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1 Heparins and heparan sulfates. Structure, distribution and protein interactions

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Abstract

Sulfated glycosaminoglycans are absent in protista, plantae and fungi. In the animal kingdom, the appearance of heparan sulfate coincides with the emergence of eumetazoa, which are animals that display true tissues. These compounds are ubiquitously found in the cell surface of all tissues and species. On the other hand, the distribution of heparin is scattered

In honor of Prof. Carl P. Dietrich

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throughout the evolutionary tree, being found only in the cytoplasmatic granules of mastocyte-like cells of some invertebrate and vertebrate species. This chapter will address glycosaminoglycans, with special emphasis on the identification of the structural elements in heparin/heparan sulfate involved in the interaction with specific proteins, leading to biological and/or pharmacological activities. Using a combination of specific endo- and exo-glycosidases, chemical methods (nitrous acid degradation, hydrazine N-acetylation and borohydride reduction) and NMR spectroscopy it was possible to propose the sequence of disaccharides in heparin and heparan sulfate polymers. The molecular interactions involved in the binding have been characterized to various degrees, including identification of heparin-binding motifs in some proteins as well as the minimum saccharide sequence in the heparin/heparan sulfate polysaccharide chain. The physiological significance of these bindings is discussed.

1. Introduction

Heparin was first isolated due to its ability to prolong the clotting time of blood. The anticlotting effect results through the interaction with specific proteins, such as antithrombin and heparin cofactor II [1]. It is interesting that heparin was discovered and defined as an anticoagulant compound in 1918 by MacLean [2] much earlier than the description of the antithrombins [3, 4]. Based on this ability of heparin to interact with some proteins, it was possible to purify antithrombin [5], as well as a whole set of different proteins involved in different cellular events. Figure 1 highlights some of the physiological processes influenced by proteins that are modulated by heparin/heparan sulfate.

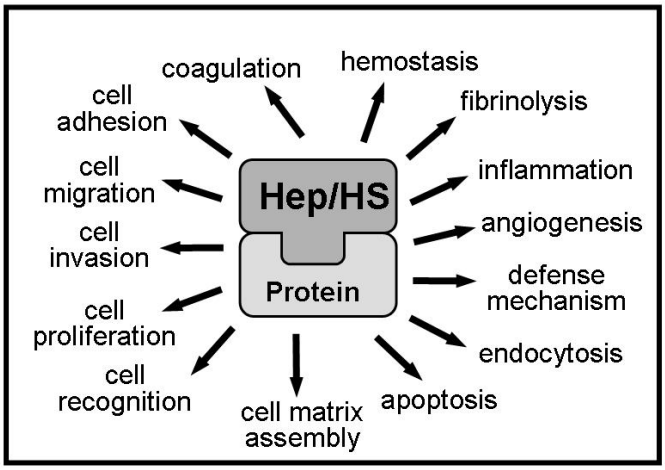

Figure 1. Physiological processes modulated by the interaction of proteins with heparin or heparan sulfate. Hep, heparin; HS, heparan sulfate.

Among the natural occurring anionic polymers, heparin is the compound with the highest density charge. Due to this negative charge, heparin as well other anionic polysaccharides can bind to basic regions on a protein surface due to electrostatic attraction, and these interactions are not likely to have physiological significance. Nevertheless, the binding of heparin to proteins, like the interaction of a ligand to its receptor, is specific involving hydrogen bonding and van der Waals interactions, besides charge and dipole attraction.

Specificity of interactions is also described for other glycosaminoglycans. The proper binding of a glycosaminoglycan with a certain protein changes its conformation leading to the biological effect. So, in order to understand the binding of glycosaminoglycans to different proteins one has to know the fine structure of these polysaccharides. This chapter will address glycosaminoglycans, with special emphasis on the identification of the structural elements in heparin/heparan sulfate chains involved in the interaction with specific proteins, leading to biological and/or pharmacological activities.

2. Glycosaminoglycans: Structural characteristics and distribution

Except for hyaluronan (also known as hyaluronic acid), all glycosaminoglycans occur in the tissues as proteoglycans, where the polysaccharide chains are covalently linked to a core protein. The glycosaminoglycans are polydisperse linear polysaccharides composed of alternate units of hexosamine and uronic acid connected by glycosidic linkages (Fig. 2A). The type of hexosamine: D-glucosamine (GlcN) or D-galactosamine (GalN); the type of uronic acid: D-glucuronic acid (GlcA) or L-iduronic acid (IdoA); the type (α or β) and the relative position ($1\rightarrow3$ or $1\rightarrow4$) of the glycosidic linkages distinguish the different glycosaminoglycans (Fig. 2B). Sulfation occurs at different positions of the disaccharide units. Table 1 summarizes some structural characteristics of this class of polymers. The sulfated glycosaminoglycans are not homopolymers. In fact, they are composed of different proportion of various disaccharides (Table 1). Nevertheless, the type of hexosamine and the inter-disaccharide glycosidic linkage are always the same throughout the polymer chain.

Sulfated glycosaminoglycans are ubiquitous to the animal kingdom of the eukarya domain, whereas in the bacteria kingdom only non-sulfated chains of glycosaminoglycans are found. Heparanosan (GlcNAc and GlcUA) and chondrosan (GalNAc and GlcA), non-sulfated versions of heparan and chondrotin sulfates are found in the capsules of some pathogenic bacteria, thus acting as molecular camouflages protecting the microbe and enhancing infection. Hyaluronan that is also present in some pathogenic bacteria play similar function [5].

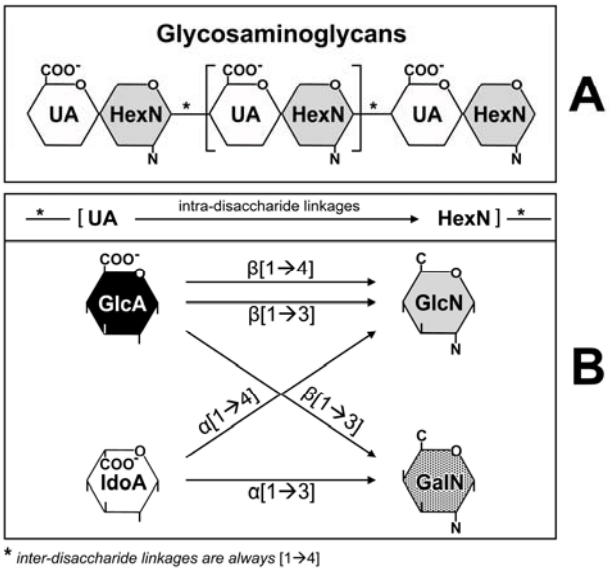

Figure 2. General structure of the glycosaminoglycans backbone. UA, uronic acid; HexN, hexosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcN, D-glucosamine; GalN, D-galactosamine.

Table 1. Main types of disaccharides present in glycosaminoglycans.

Glycosaminoglycans	**Disaccharides**				
Hyaluronan	D-GlcA	[β1→3]	D-GlcN **Ac**	**β [1→4]**	**inter-disaccharide linkage**
Chondrosan	D-GlcA	[β1→3]	D-GalN **Ac**		
Chondroitin sulfate	D-GlcA	[β1→3]	D-GalN **Ac**		
	D-GlcA	[β1→3]	D-GalN **Ac (4S)**		
	D-GlcA	[β1→3]	D-GalN **Ac (6S)**		
Dermatan sulfate	D-GlcA	[β1→3]	D-GalN **Ac (4S)**		
	D-GlcA	[β1→3]	D-GalN **Ac (6S)**		
	L-IdoA	[α1→3]	D-GalN **Ac (4S)**		
	L-IdoA **(2S)**	[α1→3]	D-GalN **Ac (4S)**		
	L-IdoA **(2S)**	[α1→3]	D-GalN **Ac (6S)**		
Heparanosan	D-GlcA	[α1→4]	D-GlcN **Ac**	**α [1→4]**	
Acharan sulfate	L-IdoA **(2S)**	[α1→4]	D-GlcN **Ac**		
Heparan sulfate	D-GlcA	[β1→4]	D-GlcN **Ac**		
	D-GlcA	[β1→4]	D-GlcN **Ac (6S)**		
	D-GlcA	[β1→4]	D-GlcN **(S)**		
	D-GlcA	[β1→4]	D-GlcN **(S,6S)**		
	L-IdoA	[α1→4]	D-GlcN **(S)**		
	L-IdoA **(2S)**	[α1→4]	D-GlcN **(S)**		
Heparin	D-GlcA	[α1→4]	D-GlcN **(S)**		
	D-GlcA	[α1→4]	D-GlcN **(S,6S)**		
	L-IdoA **(2S)**	[β1→4]	D-GlcN **(S)**		
	L-IdoA **(2S)**	[β1→4]	D-GlcN **(S,6S)**		

Sulfated glycosaminoglycans are absent in protista, plantae and fungi. In the animal kingdom, the appearance of heparan sulfate and chondroitin sulfate coincides with the emergence of eumetazoa, which are animals that display true tissues. These compounds are ubiquitously found in all tissues and species analyzed. The occurrence of dermatan sulfate is a late event in the evolutionary glycosaminoglycan tree, being restricted to the appearance of deuterostomes [6]. On the other hand, the distribution of heparin is scattered throughout the evolutionary tree, being found in some invertebrate and vertebrate species. An updated phylogenetic normal tree of the distribution of sulfated glycosaminoglycans in the animal kingdom is shown in Figure 3.

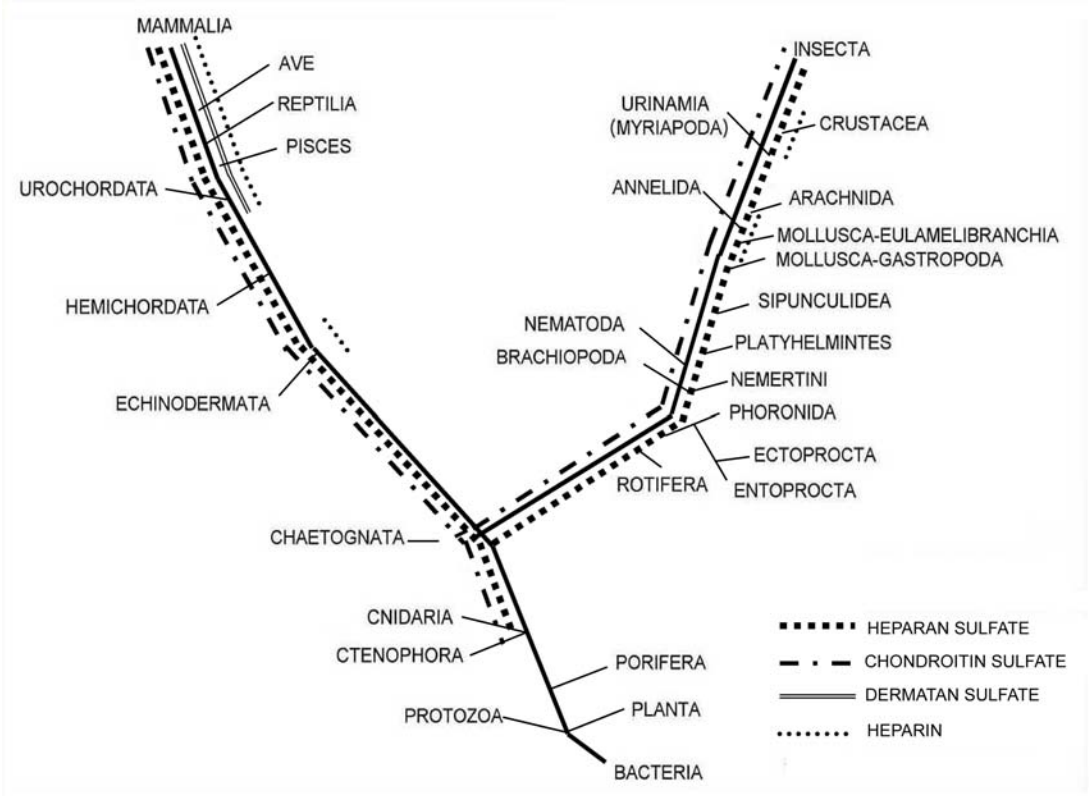

Figure 3. Distribution of sulfated glycosaminoglycans in the animal kingdom. Data from references [7-43].

3. Heparins and heparan sulfates

3.1 Distribution and biological role

The most distinguishing features between heparin and heparan sulfate, besides structural peculiarities, are their cellular localization, their occurrence in the animal kingdom, and thus their biological functions. Heparin and heparan sulfate are attached to different core proteins and found in different cellular compartments. Heparin is found exclusively inside storage vesicles of

mast cells of some animal species [22, 29, 38-43] whereas heparan sulfates are ubiquitous to the cell surface of both vertebrate and invertebrate specie [10-17]. Unlike heparan sulfate, heparin is present only in some tissues of vertebrates and invertebrates. For instance, heparin is absent in brain and is present in tissues that are in direct contact with the environment such as lung (branchia or ctenidium), skin (mantle) and intestine. Of particular significance was the observation that rabbit tissues do not contain heparin. Among mice with different genotypes regarding the mast cell precursors, the ones that have no mast cells also lack the presence of heparin. Heparin is not replaced by other sulfated glycosaminoglycan in those animals where the compound is absent, and the structure of the heparan sulfate is maintained when the mutant and the wild strains are compared. In studies using genetically selected mice for antibody production it was observed an inverse correlation between heparin content and antibody response. These and other results led to the proposition that heparin may have a role in defense mechanisms against pathogens without the direct involvement of antibody response. It is unlikely that the biological function of heparin might be related to prevention of blood coagulation, since species that lack heparin show normal blood clotting mechanisms and species, where no anticlotting system dependent on antithrombin has been described so far, contain large amounts of heparin.

Heparan sulfates are present in all species that show tissue organization (Fig. 3), and display peculiar structural variabilities according to the tissue and species of origin. Its cellular localization, structural diversity, and fluctuation of expression in different physiological conditions, such as cell division, cell growth, cell adhesion, cell migration, cell differentiation, among others, led to the proposition that heparan sulfate plays a specific role in cellular interactions.

3.2 Structure

The heparin chains are covalently bound to serglycin core protein, whereas the HS chains at the cell surface are attached to syndecan (transmembrane) or glypican (glycosylphosphatidylinositol-anchored) core proteins [44- 48]. This review will focus on the polysaccharide nature of these proteoglycans, since many of the functions of these compounds are exerted by the glycosaminoglycan side chains.

Heparin and heparan sulfates share common structural features, such as the type of hexosamine (GlcN), the types of uronic acid (GlcA/IdoA), as well as inter- ($\alpha 1 \rightarrow 4$) and intra-disaccharide ($\beta 1 \rightarrow 4/\alpha 1 \rightarrow 4$) glycosidic linkages. Nevertheless, they can be differentiated by the presence of N-acetyl groups in the glucosamine moiety and the total sulfate content. Acetyl groups correspond to less then 5% in heparins, where most of the glucosamine is N-sulfated. Furthermore, heparin shows higher degree of sulfation (2.3 - 2.8 sulfates/disaccharide) when compared to heparan sulfates (0.6 - 1.5

sulfates/disaccharide). The main types of disaccharide units found in both compounds are shown in Table 1.

Using a combination of enzymatic and chemical approaches it is possible to establish the sequence of the different disaccharides in heparin and heparan sulfate polymers.

Figure 4 depicts the general mode of action of bacterial glucosaminoglycan lyases and the animal endo-hydrolases described so far. Heparan sulfate and heparin chains can be degraded by a class of endo-hydrolases known as heparanases, which are endo-β-glucuronidases that cleave β-D-glucuronyl (1→4) D-glucosamine N-acetylated. Glycosaminoglycan lyases act upon the polysaccharide chain cleaving the glycosidic bond by a beta elimination mechanism leaving an unsaturated uronic acid (C4-C5 double bond) at the non reducing end. Among the lyases that degrade heparin and heparan sulfate, heparitinase I and heparinase show selective substrate specificity requirements for their activities. Heparinase acts upon α-D-glucosaminido-iduronic acid linkages. The sulfation at the C-2 position of the iduronic acid residue is obligatory for activity, and N-acetylation of the glucosamine moiety is impeditive for enzyme action. Heparitinase I acts only upon α-D-glucosaminido-glucuronic acid linkages, and sulfation at the C-6 position of the glucosamine is impeditive for enzyme activity. On the other hand, heparitinase II is a lyase with broad specificity acting upon heparin and heparan sulfate chains. Nevertheless, this enzyme shows no action upon glucosaminido-glucuronic acid linkages when the glucosamine is N-acetylated as well as in clusters of trisulfated disaccharides [49-54].

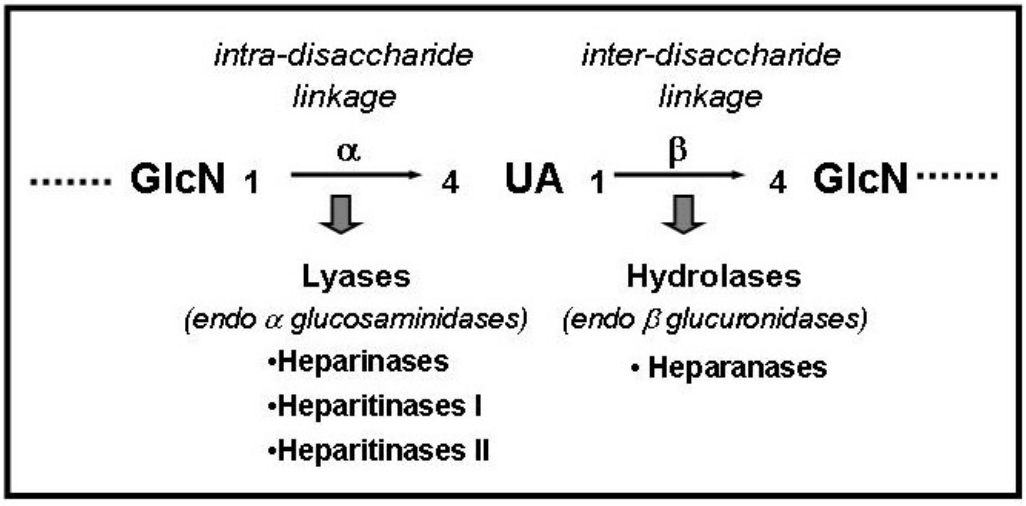

Figure 4. Endo-glycosidases that act upon heparin and heparan sulfate chains. More recently, heparinase and heparitinase II were named as heparinase I and heparinase II. It should be pointed out that heparinase III does not correspond to heparitinase I. UA, uronic acid; GlcN, D-glucosamine.

