially folded, molten-globule state. The ratio at which this conformational transition occurs is the same ratio at which HIT antibody binding is observed, suggesting that the HIT antibody recognizes a less folded, lower aggregate state of the protein (Figure 8). Another study that looked at the interaction of heparan sulfate with PF-4 identified a large heparan sulfate oligosaccharide that was involved in binding the PF-4 tetramer. The authors of this study propose that this oligosaccharide chain is capable of wrapping around the PF-4 molecule, contacting the positively charged residues involved in the interaction on all four monomers. The 2-O-sulfodurionate residues in this oligosaccharide were identified to be important for binding to PF-4. Basic residues in the circular array of charges found on the surface of PF-4 are conserved within the family of CXC type chemokines, including IL-8. Studies on the interaction of IL-8 with heparan sulfate suggest that a chain length of about 20 saccharide residues is essential for binding with a binding mode in which the heparan sulfate chain binds in a horseshoe fashion to the dimeric protein.

The stromal cell derived factor-1α (SDF-1α) is another member of the CXC chemokine family. It is a pro-inflammatory mediator, a potent chemo-attractant for a variety of cells, like monocytes and T-cells, and also a potent inhibitor of the cellular entry of HIV. Studies have shown that heparan sulfate is involved in the binding and localizing of SDF-1α to the cell surface. Site-directed mutation studies on SDF-1α have identified several residues in the protein important for this interaction. Molecular docking studies suggest that a heparin dodecasaccharide or tetradesaccharide is required for binding to SDF-1α. These large binding sites in PF-4, IL-8, and SDF-1α may be the result of chemokine oligomerization. A heparin-binding consensus motif of the type BBXB is also identified as being important for the interaction. This motif is also implicated as the principle site for heparin binding in a chemokine from the CC family called RANTES (regulated on secretion, normal T-cell expressed and secreted). Other members in this family include monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory peptides-1 (MIP-1). Studies have shown that all these chemokines also bind to heparin although with varying affinity and specificity. Due to the low circulating concentrations of these chemokines, heparan sulfate PGs may play an important role in sequestering these molecules on the cell surface, thus increasing their effective concentration in the vicinity of their low-affinity receptor sites. Data also suggest that cell surface heparan sulfate PGs play a different role than free glycosaminoglycans. Soluble glycosaminoglycans can complex with chemokines in solution and prevent their binding to the receptor, inhibiting downstream receptor-mediated cell responses.

The recently identified C chemokine family and the CX₃C family including lymphotactin and fractalkine appear to bind heparin, but the in vivo role of this interaction is not well understood. Thus, heparin–chemokine binding appears to be
involved in the regulation of a number of different important physiological processes.

6. Interaction of Heparin with Lipid- or Membrane-Binding Proteins

The interaction of heparin with proteins such as antithrombin III and the fibroblast growth factors has been very well studied and characterized in terms of both structural and functional aspects. While there is extensive literature on the interaction of heparin with various families of proteins, less is known about heparin interaction with lipid- or membrane-binding proteins.