Nitrous acid treatment at different pHs is an useful method to depolymerize both heparan sulfate and heparin. The N-sulfated glucosamines found only in heparin and heparan sulfates are sites that are unique in their susceptibility to cleavage by nitrous acid at room temperature and at low pH (~2.0). Thus, when these polymers are treated with nitrous acid, they are specifically cleaved into fragments with ranges of molecular weights that depend on the distributions of the N-sulfated glucosamine residues in the chain. Since N-acetylated amino sugars are not affected by the nitrous acid treatment, the produced fragments will contain clusters of N-acetylated glucosamines [55].

Figure 5 summarizes the mode of action of different lyases, hydrolases and chemical depolymerization upon a hypothetical glucosaminoglycan chain.

Another approach to structural elucidation of heparin and heparan sulfate is NMR spectroscopy, which requires the full assignment of both ^{1}H and ^{13}C NMR spectra of oligosaccharides. Characteristic ^{1}H and ^{13}C chemical shifts have been identified for the individual residues, and the relative abundance of these moieties can be quantitatively determined by integrating the proton signals. The anomeric proton signals of the glucosamines can be resolved further to elucidate the linkage to the neighboring uronic acid, and thus defining the presence of β-D-glucuronic acid or α-L-iduronic acid, as well as the sulfation state [23, 28, 31, 52, 53, 56, 57]. By a combination of two-dimensional NMR techniques such as correlated spectroscopy (COSY), nuclear overhauser effect (NOESY) and total correlation spectroscopy (TOCSY) for ^{1}H, and heteronuclear single-quantum coherence (HSQC) for ^{13}C some of the sequences can be determined [23, 28, 31, 52, 53, 58 - 60].

Other analytical tools have also been successfully developed for sequencing oligosaccharides, including gel electrophoresis [61], capillary electrophoresis [62], high-performance liquid chromatography [53, 63], matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [64], nanoelectrospray mass spectrometry [65], among others [66].

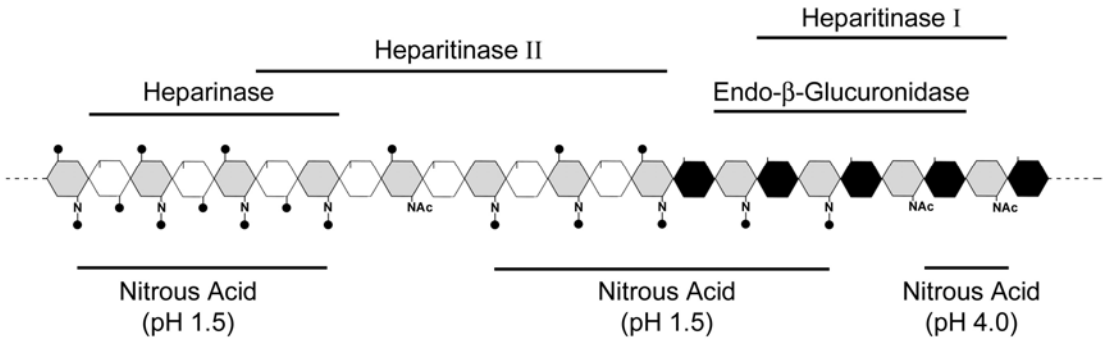

Figure 5. Hypothetical heparin/heparan sulfate chain and site of action of different enzymatic and chemical depolymerization. ⬡ Glucosamine; ⬢ Glucuronic acid; ⬡ Iduronic acid; ● Sulfate.

These analytical tools have been applied to study the sequence of disaccharides in the fragments produced by depolymerizing enzymes and chemical methods. After incubation with a mixture of heparinase and heparitinases, heparin or heparan sulfates are totally degraded to different disaccharides that can be separated and identified. The use of a single enzyme leads to the formation of oligosaccharides with variable sizes, which in turn can be used as substrate to another treatment. So, using specific endo- and exo-glycosidases, chemical methods (nitrous acid degradation, hydrazine N-acetylation and borohydride reduction) and NMR spectroscopy it was possible to propose the sequence of disaccharides in heparin and heparan sulfate from different origins. The disaccharide distribution of some heparan sulfates and heparins from different tissues and species are summarized in Figure 6 and Table 2.

It can be concluded from these studies that heparan sulfates from both vertebrate and invertebrate cells contain common structural features such as N-acetylated and N-sulfated glucosamine domains consisting of glucuronic acid-containing disaccharides (susceptible to heparitinase I, endo-β-glucuronidase, and nitrous acid pH 4.0) and a more sulfated region consisting of iduronic acid-containing disaccharides (susceptible to heparitinase II and nitrous acid pH 1.5). A peculiar tetrasaccharide is positioned between the two regions and was identified in all heparan sulfates. It was also shown that the non-reducing ends always contain the monosaccharide glucosamine N-sulfate or glucosamine N,6-disulfate[23,53,67-70].

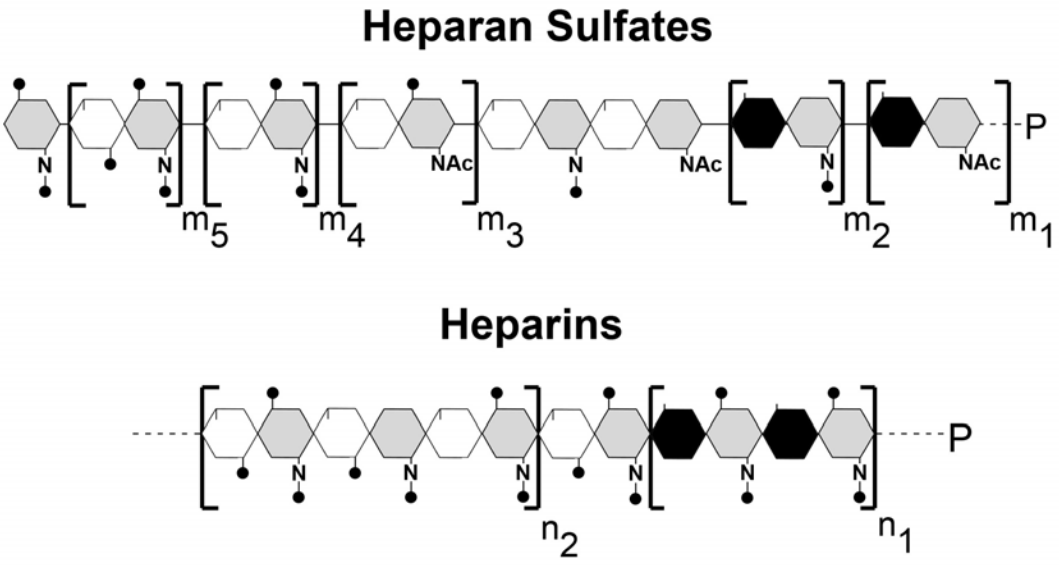

Figure 6. Proposed structure for heparan sulfate and heparin from different origins. ⬡ Glucosamine; ⬢ Glucuronic acid; ⬡ Iduronic acid; ● Sulfate; P, protein core m and n, number of building blocks. See Table 2.

Table 2. Proposed structure for heparan sulfate and heparin from different origins.

	Species/Origins		sulfate ratio[a]	number of blocks/polymer[b]						
				m_1	m_2	m_3	m_4	m_5	n_1	n_2
Heparan Sulfate[d]	Vertebratae	Bovine liver	1.1	5	3	2	2	2	-	-
		Bovine brain	1.2	7	3	6	5	3	-	-
		Bovine lung	1.2	7	4	5	5	1	-	-
		Bovine pancreas	0.6	11	4	3	4	1	-	-
		Hog liver	1.0	11	5	5	6	2	-	-
		Hog brain	0.9	4	3	2	3	1	-	-
		Dog liver	1.4	5	2	1	2	2	-	-
		Rabbit liver	1.0	9	4	2	4	3	-	-
		Chicken liver	1.1	5	3	2	2	2	-	-
		RAEC	n.d.	6	5	2	1	1	2	
	Mollusca	*Anomantidae sp.*	0.9	10	3	3		1	-	-
	Crustacea	*Artemia sp*	1.4	1	4	3	9	1	-	-
Heparin[c]	Mammalia	Bovine lung	2.8	-	-	-	-	-	1	6
		Bovine intestine	2.6	-	-	-	-	-	4	6
	Mollusca	*A.brasiliana*	2.5	-	-	-	-	-	8	3
		Donnax striatus	2.4	-	-	-	-	-	5	4
		Tivela macroides	2.3	-	-	-	-	-	5	3
	Crustacea	*P. brasiliensis*	n.d.	-	-	-	-	-	6	4
	Echinodermata	*Mellita sp*	n.d.	-	-	-	-	-	1	1

n.d., not determined; RAEC, rabbit aorta endothelial cell; *Artemia sp*., *Artemia franciscana*; *A. brasiliana*, *Anomalocardia brasiliana*; *P. brasiliensis*, *Penaeus brasiliensis*; *Mellita sp*., *Mellita quinquisperforata.*

[a] sulfate ratio, moles of sulfate/mol of disaccharide; [b] See figure 6; data from references [c] [18, 20-22, 28-29, 31, 32, 52, 53] and [d] [23, 53, 67-70].

Heparin, as heparan sulfate is also composed of two different regions, but the types and sequences of disaccharides diverge from heparan sulfate. Heparins shows a highly charged domain which is susceptible to heparinase, whose action upon the compound produces tri- and di-sulfated iduronic acid containing-disaccharides, and another less sulfated region which is susceptible to the action of heparitinase II, releasing glucuronic acid-containing disaccharides. The length and abundance of these two regions vary according to the origin of heparin [18,20-22,28-29,31,32,52,53]. The estimated abundance of the two regions in heparins from vertebrate and invertebrate is shown in Figure 6 and Table 2. Besides these disaccharides other units which occur in small amounts in the molecule have also been identified, such as disaccharides containing 3-O-sulfated residues in the glucosamine moiety as well as N-acetylated glucosamine [20,71,72].

3.3 Interaction with proteins

Glycosaminoglycans are linear polymers that exist primarily as right-handed helical structures [73]. Unlike proteins, they do no display or fold into tertiary structures. It is known that these compounds can interact with a diverse range of proteins. Furthermore, there seems to be specificity in these interactions regarding the type of glycosaminoglycan and protein. It has been shown that heparin binds to cell surface and matrix components, circulating proteins, enzymes and inhibitors, growth factors and chemokines, among others (Table 3) [74-94]. These proteins show different cellular localization and thus should modulate different biological processes, and contain relatively large numbers of the basic residues lysine and arginine, and in some cases histidine.

The molecular interactions involved in the binding have been characterized to various degrees, including identification of heparin-binding motifs in some proteins (Table 4). Cardin and Weintraub [95] after examining a series of heparin binding sequences, proposed two consensus sites XBBXBX or XBBBXXBX, where B represents basic amino acids and X, hydropathic (neutral or hydrophobic) residue. Molecular modeling studies of these consensus sequences regarding their secondary structural conformations, suggested that when XBBXBX is folded in β-strand the basic amino acids would be aligned in one face of the strand and when XBBBXXBX sequence is arranged in α-helix the basic residues would be oriented on one surface of the helix. Another consensus sequence, XBBBXXBBBXXBBX, was further proposed based on studies with vonWillebrand Factor [96] and an additional motif TXXBXXTBXXXTBB (where T defines a turn) was found to occur in heparin binding sites of growth factors [77,80]. Multiple clusters of basic amino acids in a protein that has the proper spacing should result in much stronger binding.

Several studies seriously challenged the universality of the consensus paradigma, indicating that heparin binding sites are not exclusively composed of linear sequences. Basic residues that are distant in linear sequence of the protein could be close in the folded state, assembling a cationic path that could interact specifically with heparin chains [77,79,80,102].

Nevertheless, the physiological relevance of the heparin–protein binding interactions has to be questioned, since heparin is found only in the secretory granules of mast cells. On the other hand, heparan sulfates share some structural characteristics with heparin and thus, the characterization of the motifs in the glycosaminoglycan molecule related to the binding to specific proteins is of interest. These motifs could be extrapolated to specific regions of the cell surface and matrix heparan sulfates.

Table 3. Examples of ligand associations of heparin and/or heparan sulfate.

Heparin /Heparan Sulfate Binding Proteins			
ECM	Collagens	**Cell Surface**	L-selectin and P-selectin
	Fibronectin		N-CAM (Neural Cell Adhesion Molecule)
	HB-GAM (Heparin Binding Growth Associated Molecule)		PECAM-1 (Platelet Endothelial Cell Adhesion Molecule)
	Laminin		FGF receptor
	Tenascin		HIP (Heparin/Heparan Sulfate Interaction Protein)
	Thrombospondin I and II		MAC-1 (Monocyte Adhesion Molecule)
	Vitronectin		
Serpins	Antithrombin (AT)	**Enzymes**	Angiogenin (ribonuclease with angiogenic activity)
	C1-inhibitor		Cathepsin B and G
	Cystatin		Chymase
	Heparin cofactor II (HC II)		DNA polymerase
	Kallistatin (tissue kallikrein inhibitor)		Factor Xa, IXa, IIa (thrombin)
	PEDF (Epithelial Factor)		Lipoprotein lipase
	Plasminogen activator inhibitor-1 (PAI-1)		Hepatic triglyceride lipase
	Protease nexin-1		Myosin ATPase
	Protein C inhibitor		Neutrophil elastase
	Secretory leukocyte protease inhibitor		Protein synthesis factors
	Tissue Factor Pathway Inhibitor (TFPI)		RNA polymerase
	Tissue Plasminogen Activator (t-PA)		Superoxide dismutase
Growth Factors	ECGF (Endothelial Cell Growth Factors)	**Cytokines/ Chemokines**	IL-1, -2, -3, -4, -5, -7, -8, -10, -12 (Interleukin)
	EGF family (Epidermal Growth Factors)		IP-10 (Interferon -γ inducible protein 10)
	FGFs 1 – 15 (Fibroblast Growth Factors)		CCL-2 (CC-chemokine ligand)
	VEGF (Vascular Endothelial Growth Factor)		GM-CSF (Granylocyte Macrophage Colony Stimulating Factor)
	HDGF (Hepatoma Derived Growth Factor)		MCP-1, MCP-4 (Monocyte Chemoatractant Protein)
	IGF (Insulin-like Growth Factor)		RANTES (Regulated on Activation Normal T cell Expressed and Secreted)
	PlGF (Placenta Growth Factor)		TNFα (Tumor Necrosis Factor)
	PDGF (Platelet-Derived Growth Factor)		MIP-1 (Macrophage Inflammatory Protein)
	TGF-β (Transforming Growth Factor-β)		PF-4 (Platelet factor 4)
	HGF (Hepatocyte Growth Factor)		
Pathogens	Herpes Simplex virus	**Others**	annexin V
	HIV-1		apoprotein B and E
	Influenza virus		β-amyloid protein
	Dengue virus		difteric toxins
	Malaria circumsporozoite		HGR (Histidine Rich Protein)
	Herpes simplex virus		Na^{+}/Ca^{2+} exchanger protein
	gC Pseudorabies virus		pryon

ECM, extracellular matrix; dada from references [74–94]

However, as already described (Figure 6 and Table 2) there are remarkable differences in molecular properties between these two classes of glycosaminoglycans. We will briefly review some of the structural characteristics, such as conformation of sugar residues, degree of polymerization and charge distribution that are involved in both heparin and heparan sulfate chains concerning specific protein binding.

Table 4. Heparin/heparan sulfate binding sites of some proteins.

		Amino acid binding motifs	
Cardin Sequences	***General consensus***	X **B B** X **B** X	X **B B B** X X **B** X
	Apoprotein B	^{2081}V **R K** Y **R** A ^{2119}T **K K** Y **R** I ^{3149}Y **K K** N **K** H	^{3363}T **R K R** G L **K** L
	Apoprotein E		^{140}L **R K R** L L **R** D
	ECGF/ FGF	^{8}Y **K K** P **K** L	^{93}S **K K H** A E **K** H
	gC PRV		^{76}S **R R K** P P **R** N
	Na^{+}/Ca^{+2} exchanger	^{228}Y **K R** Y **R** A	
	Fibronectin	^{5}X **R R** A **R** V ^{123}W **R R** P **H** E	
	Laminin 5	^{79}G **K K** L **R** I	
	Vitronectin	^{347}A **K K** Q **R** F	
	RANTES	^{41}T **R K** N **R** Q	
	Anexin II		F **K K K** Y G **K** S
Non-Cardin Sequences	**vWF**	^{569}**K** D **R K R** P S E L **R R** I ^{636}**R** Y V Q G L **K K K K**	
	Laminin	^{202}**R** I Q N L L **K** I T N L **R** I **K** F V **K** ^{641}P Y V V L P **R** P V C F E **K** G M N Y T V **R** ^{2010}**K** Q N C L S S **R** A S F **R** G C V **R** N L **R** L S **R**	
	Fibronectin	^{1906}Y E **K** P G S P P **R** E V V P **R** P **R** P G V ^{1946}**K** N N Q **K** S E P L I G **R K K** T	
	Collagen IV	^{342}**K** G G **R** G T P G **K** P G P **R** G Q **R** G P T G **R** G T	
	Collagen V	^{898}G G **R** G T P G **K** P G P **R** G Q **R** G P T G P **R** G E **R** G P **R** G I T	
	N-CAM	^{131}I W **K** H **K** G **R** D V I L **K K D** V **R** F I V L S	
	Thrombin	^{229}Y T H V F **R** L **K K** W I Q **K**	
	Antithrombin	^{124}A **K** L N C **R** L Y **R K** A N **K** S S **K** L V S A N **R**	
	Protein C inhibitor	^{264}S E **K** T L **R K** W L **K** M F **K K R** Q L E L Y	
	Platelet Factor 4	^{46}**K** D G **R K** I C L D L Q A P L Y **K K** I I **K K** L L E S	
	Kallistatin	^{303}N L L **R K R** N F Y **K K** L E L	
	TFPI	^{212}G **K** C **R** P F **K** Y S G C G G N E N N F T S **K** Q E C L **R** A C **K K** G	
	gC PRV	^{95}A H G **R K R** I ^{135}Y **R R** G **R** F **R**	
	HIV-TAT	G **R K K R R** Q **R R R** P P Q	
	FGF-1	^{110}G L **K K** N G S C **K R** G P **R** T H Y G Q **K** A I	
	FGF-2	^{119}L **K R** T G Q Y **K** L G S **K** T G P G Q **K**	
	TGF–β	^{23}D F **R K** D L G W **K** W I H E P **K** G Y H A	
	PDGF-A	^{194}G **R** P **R** E S G **K K R K R K R** L **K** P T	

B, basic residue; X, non-basic residue; ECGF,endothelial cell growth factor; gC PRV, gC Pseudorabies Virus; RANTES, Regulated on Activation Normal T cell Expressed and Secreted; vWF, von Willebrand factor; N-CAM, neural cell adhesion molecule; TFPI, tissue factor pathway inhibitor; FGF, fibroblast growth factor; FGF, fibroblast growth factor; TGF-β, transforming growth factor; PDGF, platelet derived growth factor. Data from references: [95-102].

With respect to heparin and heparan sulfates, the specificity seems to be related to the distribution and conformation of β-D-glucuronic acid and α-L-iduronic acid residues, relative amounts of N-acetyl or N-sulfate groups in the glucosamine moiety, as well as the relative amounts and the position of O-sulfation of the uronic acid and glucosamine units. Specific sequences of

disaccharides can favor the interaction of the molecule with certain proteins and not to others.

The conformational flexibility of the α-L-iduronic acid residue within heparin and heparan sulfate seems to be responsible for the wide range of specific protein interactions exhibited by these compounds to bind to a wide range of proteins facilitating the interaction of their anionic groups with cationic binding sites on proteins. It has been shown by NMR and X-ray analyses that the conformation of α-D-glucosamine and β-D-glucuronic acid residues within heparin and heparan sulfate assume the preferred $^4C_1$ conformation for the pyranose rings (Figure 7), whereas the conformation of the iduronic acid residue varies, depending on its location in the polysaccharide chain as well as the sulfation pattern and the substituents on adjacent glucosamine residues. Internal iduronic acid residues can assume both $^1C_4$ chair and the $^2S_0$ skew boat conformation, and at the reducing end of the chain the $^4C_1$ conformation can also be expected. It is possible for the iduronic ring to change between the $^2S_0$ and $^1C_4$ forms so that in the protein-bound state it can make favorable electrostatic interactions with basic amino acids on the protein [103-107].

Using heparin derived oligosaccharides and chemically modified molecules, the role of N-sulfated and N-acetylated domains, as well as the position of the O-sulfates, and the conformation of the uronic acid residue were investigated. Table 5 summarizes some important sequences for specific heparin/heparan sulfate-protein interactions, which are dependent mostly on

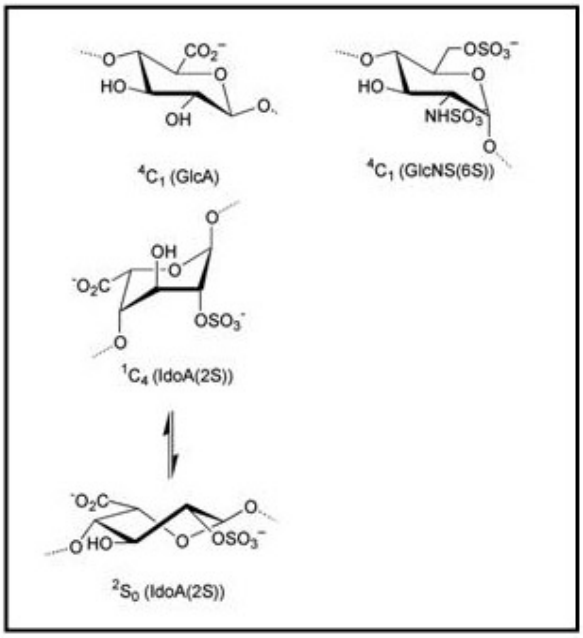

Figure 7. Conformation of the glucuronic acid, iduronic acid and glucosamine pyranose rings found in heparin and heparan sulfates.

Table 5. Characteristics of some heparin/heparan sulfate sequences for the binding to specific proteins.

Protein	Heparin/Heparan Sulfate Sequence	Ref.
AT	mininum structure: GlcNAc,6S*- GlcA- GlcNS*,3S*,6S- IdoA,2S- GlcNS*,6S (*critical for binding) GlcNS,6S*- GlcA- GlcNS*,3S*,6S- IdoA,2S- GlcNS*,6S (*critical for binding) GlcNS,6S*- GlcA- GlcNS*,3S*,6S- IdoA,2S- GlcNS*,3S^{1},6S (^{1}enhances binding)	[71,72,108]
Annexin V	minimum structure: ΔU,2S-GlcNS,6S-IdoA,2S-GlcNS,6S Calcium ions are essential for binding	[109-111]
Annexin VI	requires no N-sulfate groups, calcium independent	[109]
Apo-E	mininum structure: ΔU,2S-GlcNS,6S-IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS,6S- GlcA-GlcNS,6S	[112]
Lipoprotein lipase	(-IdoA,2S-GlcNS,6S-) rich domains	[113]
HIV-I Tat Protein	mininum structure: IdoA,2S-GlcNS-IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS,6S	[114 -116]
CCL-2	mininum structures: ΔU,2S-GlcNS-----IdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS,6S ΔU,2S-GlcNS,6S-IdoA,2S-GlcNS------IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS,6S ΔU,2S-GlcNS,6S-IdoA,2S-GlcNS,6S--IdoA,2S-GlcNS------IdoA,2S-GlcNS,6S ΔU,2S-GlcNS,6S-IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS,6S--IdoA,2S-GlcNS	[117]
PDGF-A	mininum structure: IdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S	[118]
FGF-2	mininum structures: IdoA,2S-GlcNS-IdoA,2S-GlcNS-IdoA,2S-GlcNS-IdoA,2S-GlcNS-IdoA,2S-GlcNS IdoA,2S-GlcNS-IdoA,2S-GlcNS-IdoA,2S-GlcNS HexA-GlcNS-HexA-GIcNS,6S-IdoA,2S (IdoA,2S-GlcNS)3-5	[119] [120] [121] [122,123]
FGFR-2	mininum structure: octasaccharide containing 2-O and 6-O-sulfate for binding and dodesaccharide for signaling	[122]
FGFR-4	mininum structures: GlcA-GlcNS----- IdoA,2S-GlcNS,6S- IdoA,2S-GlcNS--GlcA- AnMan GlcA-GlcNS----- IdoA,2S-GlcNS----- IdoA,2S-GlcNS--IdoA- aMan,6S GlcA-GlcNS,6S- IdoA,2S-GlcNS----- IdoA,2S-GlcNS--IdoA- aMan,6S GlcA-GlcNS,6S- IdoA,2S-GlcNS----- IdoA,2S-GlcNS--GlcA- aMan,6S	[124]
HGF	mininum structure: HexA-GlcNS (NAc)-IdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S- HexA-GlcNS(NAc),6R HexA-GlcNS(NAc),6R-HexA-GlcNS(NAc),6R-IdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S HexA-GlcNS (NAc),6R-IdoA,2S-GlcNS,6S-HexA-GlcNS (NAc),6R-IdoA,2S-GlcNS,6S Sequences rich in IdoA-GlcNS,6S and IdoA,2S-GlcNS,6S and not with HexA-GlcNAc,6S R = H or sulfate	[125] [126]

IdoA, iduronic acid; GlcA, glucuronic acid; HexA, hexuronic acid; aMan, anidromanose.

the presence of iduronic acid, as well as the sulfation of the iduronic acid, as well as the N-sulfation of the glucosamine moiety. Also, the protein interaction depends on the size of the chain, and the minimum fragment varies from a tetrasaccharide described for annexin V up to a dodecasaccharide for the FGF-2 receptor.

The structural requirements of the glycosaminoglycans differ for each protein. For instance, the heparin-derived tetrasaccharide that interacts with annexin V shows iduronic acid on the $^{2}S_{0}$ conformation while the non-interacting tetrasaccharide the $^{1}C_{4}$ conformation [109-111]. Regarding the sulfation requisites, the binding of heparin/heparan sulfate to the growth factor PDGF-A is dependent mostly on the amounts of 2-O-sulfate in the iduronic acid residues [118]. On the other hand, for CCL-2 both 2-O-sulfate in the

iduronic acid and N-sulfation of the glucosamine are required, whereas for FGF-4 receptor the affinity of the interaction depends more on the number of 6-O-sulfate groups then on their precise location [117,124]. The size of the sugar chain is an important determinant for the binding. For instance, one intact heparin (~ 12 kDa) can bind up to 4-6 molecules of Tat protein [114-116].

It is well established that fibroblast growth factors and their receptors are dependent on binding to heparan sulfate, and this interaction is an absolute requirement for full signaling. However most of the studies regarding the minimum structural features of the polysaccharide needed for the binding were concluded using chemicoenzymatically heparin derivatives as heparan sulfate analogs. Even though these heparin derivatives can be produced in high amounts, and thus can be used to elucidate some of the binding characteristics, they do not substitute the high diversity and thus the specificity that is found in the heparan sulfate polymers [127-135].

However, heparan sulfates show large sequences of glucuronic acid linked to N-acetylated glucosamine which are not present in heparins. Also, heparan sulfates show lower degree of sulfation, even in the iduronic acid residue, requisites that are described as important for the protein binding. So, this raises questions on how the binding could be affected by these domains in the heparan sulfate chains, which are the postulated polysaccharide for most of these biological interactions.

The structural requirements for heparan sulfate-protein interactions can be illustrated by experiments using FGF-1 and heparan sulfates from various sources that exhibit different disaccharides assembly (Table 6 and Figure 8) [136]. The mitogenic activity of FGF-1 is potentiated by the highly sulfated hexasaccharide derived from heparin. On a mass basis, the effect of both heparin and oligosaccharide are equivalent whereas on a molar basis, heparin, which contains about seven hexasaccharide repeats, is more efficient. On the other hand, a pentasulfated tetrasaccharide or di- and tri-sulfated disaccharides show negligible effect. This *in vivo* mitogenic activity using 3T3 cells in culture is substantiated by *in vitro* assays regarding conformation stability of FGF-1.

Heparan sulfates extracted from various mammalian tissues were also able to potentiate FGF-1 mitogenic activity. On a molar basis these compounds were less efficient than intact heparin. Nevertheless, when comparing the intact heparan sulfates with the heparin-derived hexasaccharide, there is an increase from 2 to 5 times in the mitogenic effect obtained with heparan sulfates. The glucuronic acid domain can be removed from heparan sulfates after digestion with heparitinase I (see scheme on Figure 6 and Table 2). The iduronic acid rich derived oligosaccharide is more active than the intact molecule, reaching a potentiating effect equivalent to that of heparin, whereas an N-acetylated oligosaccharide isolated after nitrous acid degradation is inactive. These data suggest that the mitogenic activity of FGF-1 is primarily potentiated by

Table 6. Concentration of heparin, heparan sulfate and derived oligosaccharides necessary to potentiate 50% (ED_{50}) of the maximum mitogenic activity of FGF-1

	ORIGIN	ED_{50} (nM)
HEPARIN	bovine lung	
	intact molecule	20
	hexasaccharide [a] ΔIdoA,2S-GlcNS,6S-IdoA,2S-GlcNS,6S-GlcA-GlcNS,6S	225
	tetrasaccharide [a] ΔIdoA,2S-GlcNS,6S-GlcA-GlcNS,6S	>1,000
	disaccharide [a] ΔIdoA,2S-GlcNS,6S	>1,000
HEPARAN SULFATE	3T3 cells	<4
	bovine liver	11
	bovine lung	40
	rabbit liver	110
	bovine spleen	105
	bovine pancreas	
	intact molecule	100
	oligosaccharide I [b] GlcNS,6S-IdoA,2S-GlcNS,6S-$(IdoA-GlcNS,6S)_4$-$(IdoA-GlcNAc,6S)_3$-Ido-GlcNAc-IdoA-GlcNS	<15
	oligosaccharide II [b] ΔGlcA-GlcNS-$(GlcA-GlcNS)_3$-$(GlcA-GlcNAc)_{11}$	>1,000

[a] hexa-, tetra- and di-saccharides were obtained after incubation of heparin with heparinase. [b] Oligosaccharides I and II, oligosaccharides obtained after digestion of heparan sulfate from bovine pancreas with heparitinase I and nitrous acid treatment pH 2.0, respectively. Data modified from ref. [136]. See figure 8.

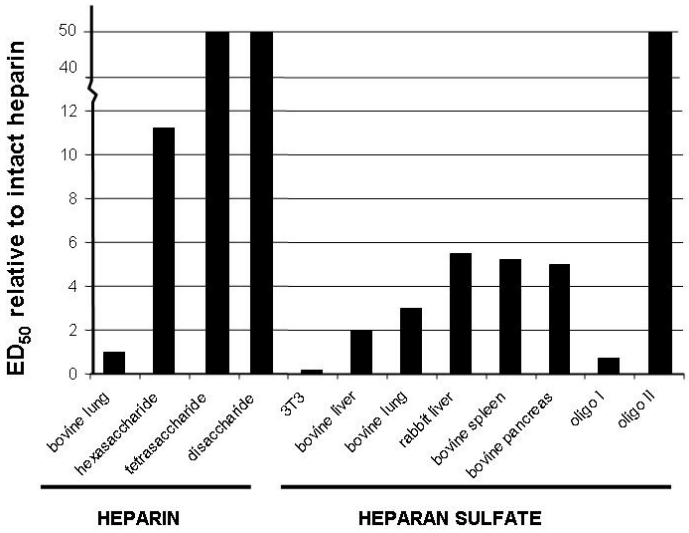

Figure 8. ED_{50} relative to heparin of the maximum mitogenic activity of FGF-1 elicited by different compounds using 3T3 cells. Hexa-, tetra- and di-saccharides were obtained after incubation of heparin with heparinase; oligo I and II, oligosaccharides obtained after digestion of heparan sulfate from bovine pancreas with heparitinase I and nitrous acid treatment pH 2.0, respectively. See also Table 6. Data from reference [136].

interacting with highly sulfated regions of heparan sulfates chains. Also, it can be concluded that the glucuronidic domain in heparan sulfates hampers the active oligosaccharide domain to interact with the protein.

Furthermore, heparan sulfate purified from 3T3 fibroblasts were 5 times more efficient than heparin on a molar basis when tested for mitogenic activity upon 3T3 cells. This result indicates that endogenous heparan sulfate is the best elicitor for the FGF-1 mitogenic activity, indicating a highly specific interaction.

4. Concluding remarks

The physical properties of heparin and heparan sulfate derive from its primary chemical structure, conformation, chain flexibility, molecular weight and high charge density. The first X-ray diffraction of heparin was obtained in the early 70's [73] and helped to show the helical conformation of the glycosaminoglycan chain. Since then, there has been a great advance in NMR spectroscopy, X-ray diffraction, mass spectroscopy, among other physical methods, that has helped the understanding of both sugar chain and protein conformation, besides the refinement in the enzymatic and chemical methods used to analyzed the compounds [133, 137-141].

It is important to take into consideration that heparin, and specially heparan sulfate are very heterogenous polysaccharides, even though their structure regarding the sequence of disaccharides is highly conserved for the each cell type under the same physiological condition. This implicates that the biosynthesis of these complex polysaccharides is under strict enzymatic control. So, to really understand the role of heparan sulfate in protein modulation, one has to take into account the origin of both polysaccharide and protein target.

Nevertheless, the data accumulated regarding synthetic oligosaccharides as well as chemically modified fragments from heparin can indicate some of the characteristics necessary for the binding, bringing some important hints in this complex scenario.

Recently, molecular dynamics studies of the interaction between specific carbohydrate sequences are being used to predict the induced fit into the protein, using the collected data available in the literature. Also, the specific contribution of important amino acid residues to the overall process can be characterized [142-145]. Altogether, molecular dynamics experiments could be used to characterize and quantify specific interactions of the sugar with the protein, and thus help on the design of compounds with pharmacological interest.

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2 Heparin and heparin derivatives and their effect on hemostasis

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Abstract

The control of blood flow is dependent on the activation or inhibition of several factors, related to different proteolytic cascades such as coagulation, fibrinolysis and inflammation. The mechanisms of these complex physiological pathways can lead to the understanding of cardiovascular disorders, thrombosis, and neoplasia, among others. The search for compounds

In honor of Prof. Carl P. Dietrich

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that may play a role in the prevention or treatment of these diseases has been the purpose of several investigations. Each individual sulfated glycosaminoglycan has a unique structure and this reflects in the modulation of their biological activity. Heparin is a highly sulfated glycosaminoglycan that possesses a wide range of different pharmacological activities. Heparin and low molecular weight heparins still is the therapeutic drug of choice for the management of thrombotic disorders, despite the recent design and development of putative single target drugs. The chapter will review the structural characteristics involved in the effect on various systems involved in hemostasis.

1. Introduction

The control of blood flow involves several coordinated physiological events that when disrupted can lead to bleeding or thrombosis. The equilibrium is dependent on the activation or inhibition of several factors, related to different proteolytic cascades such as coagulation, fibrinolysis and inflammation. It is clear that cells from the vessel wall, endothelial and smooth muscle cells, as well as blood cells, platelets and leukocytes, play important roles in this process (Figure 1). It should also be mentioned that after an injury, the normal healing depends on tissue repair, and thus of growth factors to control cell proliferation. The mechanisms of these complex physiological pathways can lead to the understanding of cardiovascular disorders, thrombosis, and neoplasia, among others.