Annexins are a family of homologous proteins (currently with 32 members) that are widely distributed and are ubiquitous in eukaryotes. A distinctive feature of annexins is their calcium-dependent binding to the surface of phospholipid membranes. Annexins have been implicated in a wide range of functions in eukaryotes, including roles in cell signaling, membrane trafficking, blood coagulation, and inflammation. Although annexins are found primarily within cells and lack signal sequences, many extracellular events are now proposed to be annexin-mediated. Calcium-dependent glycosaminoglycan binding to annexins has been reported and characterized in several in vitro studies. While there are currently over 100 proteins that bind heparin, few of these interactions are calcium-dependent; certain annexins, the serum amyloid P component (SAP) and P- and L-selectins. A qualitative study that examined the glycosaminoglycan-binding properties of annexins IV, V, and VI revealed different binding specificities for these molecules. The extracellular function of annexin V has been extensively studied: it has a role in the entry of and in the infection by influenza and hepatitis B viruses. Annexin V also exhibits potent in vitro anticoagulant activity through a proposed mechanism in which it coats placental endothelial cells to form a protective anti-thrombotic shield. Kinetic and thermodynamic studies of annexin V demonstrate that its calcium-dependent interaction with heparin and heparan sulfate involves an oligosaccharide sequence of 6–8 saccharide residues. Molecular modeling suggests three possible domains within annexin V that could be involved in heparin binding, one on the lipid-binding surface of annexin V and the other two on the exposed surface. A crystal structure analysis of annexin V with bound heparin-derived tetrasaccharides at a resolution of 1.9 Å gave the first structural information on the essential role of calcium ions in a heparin–protein interaction (Figure 9). The contact between heparin residues and calcium is indirect and involves shared hydrogen bonds with water molecules near the calcium coordination sites. The calcium dependence of the annexin–heparin interaction arises primarily through inducing the requisite conformation for heparin binding. The structure is characterized by distinct heparin-binding sites situated on opposite surfaces of the protein. Residues important for the interaction can be identified in the crystal structure, in previous modeling studies and from biochemical studies on site-directed mutants.

Based on all of these data, a model is proposed by which cell surface heparan sulfate PG wraps around the annexin V molecule making contact with all three of its heparin-binding domains (Figure 10A). The annexin V molecule is then delivered and assembled into an antithrombotic array on the cell surface phospholipid membrane.

Apolipoprotein E (apoE) is an important lipid transport protein in human plasma and brain. Biochemical, cell biological, and epidemiological studies have suggested that apoE is a major genetic risk factor in a number of diseases. The human APOE gene has three common alleles (ε2, ε3, and ε4). The three isoforms differ at amino acids 112 and 158 and vary in their metabolic properties and association with disease. ApoE4 (Arg112, Arg158) is associated with a higher risk of heart disease and is a major genetic risk factor for Alzheimer’s disease. ApoE also binds low-density lipoprotein (LDL) receptors and plays a central role in plasma lipoprotein metabolism and cholesterol transport. In the liver, apoE mediates the binding of lipoprotein remnant to the complex of heparan sulfate PG with the LDL receptor-related protein, facilitating lipoprotein uptake.

Both the N- and C-terminal domains contain a putative heparin-binding site. The high-affinity heparin-binding site of the N-terminal domain overlaps with the LDL receptor-binding region; the heparin-binding site of the C-terminal domain is only available for interaction in the lipid-free state. A third heparin-binding site has been proposed for the segment of protein that connects the N-terminal and C-terminal domains. The role of apoE and heparan sulfate PG in the brain has been examined with cultured neurons; the results suggest that the effects of Alzheimer’s disease are mediated by the interaction of apoE with the heparan sulfate PG–LDL receptor-related protein complex. Based on this model, apoE initially interacts with the heparan sulfate PG on the cell surface and is then transferred to the LDL receptor-related protein receptor for internalization. Thus, interaction with heparan sulfate represents the initial recognition step that localizes the apoE to the cell surface. The interaction of heparin with apoE isoforms has been studied using SPR and a recent study that...
characterized both the energetics and kinetics of the binding of heparin with the N-terminal domain of apoE4 identified an octasaccharide sequence as the minimum binding site within heparin.\cite{166} Analysis of these binding data suggests that a heparan sulfate chain wraps around an apoE contacting all three heparin-binding sites (Figure 10B). This model is similar to that proposed for heparin binding to the other lipid-binding protein annexin V (Figure 10A).

7. Heparan Sulfate as a Receptor for Pathogens

The initial binding of a virus to a target cell often represents a critical step in pathogenesis.\cite{167} Binding may result from a receptor-like interaction between a viral coat protein molecule and the glycosaminoglycan chain of a proteoglycan expressed on the surface of the target cells. Heparan sulfate GAGs are found on the external surface of most mammalian tissues,\cite{17, 19} therefore; it is not surprising that viruses make use of these molecules as receptors to bind to and gain entry into target cells.

The interaction of the human immunodeficiency virus type-1 (HIV-1) coat proteins with heparin and heparan sulfate has been the subject of many studies because of the potential of these sulfated polysaccharides in the therapy and prevention of HIV-1 infection.\cite{168} The physiological target cells for HIV-1 infection, CD4+ T-cells, monocytes/macrophages, and some populations of dendritic cells, all express a cellular receptor molecule CD4.\cite{169} HIV-1 binds to a loop of 20 amino acids in the first domain of CD4 by interacting with the viral surface glycoprotein, gp120.\cite{170} This interaction is believed to induce a conformational change in HIV glycoproteins resulting in the exposure of co-receptor binding sites on gp120.\cite{171} The interaction of gp120 and perhaps gp41 with CD4 and the co-receptor molecules ultimately results in the fusion of virus and cell membranes.\cite{172}

Heparin exerts its anti-HIV-1 activity by binding to the V3 loop, a major epitope of gp120.\cite{173} The V3 loop is not involved in the initial gp120–CD4 binding, but has an essential role to play in subsequent steps leading to membrane fusion.\cite{174} Studies with chemically modified heparin molecules show that O-sulfation, particularly 6-O-sulfation, and N-substitution (acetylation or sulfation) are essential for this interaction.\cite{175} Another study shows that cell lines lacking the CD4 receptor are still infected by HIV-1 due to an interaction between gp120 and cell surface heparan sulfate. This indicates that CD4 dependence for HIV-1 attachment to target cells is highly cell line specific and in some cases heparan sulfate may act as the cell surface receptor for HIV-1.\cite{176} There is also evidence that there might be a step prior to the gp120–CD4 interaction involving cell surface heparan sulfate chains and cyclophilin A (CypA), which would represent the initial step of HIV-1 localization and attachment to the cell surface.\cite{177} CypA is an abundant cytosolic protein ubiquitously expressed in eukaryotic cells and is packaged into nascent HIV-1 particles at the time of viral assembly.\cite{178} CypA is exposed on the viral membrane and also has a domain of basic residues similar to known heparin-binding motifs.

Heparin and heparan sulfate are also important in another aspect of HIV-1 infection. One of the proteins essential for HIV-1 replication is the Tat protein, which can be released from cells and has autocrine and paracrine (local signaling) activities.\cite{179} The Tat protein has the ability to enter cells and is believed to play a role in priming cells for infection. Tat is a heparin-binding protein which also interacts with heparan sulfate proteoglycans on the cell surface and in the extracellular matrix.\cite{180} The minimum-sized heparin fragment involved in Tat binding is a hexasaccharide, although the binding affinity increases with increasing oligosaccharide size, with about 18 saccharide residues required to match heparin’s affinity.\cite{181} Thus, heparin represents a “multi-target” compound capable of affecting different aspects of HIV infection.

The herpes simplex virus (HSV) causes many disease states including mucosal lesions and encephalitis. These diverse clinical manifestations reflect the capacity of the virus to infect both epithelial and neuronal cells. HSV also uses heparan sulfate PG to target\cite{182} and infect cells.\cite{183} The entry of HSV into mammalian cells is a multi-step process beginning with an attachment step in which viral coat glycoproteins gC and gB interact with heparan sulfate chains on cell surface.

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Figure 10. Model for the binding of heparin to lipid-binding proteins by wrapping around. A) Annexin V. B) Apolipoprotein E.
proteoglycans.\textsuperscript{[184]} HSV types 1 and 2 differ in their interaction with heparan sulfate, which may influence viral tropism.\textsuperscript{[185]} These observed differences are the result of different requirements of gC and gB in HSV-1 and HSV-2 for binding heparin/heparan sulfate.\textsuperscript{[186]} The second step in the entry of HSV requires the interaction of glycoprotein gD with a specific sequence within the heparan sulfate chains that have been modified by 3-O-sulfation of specific glucosamine residues. This interaction leads to the initiation of HSV-1 entry into the cell.\textsuperscript{[70]}