The search for compounds that may play a role in the prevention or treatment of these diseases has been the purpose of several investigations. Heparin was discovered by Howell and Holt in 1918 [1] as an anticoagulant compound. It was first used in Medicine to prevent blood clot during extracorporeal circulation [2]. It was introduced in clinical use in the 1940s to prevent thrombosis in

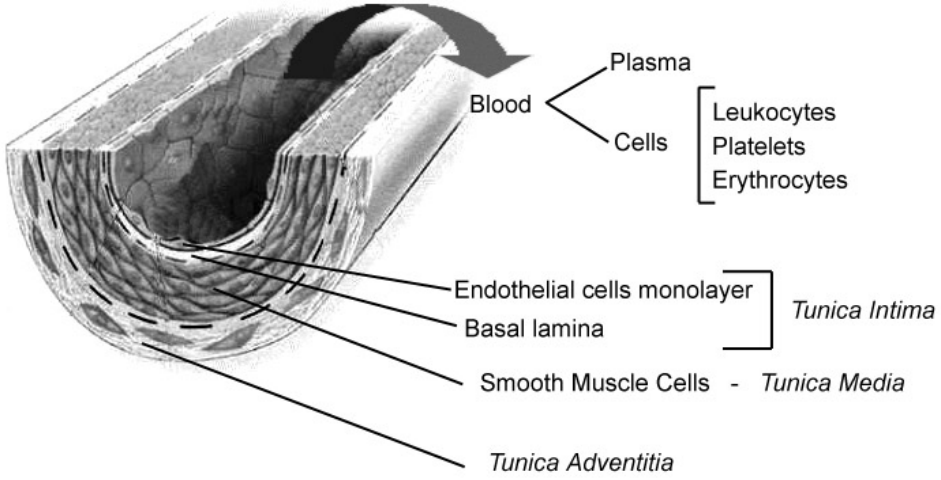

Figure 1. Possible sites of action for antithrombotic compounds.

surgical patients [3] and in the treatment of deep venous thrombosis [4,5]. Since then, its use has increased extensively in these and other clinical situations. Heparin still remains the therapeutic drug of choice for the management of thrombotic disorders, despite the recent design and development of putative single target drugs.

This chapter reviews some structural characteristics of heparin and heparin derivatives that are related to effects in the different underlying processes involved in the control of normal blood flow.

2. Hemostasis

Normal blood flow implies in the existence of a precise equilibrium of several interrelated physiological processes such as the balance of coagulation and fibrinolysis, and their natural inhibitors, inflammation, integrity of the endothelial cell monolayer, activation and inhibition of platelets and leukocytes, besides muscle contraction mechanisms.

The process of blood clotting and the subsequent dissolution of the clot, following repair of the injured tissue, is termed hemostasis, and is composed of at least 5 important events that occur in sequence following the rupture of vascular integrity:

- ✓ vascular constriction: limits the blood flow to the area of injury;
- ✓ formation of platelet plug: platelet adhesion on the sub-endothelial matrix components, and platelet aggregation at the site of injury;
- ✓ formation of clot: a fibrin mesh forms and entraps blood cells and the platelet plug;
- ✓ breakdown of the clot: dissolution of the fibrin network (fibrinolysis) in order to ensure normal blood flow;
- ✓ tissue repair: healing and cell growth.

It is important to emphasize that the endothelial cells that line the lumen of the vessel wall exhibit blood compatibility, which is they are antithrombotic in nature. After an injury, factors are exposed that trigger the coagulation system. As already mentioned, initially platelets adhere to extracellular matrix components, such as collagen and fibronectin, changing their morphology and releasing the content of the granules which induce vasoconstriction as well as adhesion and aggregation of more platelets, through the interaction with von Willebrand factor.

The serine proteases, cofactors and cell-receptor molecules that comprise the haemostatic mechanism are highly conserved modular proteins that have evolved to participate in biochemical reactions in blood coagulation, anticoagulation and fibrinolysis.

The goal of the cascade is to convert fibrinogen into fibrin to form a mesh within the platelet aggregate to stabilize the clot. This process occurs by way of amplifying a series of enzymatic reactions in which the product of each reaction converts an inactive plasma protein into an active proteinase product. So, factors circulate in an inactive form, either as zymogens (e.g. XII, XIII, II, etc) or profactors (V and VIII), which are activated by limited proteolysis. These linked reactions provide dramatic amplification and a rapid fibrin formation at the site of vascular injury. A diagram of these reactions is depicted in Figure 2.

The formation of a fibrin clot depends of both the intrinsic and the extrinsic pathways, which are independent, but not redundant and highly interconnected, and initiated by distinct mechanisms, that converges on a common pathway leading to clot formation.

Tissue factor also known as Factor III is the principal initiator of the coagulation cascade. It is a glycoprotein expressed in all cells, but endothelial and blood cells. Tissue factor is expressed in response to injury as well as to number extracellular stimuli, including LPS, TFN-α, IL-1, -2, -6, Interferon γ, lipoprotein, among others [6,7]. The damage of blood vessel walls exposes to

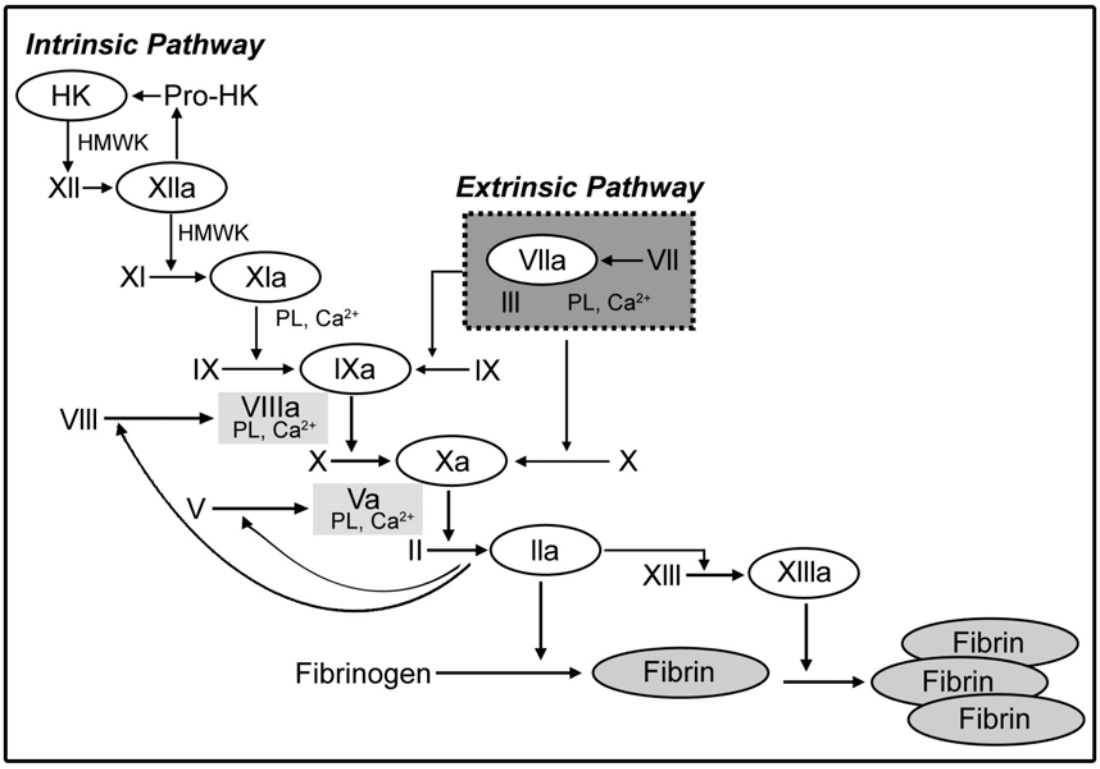

Figure 2. Coagulation cascade. II to XIII, different zymogens and pro-factors; IIa to XIIIa, different activated factors; III, tissue factor; HK, kallikrein; HMWK, high molecular weight kininogen; PL, phospholipids (phosphatidyl serine and phosphatidyl ethanolamine).

the blood tissue factor from the underlying cell layers which in turn is able to bind Factor VII in the presence of calcium ions and phospholipids, setting off the extracellular cascade (extrinsic pathway) for fibrin formation. The maintenance of the clot formation is due to the intrinsic pathway, initiated by contact activation, and leading to thrombin generation after a series of sequential steps. Both pathways convert to the formation of the prothrombinase complex, that integrates factors Xa and Va, prothrombin (Factor II), calcium and phospholipids to generate thrombin (Factor IIa) which in turn is going to cleave fibrinogen to yield fibrin.

Besides the reactions detailed on the diagram depicted in Figure 2, there are multiple other interactions for coagulation to work properly. Interaction with components of the fibrinolytic and inflammatory cascades, platelet aggregation and cell growth system are well described in the literature and relevant to hemostasis regulation.

The natural inhibitors of the coagulation cascade play a pivotal role in the control of hemostasis. This control can be exerted by the specific inhibition of the serine proteinases that are formed in the cascade, as well as by the degradation of factors. Serpins such as antithrombin (AT), heparin cofactor II (HC II) and tissue factor pathway inhibitor (TFPI) belong to the first class of compounds, whereas plasmin and PCa are involved in the degradation of activated factors (Figure 3).

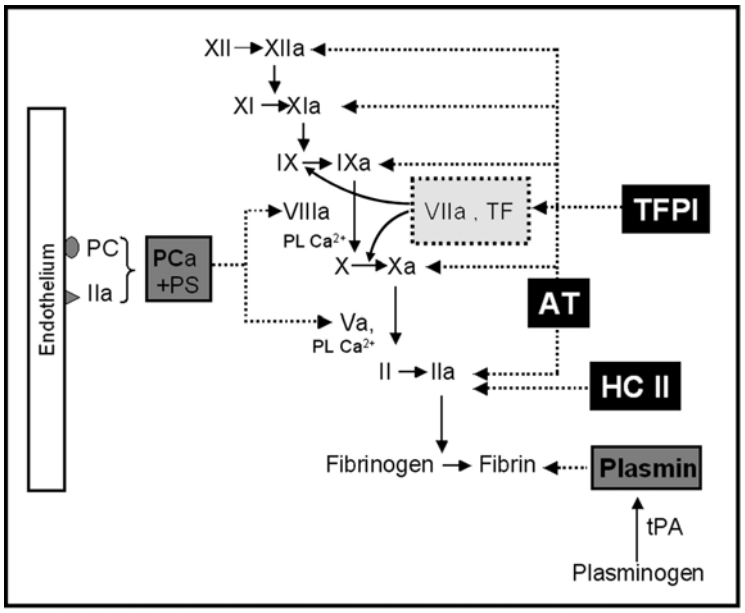

Figure 3. Natural inhibitors of the coagulation cascade. II to XII, different zymogens and pro-factors; IIa to XIIa, different activated factors; TF, tissue factor or Factor III; AT, antithrombin, HC II, heparin cofactor II; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; PC, protein C; PS, protein S. ▲, thrombomodulin (thrombin receptor). l, protein C receptor.

AT inhibits all serine proteinases of the coagulation cascade, whereas HC II is a specific inhibitor of thrombin. Both inhibitors form covalent complex with the enzyme. TFPI is a specific inhibitor of the extrinsic pathway forming a ternary complex with Factor III/Factor VIIa [8-10].

PC is a circulating zymogen of the serine proteinase PCa. Endothelial cells express receptors for PC and thrombin (thrombomodulin). When both thrombin and PC bind to their specific receptors, PC is activated by limited proteolysis. The formed PCa interacts with its cofactor, protein S (PS), and the complex inactivates Factors Va and VIIIa again by limited proteolysis, and in these conditions arresting the coagulation process. PCa is cleared from the circulation by α2-macroglobulin, α1-antitrypsin, and protein C inhibitor [11,12].

Fibrinolysis controls fibrin deposition. In the presence of fibrin, plasminogen is activated by limited proteolysis by tissue plasminogen activator (tPA) yielding plasmin that degrades the fibrin network. tPA is inhibited by plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2). Contrary to the enzymes involved in the coagulation, plasmin has a very broad specificity of action and can degrade besides fibrin, fibrinogen and other proteins, and its activity is under surveillance of inhibitors as α2-antiplasmin [13].

It is becoming more evident nowadays the cross-talk between blood coagulation and inflammation. Tissue factor has an important role in inflammation since the initiation of extrinsic pathway can trigger an intracellular inflammation signaling pathway. The activated coagulation factors FVIIa, FXa and FIIa are proinflammatory, capable of inducing an inflammatory state via proteinase-activated receptors (PAR) found on the surface of several cell types. The activation of PAR receptor can induce the expression of a variety of inflammatory molecules, such as, interleukins, adhesion molecules, and growth factors, among others. Furthermore, inflammation can promote coagulation through a feed-back on the expression of tissue factor by increasing its expression at the expenses of nuclear transcriptor factors, such as NFκB [14-16].

Plasma prekallikrein (Pro-KK) is a protein that circulates in blood as the zymogen of kallikrein (HK). It may be involved in several physiological pathways such as activation of factor XII [17] and plasminogen by direct cleavage of the zymogen [18] or by activation of pro-urokinase [19]. Kallikrein plays a key role in intrinsic blood clotting, in the kallikrein-kinin system, and in fibrinolysis. The action of HK on kininogen (HMWK) results in bradykinin release, which is a potent mediator of inflammatory responses [20].

The protein C pathway can be down-regulated by inflammatory mediators, oxidants and proteases from leukocytes. The pathway also exhibits anti-inflammatory activity, probably mediated by partially preventing NFκB nuclear translocation, and by blocking both leukocyte adhesion and activation [11,12].

Normal endothelial cells control shear stress, prevent leukocyte and platelet adhesion, synthesize NO that promotes vasodilatation, promote fibrinolysis due to the synthesis of tPA and PAI-1, synthesize and secrete antithrombotic compounds such as TFPI and heparan sulfate [21-25].

Furthermore, the blood flow in veins is slower when compared to arteries. Thus, the venous thrombi are composed mainly of fibrin aggregates with trapped red blood cells whereas arterial thrombi consist mainly of platelet aggregates held together by fibrin. So, the drugs used to prevent or treat arterial and venous thrombosis differ accordingly, even though anticoagulant drugs can be used in both cases.

Among the anticoagulant drugs used to prevent thrombosis, unfractionated heparins and low molecular weight heparins are the drug of choice, despite the recent design and development of putative single target drugs that act at specific sites of hemostasia. Due to the special pattern of negative charge distribution, heparin can interact and modulate the activity of some specific proteins, many of them involved in hemostasis. Figure 4 depicts some of the proteins related to hemostasis that are modulated specifically by the binding to heparin.

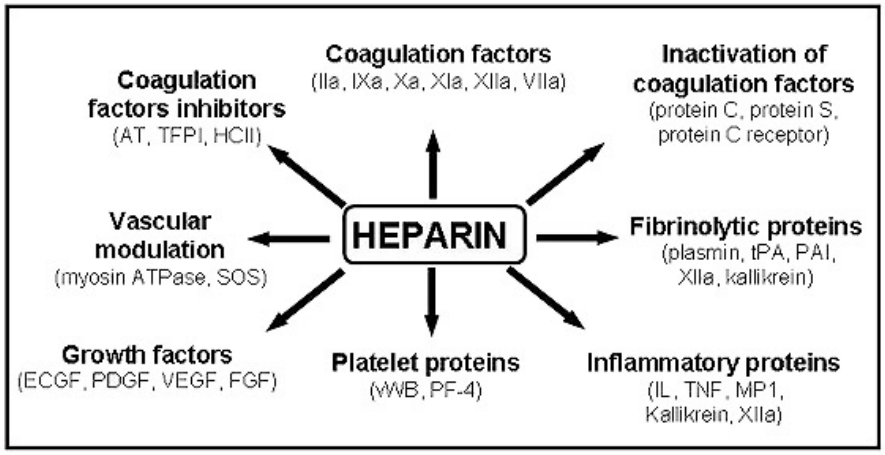

Figure 4. Proteins involved in hemostasis and related processes that can be modulated by the binding to heparin. Abbreviations as in Figs. 2 and 3; vWF, von Willebrand factor; PF4, platelet factor 4; ECGF, endothelial cell growth factor; PDGF, platelet derived growth factor; VEGF; vascular endothelial growth factor; FGF, fibroblast growth factor; IL, interleukins; TNF, tumor necrosis factor; SOS, superoxide desmutase. See references [26-44].

3. Heparin

Heparin, as described in the previous chapter is a highly sulfated glycosaminoglycan located exclusively in granules of mast cells. The use of several techniques for defining heparin became extremely important in view of the findings that heparin preparations possess a wide range of different

pharmacological activities. The advance of the chemistry and the increasing knowledge of the physicochemical properties of heparin have allowed it to be distinguished from other sulfated glycosaminoglycans. This identification has been based on a number of parameters including anticoagulant activity.

Among the different physicochemical and chemical characteristics used to distinguish heparin from other glycosaminoglycans it can be mentioned: 1) all heparins show the highest interaction with different diamines which in turn reflects in the slowest electrophoretic migration using these buffers [45,46]; 2) heparins are the only sulfated glycosaminoglycans that can be precipitated according to the pH by the use of anfolites [47-49]; 3) all heparins, regardless of the molecular size are insoluble at 2 M potassium acetate pH 5.7 at 4^{o} C [50]; 4) heparins form insoluble barium salts, and can be fractionated using barium acetate at different temperatures, that is, the highest molecular weight fractions are insoluble at room temperature, whereas the lowest ones precipitate only at 4^{o} C [51,52]; 5) heparins can be easily precipitated from low-ionic strength aqueous solution by addition of small volumes of solvents, such as ethanol and acetone (0.4 to 0.7 volumes) [53,54]; 6) among the glycosaminoglycans, heparin and heparan sulfate show positive optical rotation [55]; 7) characteristic signals in the NMR spectra; 8) degradation products after incubation with different lyases; 9) relative amounts of α-L-iduronic/β-D-glucuronic acid residues and total amount of sulfate esters; 10) distribution of the different types of disaccharides along the polymer chain (for itens 7 to 10, see previous chapter).