Dengue virus, a mosquito-borne flavivirus, responsible for yellow fever is also believed to target cells by the interaction of an envelope protein with a highly sulfated heparan sulfate (in the liver), which could give an explanation for the tropism of the virus.\textsuperscript{[187]} Two putative heparin-binding motifs at the C-terminus of the dengue envelope protein sequence have also been identified (Figure 11). Heparin and a number of smaller analogs are being investigated as potential pharmaceutical agents to prevented dengue virus infection.\textsuperscript{[187, 188]}

Other viruses, such as the respiratory syncytial virus,\textsuperscript{[189]} the cytomegalovirus,\textsuperscript{[190]} the adeno-associated virus,\textsuperscript{[191]} and the foot-and-mouth disease virus,\textsuperscript{[192]} all employ heparan sulfate chains of cell surface proteoglycans in their initial step of infection.

Heparin and heparan sulfate are also believed to play an important role in the virulence of other pathogens. The circumsporozoite (CS) protein is a sporozoite cell surface protein of \textit{Plasmodium falciparum}, the parasite causing malaria. The CS protein interacts with the highly sulfated heparan sulfate present on the surface of liver cells\textsuperscript{[193]} to promote sporozoite attachment and subsequent invasion of the hepatocytes.\textsuperscript{[194, 195]} This interaction is inhibited by heparin in a dose-dependent manner.\textsuperscript{[196]} The CS protein has been cloned and purified and identified as a heparin-binding protein.\textsuperscript{[197]} The uptake of malaria sporozoites by hepatocytes may take place by the same mechanism involved in the apolipoprotein-E-mediated clearance of lipoprotein remnants from the blood by the liver.\textsuperscript{[198]} A decasaccharide, which shares the sequence of an heparin octasaccharide identified as the minimum-size oligosaccharide required for binding apoE,\textsuperscript{[199]} corresponds to the minimum sequence size for the CS-protein-binding domain.\textsuperscript{[195]}

Binding heparin is an efficient strategy utilized by microbial pathogens to recruit a diverse array of mammalian heparin-binding proteins to their surfaces, thus bypassing the need to synthesize individual receptors for each of these proteins.\textsuperscript{[199]} Indeed, the human pathogen \textit{Neisseria gonorrhoeae} binds vitronectin, an extracellular matrix protein, without the involvement of a specific vitronectin receptor on its cell surface.\textsuperscript{[200]} In this case it is believed that vitronectin binding is indirect and mediated through a heparan sulfate chain that forms stable molecular bridges between the bacterial surface adhesin OpA and vitronectin, both heparin-binding proteins.\textsuperscript{[201]}

8. Interaction of Heparin with Adhesion Proteins

The interaction of heparin and heparan sulfate with adhesion proteins has implications in various physiological and pathological processes including inflammation, nerve tissue growth, tumor cell invasion, and plaque formation in the brain.

The selectins (L-, E-, and P-selectin) are a family of transmembrane glycoproteins found on endothelium, platelets, and leukocytes.\textsuperscript{[202]} They are responsible for mediating the initial adhesive events directing the homing of lymphocytes into lymphoid organs and the interaction of leukocytes with the endothelium in inflammation and reperfusion injury states.\textsuperscript{[203]} Selectin interactions may also be involved in the metastasis of certain epithelial cancers.\textsuperscript{[204]} Although the putative ligand on the endothelium responsible for the interaction of leukocytes with selectins is the carbohydrate antigen sialyl Lewis X (SLex), heparan sulfate has also been shown to play a role in this interaction.\textsuperscript{[153, 205]} Heparan sulfate interacts with L- and P-selectin, but not E-selectin.\textsuperscript{[153, 206]} The interaction of heparin with L-selectin is calcium-dependent and requires micromolar levels of free calcium. L-selectin binds oligosaccharides that contain highly modified, heavily sulfated, iduronate rich regions and endothelial tissue-derived heparan sulfate chains that are enriched in free amino groups.\textsuperscript{[207]} The P-selectin-binding heparan sulfates include chains with less highly modified domains.\textsuperscript{[209]} Heparin tetrasaccharides specifically block the interactions of L- and P-selectins with SLex-containing ligands demonstrating anti-inflammatory activity in vivo, and also prevent colon cancer cell adhesion to L- and P-selectin.\textsuperscript{[205]}

The heparin-binding growth-associated molecule (HB-GAM), or pleiotrophin, is another protein associated with the extracellular matrix. HB-GAM is believed to play a role in enhancing neurite outgrowth by providing extracellular tracts to guide neurites in the developing brain.\textsuperscript{[208]} A heparan sulfate PG, syndecan-3, binds with high affinity to HB-GAM and may be a receptor for HB-GAM. This binding mediates neurite outgrowth promoting signals from growing neurites.\textsuperscript{[209]} Soluble heparin and heparan sulfate GAGs are inhibitory towards neurite outgrowth, and the presence of 2-O-sulfo groups is required for inhibitory effects. A heparin
decasaccharide was required for the inhibition of HB-GAM-induced neurite outgrowth, and maximum activity was observed for an octadecasaccharide. ITC and SPR were used to study the thermodynamics and kinetics of the heparin–HB-GAM interaction. ITC studies demonstrated a stoichiometry of interaction corresponding to a heparin binding site for HB-GAM of 12–16 saccharide residues. Affinity chromatography on HB-GAM-Sepharose showed that oligosaccharides with more than 18 saccharide residues gave the strongest interactions. CD and NMR spectroscopy revealed that HB-GAM undergoes a conformational change upon binding to heparin and that the binding occurs primarily through the β-sheet domains of HB-GAM. A search of sequence databases shows that the β-sheet domains of HB-GAM are homologous to the thrombospondin type I repeat (TSR). The TSR sequence motif occurs in a wide variety of proteins (including malaria CS protein) that mediate cell-to-extracellular matrix and cell-to-cell interactions, in which the TSR domain mediates specific cell surface binding.

Heparin and heparan sulfate are also believed to be involved in the formation of amyloid deposits in Alzheimer’s disease and are found in associated senile plaques and neurofibrillary tangles. The amyloid P (AP) component is a glycoprotein found in the circulation, in basement membranes, and in connective tissue and is also a universal constituent of the abnormal tissue deposits in amyloidosis, including Alzheimer’s disease. AP has been shown to bind to heparin, heparan sulfate, and dermatan sulfate in a calcium-dependent manner. AP also exhibits a calcium-independent binding of lower affinity to heparan sulfate and dermatan sulfate, and this binding is enhanced under conditions of slightly lowered pH, possibly due to the protonation of histidine residues in AP. Two heparin-binding fragments have been identified in the proteolytic digests of AP. The fragment sequences do not correspond to any previously reported heparin-binding sequences and unlike AP these peptides did not require calcium for binding activity. The binding of the peptide that exhibited the highest affinity for heparin was characterized by affinity capillary electrophoresis. This binding was found to be highly specific for heparin and heparan oligosaccharides down to tetramers and appeared stronger at a slightly alkaline pH. No binding could be demonstrated with heparan sulfate, chondroitin sulfate, and desulfated heparin. While the precise role of heparin and heparan sulfate in Alzheimer’s disease pathology is still unknown, there is a possibility that heparin or heparan oligosaccharides may be useful agents in slowing or reversing the formation of Alzheimer’s and prion-based plaques in the brain.

9. Heparin-Degrading Enzymes

Two types of enzymes act on heparin and heparan sulfate, the prokaryotic polysaccharide lyases (acting through an eliminative mechanism) and the eukaryotic glucuronid hydrodrolases (acting through a hydrolytic mechanism). Heparin lyases eliminatively depolymerize heparin affording unsaturated oligosaccharide products (Scheme 5). Three major polysaccharide lyases (heparin lyases I, II, and III), isolated from Flavobacterium heparinum, are capable of cleaving linkages present in heparin and heparan sulfate. The substrate specificity of each of these enzymes has been well studied using heparin-derived oligosaccharides and chemically modified heparins (Scheme 6A).
lyases I and III but inhibits enzymatic activity of heparin lyase II.[12]