Heparin as other sulfated glycosaminoglycans is a polydisperse compound. Fractions with different molecular weights obtained from the same commercial heparin preparations with an average molecular weight of 15 kDa have not shown striking differences in chemical composition. Hence the polydispersity of heparin could only be due to the number of disaccharide repeating units of the various individual molecules, and about 25 molecular species should be present in heparin with molecular weights ranging from 5 to 20 kDa.

It has been shown that commercial heparin preparations when submitted to electrofocusing (Figure 5A), which involves a pH gradient, are divided into different fractions that differ among each other regarding the molecular weight, that varies from 1,800 daltons (pH 3.0), that corresponds to the molecular mass of a heparin derived hexasaccharide, up to 37,500 daltons (pH 4.5), that corresponds to around 20 repetitions of the hexasaccharide unit [47-48,54,56-57]. Each of these fractions where tested for *in vitro* anticoagulant activity and *in vivo* antithrombotic activity. The fractions with high molecular weight showed anticoagulant activities up to 300 IU/mg, that is, 2 times the activity of the original unfractionated heparin (150 IU/mg). There is an inverse relation between molecular weight and anticoagulant activity. Another very important observation was the fact that the *in vivo* antithrombotic activity is

not dependent on the molecular weight of the fractions. Thus, low- and high-molecular weight fractions that differ dramatically regarding anticoagulant activities, show similar antithrombotic action. Nevertheless, the yield of these fractions showing high antithrombotic activity and low anticoagulant activity is around 15% in commercial heparin preparations which would then make the procedure to isolate these fractions not economically viable. This was the first clear evidence that anticoagulant and antithrombotic activities are dependent of different requirements regarding molecular size and reflect different actions, and the results *in vitro* do not necessarily reflect the *in vivo* effect.

Since heparin is an exclusive polyanion we have to assume that heparin species are complexing with ampholines and precipitating at different pHs in function of their molecular sizes. The pattern produced when heparin is subjected to electrofocusing is not duplicated by any other glycosaminoglycan. This technique is specific and reproducible and unequivocally distinguishes heparin from other glycosaminoglycans. The fractionation is dependent upon

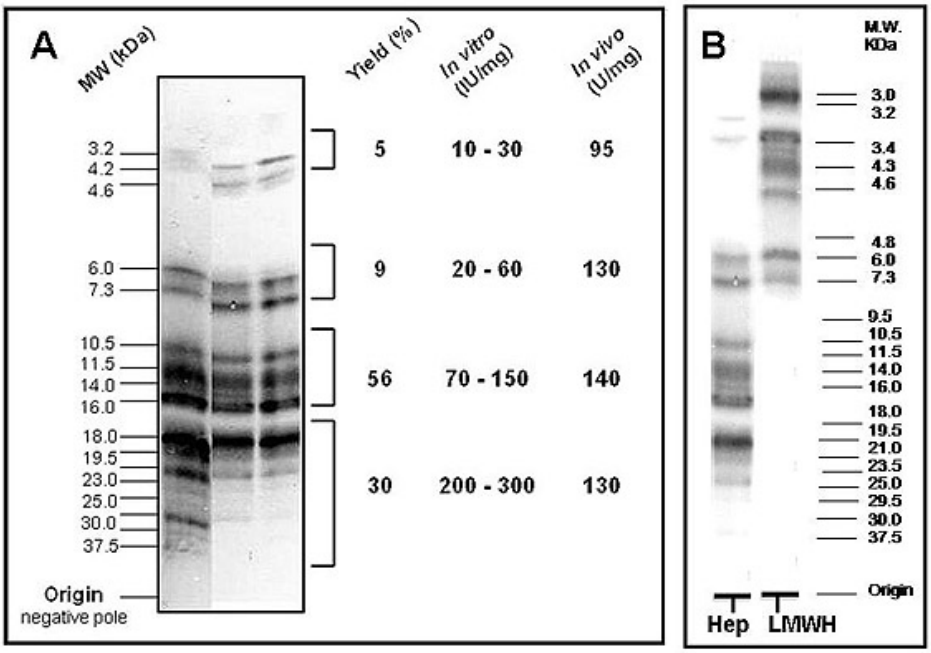

Figure 5. Fractionation of different molecular species in heparin by focalization. **A**- Three different heparin commercial preparations were applied to polyacrylamide gel slabs containing 2% ampholyte (pH 3.0 to 5.0) and submitted to a potential of 15V/cm for 18 hours. The origin corresponds to the negative pole. After electrofocalization, the gel containing the visible precipitated fractions was cut and eluted. Molecular weight, *in vitro* anticoagulant activity (USP), *in vivo* antithrombotic activity (ligature of the venous cava in rats) and yields of the fractions were determined. In another set of experiments, the gel was stained with toluidine blue, as shown in the figure. **B**- Focalization of unfractionated heparin (Hep) and the low molecular weight heparin (LMWH) prepared by the Fenton reaction.

the structure of the fractions, since N-and O-desulfation of the polymer markedly changed the focalization pattern. This implies that a special arrangement of some of the sulfate groups of the heparin molecule has to exist in order to bind with the ampholines [47-48,54,56-57].

These results led to the search for methods to depolymerize heparin. The first methodology described in the literature is based on the Fenton reaction. The method consists in producing free radicals using H_2O_2 and Cu^{2+} as catalyst [49,58-60]. The heparin molecules are fragmented in the non-sulfated glucuronic acid containing regions yielding low molecular weight heparin (Figure 6). When unfractionated heparin and the LMWH produced by this procedure are analyzed by focalization it is clear that the medium and high molecular mass fractions were depolymerized to small products (Fig. 5B). The yield of this procedure was around 60 to 70%. This protocol was later on patented by some pharmaceutical companies and corresponds to the low molecular weight heparins known as ardeparin and parnaparin.

In the early eighties, it was also shown that LMWHs could be prepared upon degradation with bacterial enzymes from *Flavobacterium heparinum*, such as heparinase and heparitinase II, that act upon different regions of the heparin molecule [58-60]. Heparinase recognizes the iduronic acid 2-O-sulfated portion and heparitinase II the non-sulfated glucuronic acid region of the molecule, as described in detail in the previous chapter. Upon these procedures, LMWHs were also produced in high yields. This methodology corresponds to the now commercially available LMWHs named tinzaparin (Figure 6).

Nowadays various laboratories prepare low molecular weight heparins by different procedures. Besides the ones described above, other methods are employed for depolymerization, such as controlled nitrous acid degradation

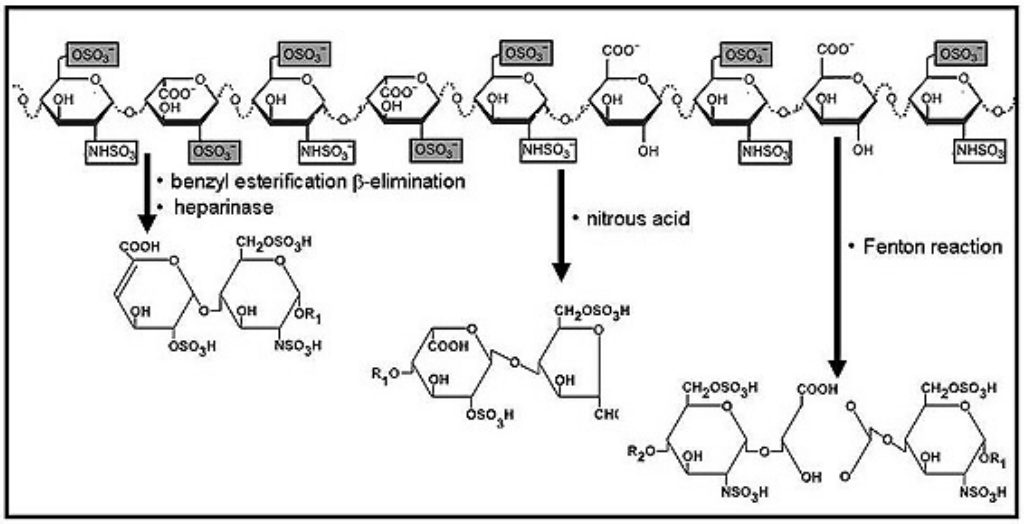

Figure 6. Low molecular weight heparins: depolymerization processes and chemical characteristics.

(dalteparin, nadroparin and reviparin), isoamyl nitrite (certoparin) and benzylation followed by alkaline hydrolysis (enoxaparin). Table 1 and Figure 6 list the different depolymerization processes and the main chemical characteristics that distinguish the low molecular weight heparins commercially available.

A comparative study of three different commercially available low molecular weight heparins has shown that exoparin exhibits the lowest molecular mass around 4,500 daltons followed by nadroparin (5,500 Da) and dalteparin (6,400 Da). The molecular weight of different LMWHs and the anticoagulant activity measured by the USP method is shown in Table 2. It can be noticed that most of the molecules are in the range of 2.5 to 7.5 kDa. Regarding enoxaparin, it

Table 1. Low molecular weight heparins (LMWHs): depolymerization processes and chemical characteristics.

LMWH	Process	Chemical change
dalteparin, nadroparin, reviparin	nitrous acid depolymerization	2,5-anhydro-D-mannose at the reducing end
certoparin	isoamyl nitrite depolymerization	2,5-anhydro-D-mannose at the reducing end
enoxaparin	benzylation followed by β-elimination	4,5-unsaturated uronic acid at the non-reducing end
tinzaparin	heparinase digestion by β-elimination cleavage	4,5-unsaturated uronic acid at the non-reducing end
ardeparin, parnaparin	peroxidative cleavage	labile glycosidic bonds

Table 2. Anticoagulant activity and molecular weight of LMWHs.

Heparin	%[a]	%[b]	Average molecular weight (kDa)	Anticoagulant activity (IU/mg)
Enoxaparin	86	-	4.5	54
peak 1		11	2.9	-
peak 2		21	3.9	-
peak 3		33	4.8	-
peak 4		35	5.6	-
Nadroparin	85	-	5.5	46
Dalteparin	73	-	6.4	75
Reviparin	68	-	3.4	48
Tinzaparin	61	-	4.8	68
Certoparin	86	-	6.3	55
Ardeparin	62	-	6.5	72
Parnaparin	-	-	7.2	84
Unfractionated	14	-	15.0	166

[a] indicates the percentage of molecular species that are comprised in the range 2.5-7.5 kDa [61-63]; [b]from reference [64].

was observed the presence of at least four major peaks with distinct molecular weights. Furthermore, analysis of the same commercial enoxaparin shows that the relative distribution of the fractions varies according to the different batches (Figure 7).

The structural difference among these LMWHs can be clearly shown by the degradation with heparinase and heparitinase II. It can be noted that enoxaparin and unfractionated heparin are degraded by heparitinase II, whereas the other two LMWHs are not substrate for this enzyme (Table 3).

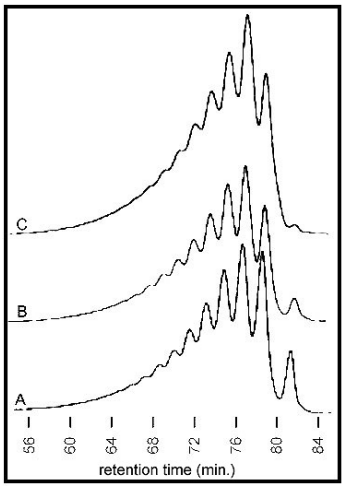

Figure 7. Distribution of molecular weight fractions in different batches of the same low molecular weight heparin. Three different batches (A, B, and C) of enoxaparin were fractionated by molecular sieving using HPLC.

Table 3. Degradation products produced by heparinase and heparitinase II upon some LMWHs.

Enzyme[a]	Products[b]	%			
		Dalteparin	**Nadroparin**	**Enoxaparin**	**UFH**
heparinase	tetrasaccharide	21	15	39	29
	ΔU,2S-GlcNS,6S	50	55	46	61
	ΔU,2S-GlcNS	23	26	5	8
	GlcNS,6S	6	4	10	2
heparitinase II	oligosaccharides	41	52	15	10
	tetrasaccharide	22	20	5	-
	ΔU,2S-GlcNS,6S	22	20	56	60
	ΔU-GlcNS,6S	15	8	24	30

[a]For details of specificity of the enzymes see the previous chapter. [b]Measured by absorbance at 230 nm and silver nitrate staining; UFH, unfractionated heparin; D, insaturation.

ΔU,2S-GlcNS,6S: O-(enepyranosyluronic acid-2-O-sulfate)-(1→4)-2-sulfoamino-2-deoxy-D-glucose 6-O-sulfate; ΔU,2S-GlcNS: O-(enepyranosyluronic acid-2-O-sulfate)-(1→4)-2-sulfoamino-2-deoxy-D-glucose; GlcNS,6S: 2-sulfoamino-2-deoxy-D-glucose 6-O-sulfate; ΔU-GlcNS,6S: O-(enepyranosyluronic acid)-(1→4)-2-sulfoamino-2-deoxy-D-glucose 6-O-sulfate; ΔU: 4,5-unsaturated uronic acid.

These and other data show differences among these LMWHs. Whereas enoxaparin seems to maintain some of the structural characteristics of the unfractionated heparin, the other two LMWHS due to the chemical depolymerization procedure have lost the non-sulfated glucuronic acid containing region, and thus the susceptibitility to heparitinase II [64].

The LMWHs differ among each other and from unfractionated heparin regarding molecular masses, chemical structure, sequence of disaccharides and their reducing and non-reducing ends. These differences reflect in their biological *in vitro* and *in vivo* activities (Table 4).

Table 4. Pharmacological *in vitro* and *in vivo* activities of different LMWHs.

COMPOUND	*In vitro* assays				*In vivo* assays			
	aPTT	aXa	Ratio aXa: aIIa	Protamine neutralization	EC	TFPI	Bioavai-lability	Bleeding
UF-Heparin	153	180	1	1.0 (1.0)	1.6	4.3	1.0	1.0 (1.0) [1.0]
LMW-Heparin								
enoxaparin	54	100	2 - 4	0.3 (1.1)	2.2	3.6	4.6	0.4 (0.5) [1.7]
tinzaparin	78	92	2 - 3	0.6 (0.8)	2.0	4.4	3.3	0.5 (0.8) [1.6]
nadroparin	45	100	2 - 4	0.3 (1.0)	1.7	3.4	4.3	0.4 (0.5) [1.9]
dalteparin	75	150	2 - 4	0.5 (1.1)	1.8	3.3	2.9	0.6 (0.8) [1.0]
reviparin	48	108	3 - 5	0.3 (1.0)	1.7	4.2	4.2	0.5 (0.5) [1.3]
certoparin	54	92	-	0.4 (1.1)	1.9	3.9	4.7	0.4 (0.7) [1.5]
parnaparin	84	85	3.0	0.7 (1.1)	-	-	2.6	0.4 (0.7) [1.1]
ardeparin	72	60	2.0	0.7 (1.1)	1.8		2.5	- (0.8) [0.9]

aPTT, as IU/mg; **aXa**, measured by an amidolytic anti-Xa method [65-68]; **protamine neutralization** of anti-Xa and anti-IIa (numbers between parenthesis) expressed relative to heparin (100% neutralization); each LMWH was supplemented into human plasma at a final concentration of 5 μg/ml and 5 μg/ml of protamine and the residual anti-Xa and anti-IIa activity determined by amidolytic assay [65-68]; **EC**, increase in the expression of the antithrombotic heparan sulfate from endothelial cells [69, 70], the numbers are expressed relative to control in the absence of the compound; **TFPI**, measured as Ag (ng/ml) and expressed relative to the values in the absence of compound (saline); **bioavailability**, measured by amidolytic anti-Xa assay (500 UI/kg sc in primates) [65-68] and values are expressed relative to unfractionated heparin; **bleeding**, expressed relative to heparin in the rat tail model (topical application of 400 μg/ml of the compound) [65-68]; for the number between parenthesis the experiment was performed after intravenous injection (1.0mg/ml) and between brackets after subcutaneous injection (2.5 mg/kg) using the rabbit blood loss model [65-68].

The different low-molecular-weight heparins due to the type of depolymerization process show specific molecular and structural attributes. Furthermore, even considering the same basic procedure of depolymerization, it is important to point out that the conditions vary according to the manufacture, so the final product will display specific characteristics that may contribute to their own unique biochemical and pharmacological profiles. The various low molecular weight heparins show a markedly decreased in aPTT

and anti-IIa activities and a slight decrease in anti-Xa activity when compared to unfractionated heparin. Thus, the anti-Xa/anti-IIa ratio is around 1 for unfractionated heparin, whereas for LMWHs it varies from 2 to 5. Protamine is the usual procedure to neutralize heparin activity *in vivo*. Studies have shown that for LMWHs this procedure is less effective, probably due to the lower binding of protamine to small molecules. LMWHs produce less bleeding than unfractionated heparin when tested after topical or endovenous administration, but they cause similar or even higher blood loss after a subcutaneous injection. The effect of LMWHs when measured by anti-Xa *ex vivo* activity is more durable than UFH and differs among the various compounds. On the other hand, the release of TPPI and the increase in the expression of endothelial cells heparan sulfate is similar for all heparins. These pharmacological *in vitro* and *in vivo* activities are shown in Table 4.

The LMWHs are administered as anti-Xa units which certainly do not reflect the differences in structure and relative amounts of fractions. Since, heparin and heparin derived fragments interact differently with various proteins it would be unlike that they would fulfill the same net biological effect.

Each LMWH has a characteristic pharmacokinetic and pharmacodynamic profile and cannot be considered bioequivalent either at doses recommended for prophylaxis or at anti-Xa adjusted doses. The unique features of the different LMWHs alert us that they are not the same drug and thus they are non interchangeable.

Heparin and low molecular weight heparins act as anticoagulant and antithrombotic drugs by interactions with different serpins. For instance, heparin interacts with antithrombin inducing a conformational change in the molecule, and thus accelerating up to 2,000 times the inhibition of thrombin, as well as other serine proteinases of the coagulation cascade. In this condition it is formed a ternary complex involving heparin, serpin and proteinase. LMWHs show the similar affinity to AT, nevertheless due to their smaller molecular weight they preferentially bind to Xa (Figure 8). Besides AT, heparin specifically binds to several proteins related to coagulation, such as heparin cofactor II, TFPI, PAI, among others. When one analyses the *in vivo* effect, it should be also taken into account that heparin and derivatives have an impact on other biological processes such as inflammation, cell growth, cell adhesion, vascular modulation, apoptosis, etc, and thus the biological effect is going to reflect a combination of these complex network of interconnected pathways (Figure 4).

The biological actions of low molecular weight heparins relevant to their anticoagulant/antithrombotic activities can be divided into mediated or not by antithrombin. Anti-Xa and anti-IIa activities are among the ones dependent upon AT. On the other hand, release of TFPI, increase in the expression of

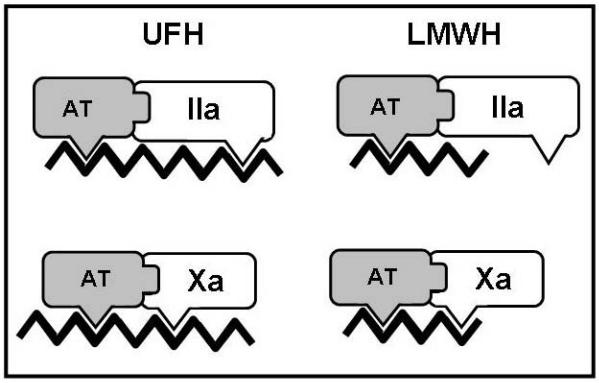

Figure 8. Interaction of unfractionated heparin and low molecular weight heparin with thrombin and Factor Xa mediated by antithrombin. AT, antithrombin; IIa, thrombin; Xa, factor Xa; UFH, unfractionated heparin; LMWH, low molecular weight heparin.

heparan sulfate from endothelial cells, interaction with heparin cofactor II, inhibition of procoagulant action of leukocytes, promotion of fibrinolysis, modulations of vascular endothelium mediated or not by receptor, among others are non-antithrombin related actions of LMWHs. Low molecular weight heparins show a longer half life than unfractionated heparin. Nevertheless, they still have no significant effect when administered orally. The introduction of special groups in heparin molecules, such as in SNAC heparin yielded a drug entitled with an oral effect [84-86].

4. Heparin mimics

Most of the now available anticoagulant drugs, including the low molecular weight heparins, have inhibitory effects not only on FXa, but also on thrombin, even though to a less extent. Factor Xa occupies a central position in the coagulation cascade, and would be an attractive target for antithrombotic treatment strategies.

Interesting results can be depicted in Table 5. Heparins from different origins, that show different molecular weight and chemical structure (see previous chapter) display differences in their pharmacological activities according to the preferential protein interaction. These differences reflect in the different parameters analyzed. The data shows a lack of correlation between *in vitro* and *in vivo* activities indicating that aPTT and anti-Xa methods cannot predict the antithrombotic activity of a heparin or a heparin related compound. This result clearly show that not only antithrombin is responsible for the antithrombotic effect of the compounds. Of special significance was the finding that a heparan sulfate isolated from bovine pancreas is a potent antithrombotic agent *in vivo* and devoid of significant pharmacological activities *in vitro*. The results show that substitution of the N-sulfate groups of the glucosamine moiety in the

Table 5. Molecular weight and pharmacological *in vitro* and *in vivo* activities of different heparins and heparin derived oligosaccharides and other sulfated glycosaminoglycans.

COMPOUND	MW (kDa)	USP	aPTT	aXa	HCII	Antithrom-botic	EC	Bleeding
UF-Heparin								
bovine lung	13	150	164	101	200	99	1.6	1
N-Acetylated Hep	13.4	10	-	-	-	-	1.0	zero
bovine pancreas	10	140	154	129	-	84	1.7	1
bovine mucosa	12.5	152	153	126	210	176	1.7	1
slow moving	17.8	163	285	134	-	64	1.7	1
fast moving	5.7	67	39	98	-	86	1.8	0.8
oligo chemical	4.5	48	37	90	-	119	2.0	0.7
oligo enzymatic	5.8	45	22	78	-	146	1.9	0.7
molecular sieving	4.6	49	31	100	-	161	1.8	0.8
mollusc								
A. brasiliana	26	320	345	150	-	120	1.9	1
T. mactroides	25	220	-	-	-	-	-	1
D. striatus	20	180	-	-	-	-	-	1
shrimp	9	55	-	100	160	110	2.1	0.8
crab	13	4	15	5	40	100	2.3	0.5
Heparan Sulfate								
bovine pancreas	25	19	2	4	14	170	1.0	zero
bovine lung	35	4	3	-	-	<10	1.0	zero
crustacea	9.2	3	5.2	<1	66	-	1.4	zero
Dermatan Sulfate	28	4	4	5	205	28	1.0	zero
Chondroitin Sulfate	17	1	1	1	10	4	1.0	zero

USP, anticoagulant activity expressed as IU/mg and determined according to the United States Pharmacopea; **aPTT,** as IU/mg; **aXa,** measured by an amidolytic anti-Xa method; **HC II**, as U/mg measured by the HEPTEST; **EC**, increase in the expression of the antithrombotic heparan sulfate from endothelial cells [69,70], the numbers are expressed relative to control in the absence of the compound; bleeding, expressed relative to heparin, using 400 μg/ml of the compound in the rat tail model [71]. Data from references [58-60,69-83].

heparin molecule by N-acetylated groups leads to a compound devoid of anticoagulant activity both *in vitro* and *in vivo.* Thus, besides molecular weight, N-sulfates are essential for the anticoagulant activity.

Glycol splitting of nonsulfated glucuronic residues leads to substantial loss of anticoagulant activity of heparin. Partial removal of sulfate groups at position 2 of iduronic acid residues followed by glycol splitting of all nonsulfated uronic acid residues yields nonanticoagulant heparins with antithrombotic and antiangiogenic activities [87-89].

In favor of the importance of the sulfation of the iduronic acid residues for antithrombotic activity are the results regarding acharan sulfate, a natural occurring glycosaminoglycan present in tissues of the mollusc *Achatina fulica* that is composed of repeating disaccharide units of α-D-N-acetylglucosamine and α-L-iduronic acid-2-O-sulfate. This compound shows *in vivo* antithrombotic activity and negligible *in vitro* anticoagulant activity

[90]. Thus, this and the other results discussed above clearly indicate that other proteins, besides antithrombin play pivotal roles in the control of hemostasis.

Specific structural characteristics of each individual sulfated glycosaminoglycan are involved in the modulation of their biological activity. Regarding heparin and heparan sulfate, the conformation of α-D-glucosamine and β-D-glucuronic acid residues assume the preferred $^4C_1$ conformation for the pyranose rings whereas the conformation of the iduronic acid residue varies, depending on its location in the polysaccharide chain as well as the sulfation pattern and the substitutions on adjacent glucosamine residues. Internal iduronic acid residues can assume both $^1C_4$ chair and the $^2S_0$ skew boat conformation, and at the reducing end of the chain the $^4C_1$ conformation can also be expected. The conformation changes between the $^2S_0$ and $^1C_4$ forms so that in the protein-bound state it can make favorable electrostatic interactions with basic amino acids on the protein [87,91-94]. Also, the substitution at the C-2 position of the glucosamine, either N-sulfate or N-acetylated groups, as well as specific O-sulfate groups can be exposed differently according to the position in the polysaccharide chain, and thus exerts different roles. When we now consider chemical or enzymatic modifications of these compounds it becomes evident that changes in their effectiveness in biological activities can be changed. For instance, hydroxyl groups, either preexisting in the original compound or exposed by desulfation, can be sulfated. Heparin/heparan sulfate can be partially or totally N-acetylated after removal of the N-sulfate groups. These types of modifications were performed in these molecules and gave interesting results reassuring the specific role of the different substitutions, besides affording novel compounds with protein binding and associated biological properties different from those of the original glycosaminoglycan.

More recently, studies have been performed using heparosan, the K5 polysaccharide from *E. coli*. The original compound is composed of repeating disaccharide units of α-D-glucosamine and β-D-glucuronic acid residues in the $^4C_1$ conformation for the pyranose rings. This polymer can be chemically modified yielding compounds with N-sulfation, O-sulfation within specific regions of the polymer affording a number of derivatives, some endowed with anticoagulant/antithrombotic activity and others with antimetastatic properties, etc [88,95-97]. Most of the activities could be correlated with typical sulfation patterns along each GAG backbone. The results indicate, similarly to other studies that the high affinity for antithombin and high anticoagulant activity depends on 3-O-sulfation of specific glucosamine residues [87,91,98]. This novel class of multi-target molecules with possible therapeutic implications constitute the forth

generation of anticoagulant drugs, that correspond to the biotechnological heparin/heparan sulfate.

Structurally defined sulfated polysaccharides can also be produced by partial synthesis using glucans of different natural origins. From the investigated glucan sulfates, mainly C-2 and C-4 sulfated linear β(1→3) polymers (20-50 kDa) were the most suitable for a potential use as heparin alternatives [99]. Dextran sulfates are other examples of chemically modified glucans with pharmacological activities. It is polymer of α-D-glucose joined by 1→6 glycodic linkages, bearing sulfates at different positions. Interestingly, the low molecular weight dextran sulfate (5kDa) is a potent inhibitor of the activation of both the coagulation and the complement systems [100,101] and unlike high molecular weight compound does not activate the fibrinolytic system [102]. These compounds like LMWHs have been shown to have direct effects on cell interactions, such as inhibition of E-selectin-mediated adhesion of neutrophils to endothelial cells [103,104].

Figure 9 shows the pentasaccharide described as the minimum structure in the heparin molecule responsible for antithrombin binding, including the critical 3-O-sulfate of the glucosamine [98,105]. The development of chemical synthesis of this and other heparin oligosaccharides has enabled to produce different modification of the pentasaccharide. This has led to the development of a new class of antithrombotic agents that acts by specific inhibition of FXa and lacks activity against thrombin. Fondaparinux is the first commercially available drug of this class of antithrombotic agents. It is purely a factor Xa inhibitor and an inhibitor of thrombin generation that requires binding to antithrombin [94,106-109].

Regarding other effects promoted by heparin, different minimum structures than the one described for antithrombin are found. For instance, a pentasulfated tetrasaccharide is the minimum structure necessary to elicit the endothelial cell response to the upregulation of heparan sulfate expression [110]. On the other hand, disaccharide bearing C4-C5 unsaturation at the uronic acid 1→4 linked to N-acetylglucosamine sulfated at the C-6 position is

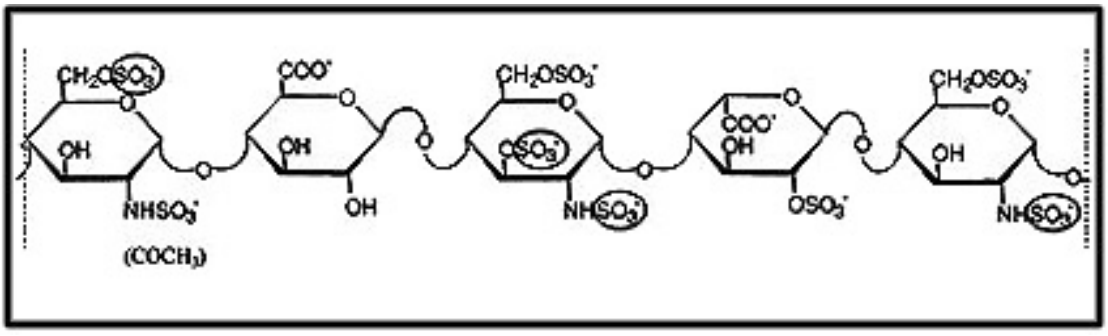

Figure 9. Heparin pentasaccharide related to antithrombin binding.

the minimum structure necessary to promote bleeding. If the sulfate is at the C-2 position of the glucosamine moiety the effect is abolished. Disulfated disaccharide containing glucosamine 2,6-sulfated and tri-sulfated disaccharide composed of uronic acid 2-sulfated and glucosamine 2,6-sulfated also show hemorrhagic effect. Previous results indicated that the bleeding was a consequence of the binding of heparin or fragments to the myosin-ATPase of wounded tissue. This effect could be reversed by addition of myosin or ATP [71,75-76,111]. Interestingly is the finding that topical application of ATP can reduce blood loss in patients submitted to cardiopulmonary bypass surgery [112]. Conformational studies of the disaccharides and ATP clearly show that the one displaying antihemostatic activity has a conformation similar to ATP (Figure 10) [113,114].

Intracellular calcium is tightly regulated and critically important for vascular smooth muscle contraction. Heparin and heparan sulfate fragments bearing C4-C5 unsaturation at the non-reducing end of the molecules promote a decrease of the intracellular calcium. Unsaturated disaccharides from chondroitin sulfated, dermatan sulfates and hyaluronan are inactive, indicating that besides the unsaturation of the uronic acid a vicinal 1→4 glycosidic linkage is needed (Figure 11). This effect is a result of the interaction of heparin and heparan sulfate fragments with the cardin sequence in XIP (exchanger inhibitor peptide) region of the Na^{+}/Ca^{2+} exchanger [115].

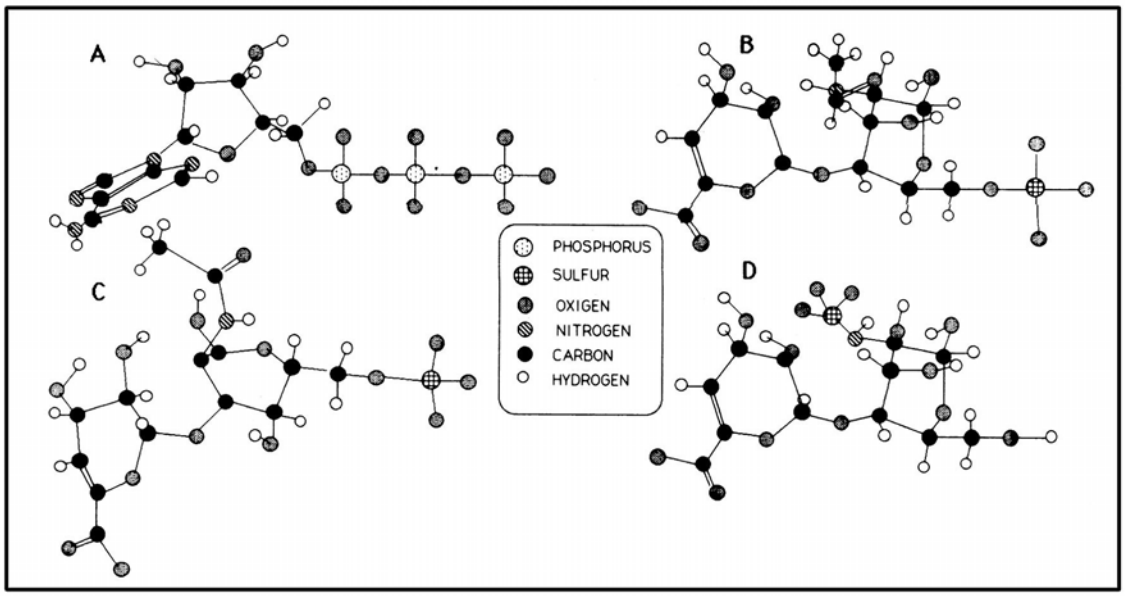

Figure 10. Conformation of ATP and of different glycosaminoglycans derived disaccharides. **A**, ATP; **B**, ΔU-(1→4)GlcNAc,6S; **C**, ΔU-(1→3)GalNAc,6S; **D**, ΔU-(1→4)GlcNS.

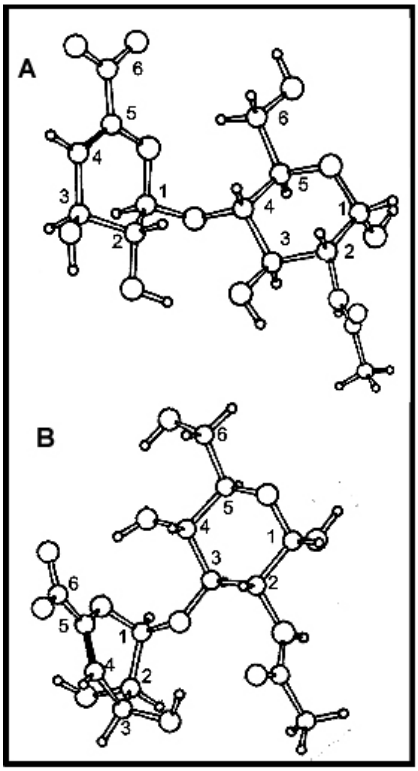

Figure 11. Conformation of disaccharides containing C4-C5 unsaturated uronic acid and N-acetylated glucosamine joined by 1→4 and 1→3 glycosidic linkages. **A**, disaccharide derived from heparan sulfate by degradation with heparitinase, ΔU-(1→3) GlcNAc, O-(enepyranosyluronic acid)-(1→4)-2-acetamido-2-deoxy-D-glucose; **B**, disaccharide derived from hyaluronan by degradation with lyase, ΔU-(1→3)GlcNAc, O-(enepyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-D-glucose; ΔU, 4,5-unsaturated uronic acid.

5. Future perspectives

This chapter has discussed several compounds that act on the control of hemostasis, with special emphasis on heparin and low molecular weight heparins. It has also been pointed out that the observed *in vitro* effect cannot predict the actual *in vivo* action. Structural characteristics of the compounds related to the various effects have been discussed, especially for low molecular weight heparins. The evolution in antithrombotic therapy is shown in Table 6.