The genes encoding the three heparin lyases in *Flavobacterium heparinum* have been cloned and expressed in *Escherichia coli*. [220, 221] Studies on the enzymology of heparin lyase I have identified a cysteine and a histidine residue as part of the catalytic domain[222] and shown that three lysine residues are involved in heparin binding close to the catalytic site.[223] Heparin lyase II has the broadest substrate specificity of the three heparin lyases. It is known to have two distinct active sites, one believed to act on heparin and the other on heparan sulfate.[224] One cysteine and three histidine residues have been identified as crucial for heparin lyase II activity.[225] Heparin lyase III, exhibiting a strong specificity for heparan sulfate, is unique in that it contains no cysteine residues[221, 226] but instead two histidine residues crucial for its activity on heparan sulfate.[227]

These enzymes are essential in the preparation of defined oligosaccharides from heparin and heparan sulfate required for the structural characterization of heparin and heparan sulfate.[14] They are also important clinically and have been used in the monitoring of heparin levels in the blood,[221] the neutralization of heparin in the blood and the production of LMW heparins for use in humans.[8, 229] Recent studies show that heparin lyase I and II are capable of cleaving the AT-III-binding site within heparin leaving only a partial site which lacks some of the functional attributes of heparin with an intact AT-III-binding site.[228] Heparin lyase I and III are also potent inhibitors of neovascularization, which is associated with the regulation of tissue development, wound healing, and tumor metastasis.[229] A novel type of heparin lyase recently purified from *Bacteroides stercoris* HJ-15, isolated from human intestine,[230] may be important in understanding how to improve the oral bioavailability of pharmaceutical heparin.[231]

Human heparanase, which is an endo-β-D-glucuronidase capable of cleaving heparan sulfate, has been purified, cloned, and characterized.[232] Heparanase is a hydrolase, distinct from the flavobacterium heparin lyases, and has been implicated in inflammation as well as in tumor angiogenesis and metastasis, making it an important target for the development of inhibitors.[233] Heparanase is known to cleave heparan sulfate into characteristic large-molecular-weight fragments.[234] Studies on the substrate specificity of this enzyme suggest the importance of 2-O-sulfo groups on uronic acids (Scheme 6B).[235] The optimal pH for the heparanase activity is 4.2, implying that it might be localized in the lysosomes.[236]

**10. New Clinical Applications of Heparin**

The interaction of heparin with various proteins that play important roles in the regulation of normal physiological processes as well as disease states has lead to an interest in using heparin in roles outside its normal application as an anticoagulant/antithrombotic agent. Randomized trials to study the effectiveness of LMW heparin as compared to unfractionated heparin in treating venous thromboembolism in cancer patients led to a surprising observation: treatment with heparin may affect survival of patients with malignan-

cy.[238] Cancer patients who had been treated with LMW heparin for their thrombosis showed a slightly improved three-month survival rate 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 the vascular endothelium, regulate the immune system and have both inhibitory and stimulatory effects on angiogenesis.[239] There is recent evidence that heparin treatment reduces tumor metastasis in mice by inhibiting P-selectin-mediated interactions of platelets with mucin ligands on carcinoma cell surfaces.[240]

Numerous proteins of physiologic and pathophysiologic importance interact with heparin and heparan sulfate. This offers a large number of potential therapeutic applications for heparin. The major limitation in utilizing heparin in new ways is that its high potency as an anticoagulant becomes a side effect that can lead to hemorrhagic complications. The introduction of LMW heparins and the preparation of heparin oligosaccharides and synthetic analogs devoid of anticoagulant activity may open up a wide variety of new therapeutic applications in the treatment of cancer, viral and bacterial infections, Alzheimer’s disease, and transplant rejection.

**11. Summary and Outlook**

Over the last few decades heparin and heparan sulfate have been shown to interact with a number of biologically important proteins, thereby playing an essential role in the regulation of various physiological processes (Figure 12). Our understanding of these interactions at the molecular level is important for the design of highly specific therapeutic agents. In addition, an understanding of the specificity of heparin and heparan sulfate will be necessary to understand normal physiologic and pathophysiologic processes. 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.

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