It can then be concluded that each compound is unique, and has a specific profile of activity. And quoting Stuart L Nightingale from the Food and Drug Administration (FDA): "The FDA is alerting physicians and other health professionals to important considerations in the use of LMWHs, most particularly to the fact that LMWHs cannot be used interchangeably, unit for unit, with heparin, nor can one individual LMWH be used interchangeably with another" [116].

Table 6. Development of heparin and related compounds.

Generation	Compound
1st	Unfractionated heparins from different mammalian sources (e.g. bovine lung, bovine and porcine intestinal mucosa)
	Different salts of unfractionated heparins (e.g. Na^+, Li^+, Ca^{2+})
2nd	Low molecular weight heparins
3rd	Synthetic heparins (e.g. fondaparinux)
	Chemically modified heparins
	Heparin derivatives (e.g. SNAC heparin)
	Heparan sulfates (e.g. bovine pancreas)
	Dermatan sulfates (e.g. porcine mucosa)
4th	Heparin from invertebrates (e.g. shrimp)
	Fucans from seaweeds
	Biotechnology derived products

Fondaparinux, synthetic pentasaccharide; SNAC heparin, N-(8[2 hydroxybenzoyl]amino) caprylate.

The Brazilian Biochemical and Molecular Biology Society Annual Meeting of 2001 was in honor of Prof. Carl P. Dietrich for his outstanding contributions to the understanding of the structure of heparin and related compounds from different origins, development of low molecular weight heparin, and their involvement in the control of hemostasis. In his magnum lecture he showed his view on the historical development and use of heparin and antithrombotic compounds as illustrated in Figure 12.

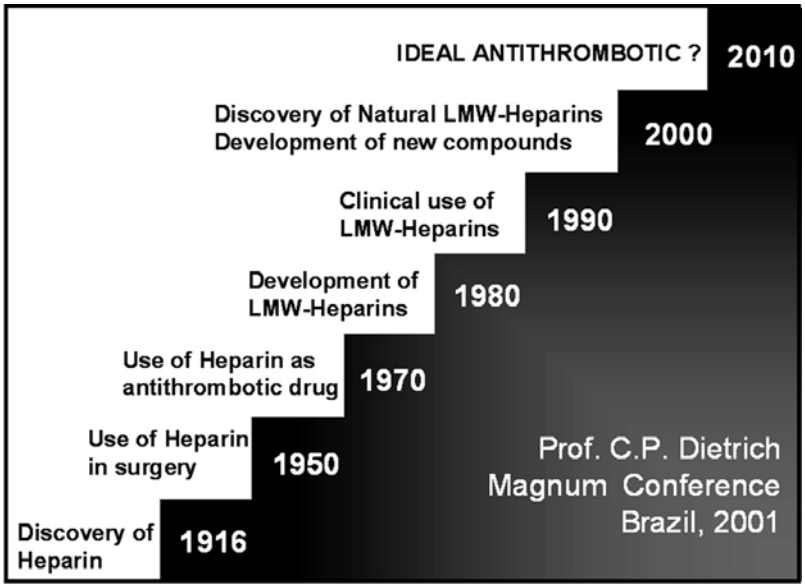

Figure 12. Historical view on the development and use of anticoagulant and antithrombotic compounds.

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3

Natural sulfated polysaccharides as antithrombotic compounds. Structural characteristics and effects on the coagulation cascade

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Abstract

Sulfated polysaccharides from marine seaweeds (green, red and brown) are one the most abundant source of non-animal anticoagulant sulfated polysaccharides in nature. In addition, there are

In honor of Prof. Carl P. Dietrich

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structural similarities between sulfated polysaccharides from seaweeds and heparin. The well known anticoagulant polysaccharides from red seaweed are homogalactans, and from brown algae are homo- and hetero-polysaccharides containing α-L-sulfated fucose called fucan and fucoidan, respectively. Anticoagulant activity is more pronounced in extracts from the brown algae compared to red and green algae. Structural studies clearly indicate that the anticoagulant effect of galactans is stereo-specific and not merely a consequence of their charge density or sulfate content. The brown seaweeds are the main source of fucan and fucoidan. The structure of these polymers varies according to the species as well as among the different parts of the seaweed. Using the same methodology for analysis, it was shown that all brown seaweeds analyzed contained three different classes of fucans. Using diamine buffers it was possible to form different complexes with the polysaccharides due to the spacing in the sulfate substitutions and sugar conformations, similarly as it was previously observed for the sulfated glycosaminoglycans from animal tissues. Some structural characteristics of these complex polysaccharides are discussed regarding anticoagulant and antithrombotic activities.

1. Introduction

Unfractionated heparins and low molecular weight heparins are the sulfated polysaccharides currently used as anticoagulant drugs. These compounds, however, show some side effects, such as heparin induced thrombocytopenia and bleeding. In addition, the commercial sources of heparins are mainly pig and bovine intestine. The possibility that prions and virus could be carried by these molecules due to the source of origin, as well as the increased needs for antithrombotic therapies, indicates the necessity to look for alternatives sources of anticoagulant agents.

Sulfated polysaccharides from marine seaweeds (green, red and brown) are one the most abundant source of non-animal anticoagulant sulfated polysaccharides in nature. In addition, there are structural similarities between sulfated polysaccharides from seaweeds and heparin. These characteristics led several groups to study algal polysaccharides as anticoagulant compounds. The well known anticoagulant polysaccharides from red seaweed are homogalactans, and from brown algae are homo- and hetero-polysaccharides containing α-L-sulfated fucose [1, 2] called fucan and fucoidan, respectively. Anticoagulant activity is more pronounced in extracts from the brown algae compared to red and green algae. Few studies describe the presence of anticoagulant sulfated polysaccharides in green algae.

2. Sulfated galactans

Sulfated galactans are among the most abundant non-vertebrate sulfated polysaccharides found in nature. They occur in high concentration, not only in

red [3], brown [4], and green [5] seaweeds, but are also found in marine invertebrates and sea grass [6].

The first report on sulfated polysaccharide from seaweed (*Iridae laminarioides*) possessing blood anticoagulant properties was by Chargaff *et al.* [7].

Sulfated galactans from red algae (also know as carrageenans or agarans) are composed of alternating 3-linked β-galactose and 4-linked α-galactose units bearing sulfate substitutions in different positions [8]. Figure 1 shows a schematic representation of the main structural features of sulfated galactans.

However, considerable structural variation occurs among the polymers purified from different sources. A substantial or even all α-galactose residues may be replaced by 3,6-anhydro-α-L-galactose. In addition, besides the sulfate hemi-esters, pyruvate acetal and/or methyl ethers can also substitute the hydroxyl group of galactans. The different number and positions of the sulfate groups together with the presence or absence of 3,6-anhydride form of the 4-linked α-galactose in the structure lead to 42 theoretical different disaccharide units among which only 15 have been identified in algal galactans and assigned Greek letters (Table 1) [9].

These compounds present anticoagulant activity, and λ-carrageenans display the highest action even though weaker than that observed for heparin [10]. The proposed mechanisms of action of these compounds are predominantly related to the *in vitro* inhibition of thrombin mediated by antithrombin (AT) and/or heparin cofactor II (HC II) [11]. Also, carrageenans are able to induce human platelet aggregation through a phospholipase C-dependent mechanism [12].

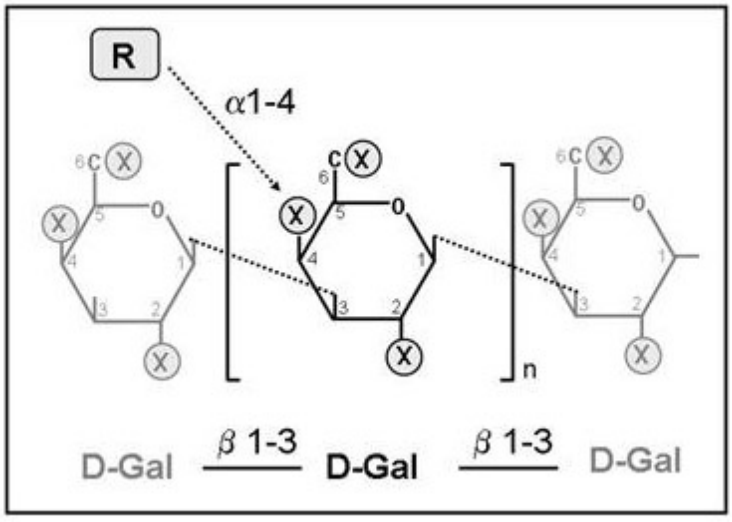

Figure 1. Basic unit in sulfated galactan. X, sulfate or H; R, 3,6-anhydro-α-D-galactose or α-D-galactose bearing different substitutions. See Table 1.

Table 1. Disaccharide repeating structures of galactans from red seaweeds[a].

Family	Greek symbol	1,3-linked	1,4-linked
kappa			
	Kappa (κ)	β-D-galactose 4-sulfate	3,6-anhydro-α-D-galactose
	Iota (ι)	β-D-galactose 4-sulfate	3,6-anhydro-α-D-galactose 2-sulfate
	Mu (μ)	β-D-galactose 4-sulfate	α -D-galactose 6-sulfate
	Nu (ν)	β-D-galactose 4-sulfate	α -D-galactose 2,6-di-sulfate
	Omicron (ο)	β-D-galactose 4-sulfate	α -D-galactose 2-sulfate
Beta			
	Beta (β)	β-D-galactose	3,6-anhydro-α-D-galactose
	Gamma (γ)	β-D-galactose	α -D-galactose 6-sulfate
	Omega (ω)	β-D-galactose 6-sulfate	3,6-anhydro-α-D-galactose
	Psi (ψ)	β-D-galactose 6-sulfate	α -D-galactose 6-sulfate
Lambda			
	Delta (δ)	β-D-galactose	α -D-galactose 2,6-di-sulfate
	Alfa (α)	β-D-galactose	3,6-anhydro-α-D-galactose 2-sulfate
	Lambda (λ)	β-D-galactose 2-sulfate	α -D-galactose 2,6-di-sulfate
	Theta (θ)	β-D-galactose 2-sulfate	3,6-anhydro-α-D-galactose 2-sulfate
	Xi (ξ)	β-D-galactose 2-sulfate	α -D-galactose 2-sulfate
	Pi (π)	β-D-galactose P,2-sulfate	α -D-galactose 2-sulfate

[a]Adapted from Lahaye [8], P: pyruvate acetal

Several studies have been performed to determine structural requirements involved in the anticoagulant activity of sulfated galactans. Comparison with several well defined sulfated homogalactan from marine invertebrates and from the red seaweed *Botryocladia occidentalis* (λ-carrageenan like) have shown that galactans with one-third of the galactose residues 2,3-di-O-sulfated have similar anticoagulant activity as unfractionated heparin, whereas galactans sulfated at either 2-O or 3-O position of galactose residues (but not at both positions) have weak anticoagulant activity [13]. On a subsequent study [3], it was shown that a galactan from *Gelidium crinale* (15% of the total α-units are 2,3-di-sulfated and another 55% are 2-sulfated) had a lower anticoagulant activity on a clotting assay in comparison to galactan from *B. occidentalis*, that shows 30% of disulfated units. However, there were not differences between them regarding thrombin inhibition assays mediated by AT, but when HC II replaced AT, the galactan from *G. crinale* was a less effective compound. In contrast, when factor Xa instead of thrombin was used as a target protease, the galactan from *G. crinale* was a more potent agent. The comparison between the anticoagulant activity of these two polymers reveals that 2,3-disulfated α-galactose units are important requirement for interaction of the sulfated galactans with coagulation inhibitors. The results indicate that the optimal proportion of these units for the effect of the polysaccharides on coagulation assays varies depending on the protease and coagulation inhibitor [3].

These data clearly indicate that the anticoagulant effect of galactans is stereo-specific and not merely a consequence of their charge density or sulfate content.

3. Fucans and fucoidans

Brown algae contain a wide variety of acid polysaccharides such as the alginic acids, consisting exclusively of uronic acid, the homo-fucans, consisting of sulfated fucan, and the heterofucans, that contain portions of other neutral sugars and uronic acids in addition to sulfated fucose [14,15]. In these cases, besides the presence of branches at different sugar residues and positions, it is also observed a complex distribution of sulfate and occasionally acetyl groups [16,17].

Fucan is the term used to define a polysaccharide composed mainly on sulfated L-fucose and containing less than 10% of other monosaccharides. The term fucoidan can be used to define heterofucans, even though several times, the terms fucan and fucoidan are employed as synonyms. These polysaccharides have been isolated only from brown seaweed [18], egg jelly coat of sea urchins and body wall of sea cucumber [19]. Recently, a fucoidan-like was isolated from the sponge *Mixylla rosacea*. However, the authors decided to name rosacelose this sulfated polysaccharide [20]. Thus, the main natural source of fucans are the brown seaweeds.

Indeed, all algal fucans have complex structures but recent studies have revealed ordered repeated units in homofucans from several species. These studies clearly show that several homofucans have large proportions of both α-(1→3) and α-(1→4) glycosidic linkages with sulfate groups at C-2, without excluding the presence of other sulfates, acetyl groups or branches at positions 2, 3 or 4 [17,21,22]. Figure 2 shows a schematic representation of the main structural features of homofucans.

Heterofucans are more complex than homofucans. Furthermore, little is known about the structural features of the heterofucans. Most of the difficulties of structural studies arise from the fact that these compounds are very heterogeneous, yielding complex NMR spectra with broad signals and thereby interfering with resolution. In fact, for these algal polysaccharides even high-field NMR provides data of limited value, and complete descriptions of their structures are not available [23].

Nagaoka *et al*. [24] proposed that a fucan from *C. okamuranus* contains a linear backbone of (1→3) linked fucose. Partly substituted with (1→2) linked α-glucuronic acid. Abdel-Fattah *et al*. [25] isolated a fucan from *S. linifolium* containing a central core composed of β-D-glucuronic acid and β-D-mannose and Leite *et al*. [26] showed a xylofucoglucuronan composed of a core of (1→3) linked β-D-glucuronic acid with branches at C-4 of (1→3) linked

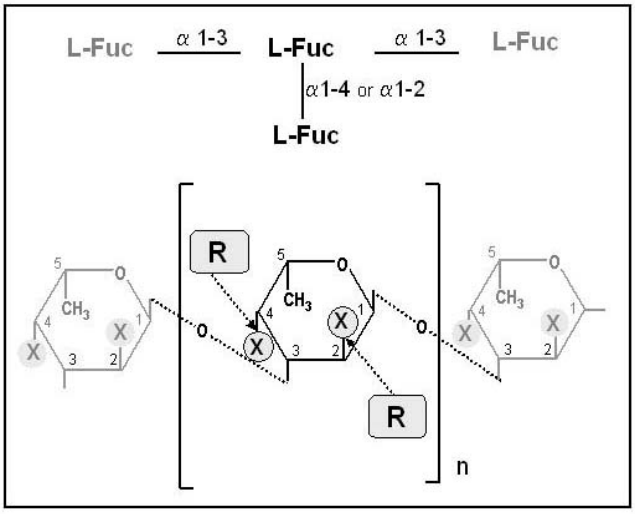

Figure 2. Basic unit of a homofucan. X, sulfate or H; R, branches of α-L-fucopyranose bearing different sulfate substitutions. Data from references [21,22].

a-fucose chains. More recently, a fucan from *Padina gymnospora* showed that the fucose was mostly substituted at C-2 with chains of (1→4) linked β-D-xylose [27].

These differences in structure could be related to the methods employed for extraction and purification, and/or source of origin. Indeed, the structure of these polymers varies according to the species [28,29] as well as among the different parts of the seaweed [29]. Using the same methodology for extraction, purification and analyses, Dietrich et al. investigated the type of fucoidans present in tissues of different species of brown seaweeds. It was shown that all brown seaweeds analyzed were composed of three different classes of fucans. These was clearly observed when the electrophoretic migration was investigated using 1,3-diamino propane buffer [30]. The diamine forms different complexes with the polysaccharides due to the spacing between the negative charges, mainly due to sulfation substitutions in a specific conformation of the sugar residue. This type of complex formed between sulfated polysaccharides and diamine is similar to what as was previously described for the sulfated glycosaminoglycans from animal tissues [30]. Thus the fucans from brown seaweed display structural variability but nevertheless they can be divided into three different classes, according to their electrophoretic behavior, which indeed reflects the structure, regarding the proportion and sequence of sugar residues as well as the sulfation pattern. So, each polysaccharide purified from brown marine algae is a new compound with unique structure, like it has been described for the sulfated glycosaminoglycans (chondroitin sulfate, dermatan sulfates, heparan sulfate and heparin) from animal species.

Figure 3 displays a general structure for two classes of algae fucoidan and Figure 4 shows the full assembly of the units in the structure of two fucans purified from *Spatoglossum schröederi*. Fucan A is composed of a central core of β-D-glucuronic acid bearing 1→4 branches of α-L-sulfated fucose containing oligosaccharides which in turn show branches of β-D-xylose [26]. Fucan B is composed of a central core of 1→4 linked β-D-galactose that may bear sulfate substitutions at C-3 and at some C-2 position a branch composed of α-L-fucose (sulfated or not) and β-D-xylose [18].

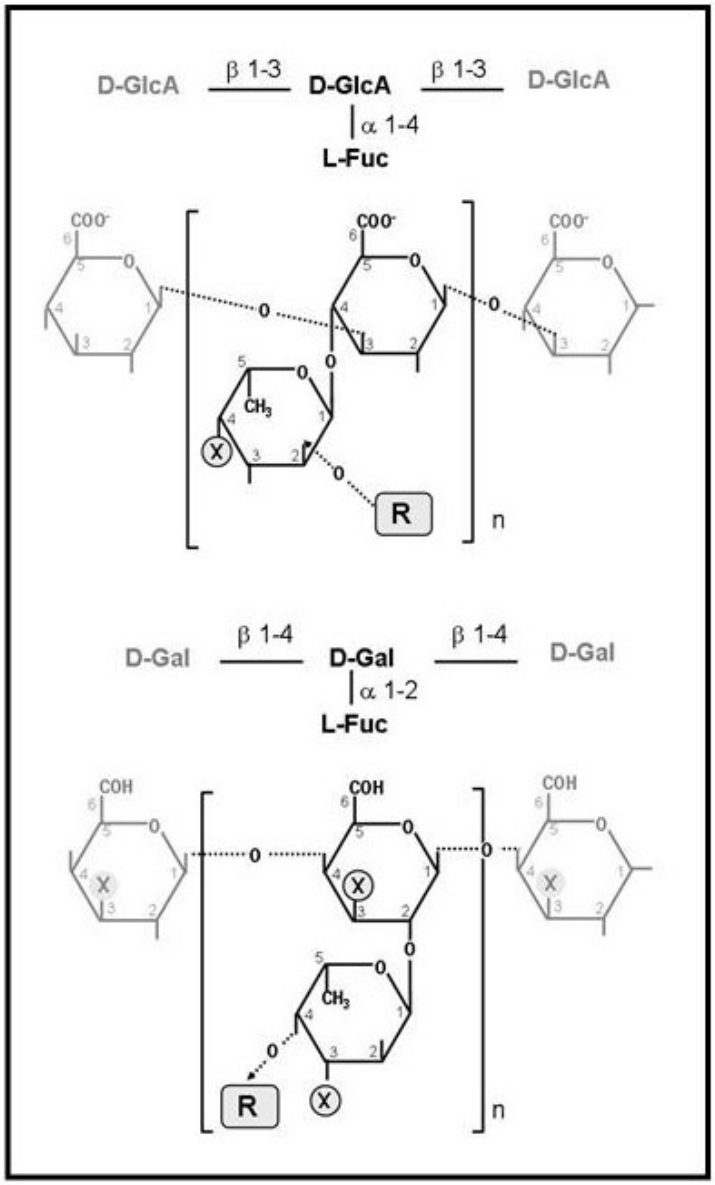

Figure 3. Basic units of heterofucans. X, sulfate or H; R, branches of α-L-fucopyranose and β-D-xylopyranose with different degree of polymerization and sulfation.

In recent years, sulfated fucans have emerged as an important class of natural biopolymers. Each new polysaccharide has a potential novel biological activity. In fact, fucan and fucoidan have a wide range of activity when tested in different biological systems (Table 2). The most extensive studies have been conducted on anticoagulant activity of several fucan and fucoidans.

The strong anticoagulant activity of fucoidan from *Fucus vesiculosus* was demonstrated in an early study [68]. More recently, it has been shown that this compound exerts the activity by potentiating mainly HC II [69-71]. This fucoidan, like heparin, stimulates HC II primarily by an allosteric mechanism

Table 2. Biological activities of fucans from brown algae.

Biological activity[a]	Seaweed	Reference
Anti-angiogenic	*Fucus vesiculosus*	[31]
Angiogenic	*Ascophyllum nodosum*	[32]
Anticomplement	*Laminaria cichorioides, L. japonica, Fucus evanescens, F. Vesiculosus, A. nodosum*	[33,34]
Inhibition of leukocyte rolling	*F. vesiculosus*	[35]
Regulation of cytokine production	*Undaria pinnatifida, Cladosiphon okamuranus, F. vesiculosus*	[36,37]
Stimulation of nitric oxide production	*F. vesiculosus*	[38]
Stimulation of protein tyrosine phosphorylation	*F. vesiculosus*	[39]
Blocking of P- and L-selectin	*F. vesiculosus*	[40]
Antioxidant	*F. vesiculosus*	[41]
Antiproliferative	*A. nodosum, F. Vesiculosus, Turbinaria ornata*	[42-44]
Antimetastic	*F. vesiculosus, Sargassum thunbergii, S. fuvellum, S. kjellmanianum, Eisenia bicyclis, U. pinnatifida*	[45-50]
Antiadhesive	*A. nodosum, L. brasiliensis, Spatoglossum schröederi*, *Sargassum stenophyllum*	[51-53]
Induction of apoptosis	*Cladosiphon Okamuranus, F. Vesiculosus*	[54,55]
Antiviral	*Sargassum horneri*, *S. patens, F. Vesiculosus, Adenocystis utricularis, U. Pinnatifida, Leathesia difformis, Laminaria abyssalis*	[56-62]
Interaction with growth factors	*F. vesiculosus*	[63]
Fibrinolytic activity	*Ecklonia kurome, F. vesiculosus*	[64,65]
Contraceptive	*F. vesiculosus*	[66]
Anti-ulcer	*Cladosiphon okamuranus*	[67]

[a]Anticoagulant and antithrombotic activities are described below

requiring the N-terminal acidic domain of the protein. Thus, it forms a ternary complex with HC II and thrombin [72]. On the other hand, the fucoidan from *Fucus evanescens* binds to AT and shows an anticoagulant activity similar to that of heparin [73], whereas fucoidans from *Eklonia kurome* [74], *Pelvetia caniculata* [75] and *Ascophyllum nodosum* [76] have activity mediated by both AT and HC II.

The relationships between structure and anticoagulant activity of fucoidans are not yet clearly established. The importance of the molecular size and sulfate content has been reported [77,78]. However, it is most likely that some structural features are required for anticoagulant activity, especially sulfate clusters to ensure interactions with cationic proteins.

Structural analyses of fucoidan derived-oligosaccharides from *A. nodosum* have shown that all O-2 and some O-3 positions of the fucose residues are obligatory for anticoagulant activity, whereas O-4 sulfates seem to be unnecessary. In addition, 3- and/or 4-linked fucose residues are always present [79]. Recent studies using a high heterogeneous fucoidan from another brown seaweed (P. *gymnospora*) showed that the presence of sulfate groups only at O-3 position of the fucose residues were important for the anticoagulant activity [2,27].

Fucoidan from *A. nodosum* has also shown antithrombotic activity, as a result of the binding to both AT and HC II. These serpins act against several proteases of the coagulation cascade, including thrombin, Factor Xa and Factor IXa. All these factors may be involved in the ability of fucoidan to prevent venous thrombosis [76,80].

Fucans from *Fucus vesiculosus* and *Ascophyllum nodosum* displayed activity both on thrombin generation promoted by the initiation phase through TF (Tissue Factor) and by the augmentation stage, which is initiated when blood comes into contact with an anionic surface. Also, they inhibited thrombin-induced platelet aggregation better than heparin. This might suggest that the fucoidan also binds, besides exosite 2, exosite 1 and that the fucan could be a heparin substitute endowed with antithrombotic and anti-inflammatory activities [81,82].

The exerted antithrombotic effect of fucoidan has been suggested to be mainly mediated by enhancing thrombin inhibition via different mechanisms [83,84] whereas others have proposed that fucoidan primarily stimulate the release of tissue factor pathway inhibitor from endothelial cells [85]. Sulfated polysaccharides are also known to bind and block the function of members of the selectin family of adhesion molecules. Notably, fucoidan is known to bind and interfere with the activity of both P- and L-selectin [21,86,87] which, thus, may be involved in any antithrombotic effect of fucoidan *in vivo.* Another paper, using a different approach, suggested that fucoidan effectively prevents microvascular thrombus formation induced by endothelial damage in arterioles and venules *in vivo*, but the effect is not attributable to inhibition of P- and

L-selectin function but may instead be related to the anticoagulative capacity of fucoidan [88]. These discrepancies in the results are certainly related to differences in the fucoidan structure, due to source of origin as well as the method of extraction.

Recently, the purification, structural characterization, and pharmacological activities of a new sulfated polysaccharide from the brown alga *Spatoglossum schröederi* has yielded a fucoidan with a unique structure, composed of a central core of 4-linked, partially 3-sulfated-galactose units (Fig. 4B). Approximately 25% of these units contain branches of oligosaccharides formed by non-sulfated-xylose and 3-sulfated-fucose units linked to the O-2 position of the central core. Of particular significance was the finding that this sulfated galactofucan has no anticoagulant activity in several *in vitro* assays but shows a potent *in vivo* antithrombotic activity with no hemorrhagic effect. The antithrombotic effect was time-dependent, reaching a maximum 8 hours after its administration compared to the more transient action of heparin. The authors attributed the antithrombotic activity of this fucoidan to its potent effect on the stimulus of synthesis of a highly sulfated heparan sulfate by endothelial cells of the vascular wall that displays important antithrombotic activity [18]. In addition, two other fucoidans isolated from the same alga were able to stimulate the synthesis of the antithrombotic heparan sulfate. They show no anticoagulant activity using *in vitro* assays, as well as no *in vivo* antihemostatic activity, but their antithrombotic activity was not analyzed yet [26,89].

Fucoidans can also exert their antithrombotic effect using other serpin-independent mechanisms. For instance, fucoidan from *F. vesiculosus* is more potent then heparin in the release of TFPI (tissue factor pathway inhibitor) from endothelial cells more [85], endothelial cell migration [90], or in the stimulation of fibrinolysis by potentiating plasminogen activators [91].

Other activities attributed to heparin not directly related to coagulation are also shared by fucoidans. Maintenance of the equilibrium between deposition and degradation of extracellular matrix is essential to tissue homeostasis and remodeling. Moreover, their weak bleeding effect and non-animal origin is in favor of their use in clinic for humans. It has been shown that these polysaccharides stimulate dermal fibroblast proliferation and extracellular matrix deposition. More recently, a 16kDa fucoidan fraction was able to inhibit metalloproteinases (MMPs), increasing the rate of association of the protease with the specific inhibitors (TIMPs). The combined experiments suggested that fucoidans can be used in clinical situations where uncontrolled extracellular matrix degradation takes place [92]. Fucoidans when tested animal models of restinosis showed a comparable effect to that described for heparin [93]. It has been also shown that fucoidan from *Ascophyllum nodosum* and heparin share some similar mechanisms of action, such as smooth muscle cells growth inhibition, binding, and internalization [94].

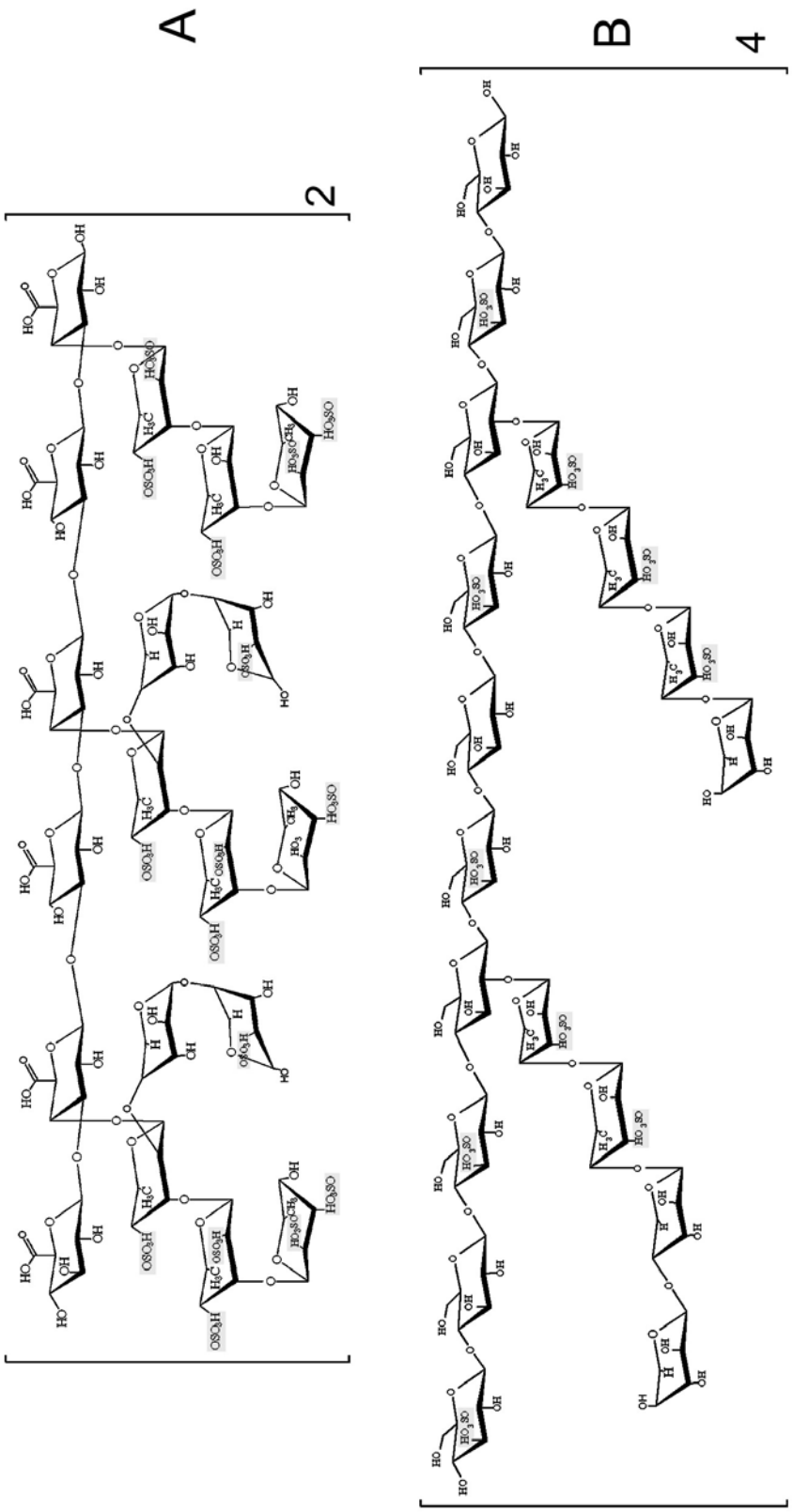

Figure 4. Structure of sulfated fucans from *Spathoglossum schröederi.* For details see Ref. [18,26].

Sulfated fucans show anti-adhesive activity affecting the attachement of cells to fibronectin. This effect is sulfate dependent, since desulfation of the fucan abolished the effect. The results suggested that the fucan showed anti-adhesive activity by binding directly to FN, and blocking FN sites that are recognized by cell surface ligands, possibly the integrin family [52].

It has also been shown that fucoidan from *Ascophyllum nodosum* and heparins share some similar mechanisms of action, such as SMC growth inhibition, binding, and internalization [94]. Experiments using FGF-2 induced proliferation and differentiation of human umbilical vein endothelial cells showed that at antithrombotic concentrations, contrary to heparin, fucoidan can enhance vascular tube formation induced by FGF-2 with a modulation of the expression of surface proteins (mainly α_6) involved in angiogenesis [83].

4. Anticoagulant sulfated polysaccharides from green algae

The green algae were not recognized to contain anticoagulant polysaccharides for a long time. After Deacon-Smith *et al.* [95] reported the presence of anticoagulant polysaccharides in *Codium fragile*, much effort has been focused on green algae, and many anticoagulant polysaccharides have been described. Chemical analyses have shown that these polymers are heteropolysaccharides, although an anticoagulant arabinan (homopolymer of arabinose) was purified from the *Codium dwarkense* [96]. There is a predominance of one monosaccharide at the expense other monosaccharides in several algal orders e. g., the Codiales polysaccharides are manly composed of arabinose or galactose, and the Caulerpales and Ulvales polysaccharides are mainly composed by galactose and rhamnose, respectively. However, there is insufficient evidence yet to establish any systematic correspondence between structure, including monosaccharide composition, and algal order.

Comparison among anticoagulant sulfated polysaccharides isolated from eight green algae has demonstrated that these compounds exert their activity through HC II. In addition, arabinose-rich polysaccharides were more potent thrombin inhibitors than galactose-rich polysaccharides. Also, HC II binding site for each of these eight compounds seems to be different from that of heparin or dermatan sulfate [97].

Other polysaccharides from green algae have shown different mechanism of action. A sulfated polysaccharide composed mainly of glucose with minor amounts of arabinose and galactose showed anticoagulant activity using activated partial thromboplastin time (aPTT) and thrombin time (TT) *in vitro* assays. Its anticoagulant mechanism was due to the direct inhibition of thrombin and potentiation of AT [98]. A sulfated galactan shows effect on TT and aPTT clotting times, with no action on PT. In addition, the effect is not

related to AT and HC II. Probably, it would inhibit either thrombin activity directly or fibrin polymerization. This compound shows a branched structure (1,3-,1,3,4- and 1,3,6-linked α-D-galactose). The 1,3 and 1,3,6-linked α-D-galactose can be highly 4-O-sulfated [5].

Table 3 summarizes some of the structural features of algal anticoagulant polysaccharides that interact with proteins involved in coagulation control (enzymes, like thrombin, and serpins such as AT and HC II).

Table 3. Structural features of anticoagulant polysaccharides from marine alga.

Polysaccharide	Main structural features responsible for anticoagulant activity	Site of action
Galactans (red algae)	-4-α-D-Gal-1→3-β-D-Galp-1 2,3-di- and 2-sulfated galactose	HC II and AT
Fucans and Fucoidans (brown algae)	–4–α-L-Fuc-1→3–α-L-Fuc-1 2,3-di- 2 and -sulfated fucose	HC II and/or AT
Galactans (green algae)	3-α-D-Galp-1 4-sulfated galactose	Thrombin

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9 Heparan sulfate proteoglycan as regulator of protein functions

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Abstract

Heparan sulfate is a ubiquitous glycosaminoglycan of animal cells. Heparan sulfate occurs at the cell surface and in extracellular matrix as proteoglycans. Most of cellular heparan sulfate derives from the syndecans and glypicans proteoglycans. The syndecan family is associated with the cell membranes via transmembrane core proteins and the glypican family

In honor of Prof. Carl P. Dietrich

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is anchored by glycosilylphosphatidylinositol anchor core proteins. Also, heparan sulfate proteoglycans are present in basement membranes performing the perlecan family. Heparan sulfate proteoglycans play a complex role in the cell surface, regulating a wide variety of biological process, including hemostasis, inflammation, angiogenesis, cell growth, differentiation, adhesion, endocytosis and others. This work focuses on heparan sulfate proteoglycans as a regulator of proteins functions.

1. Introduction

Proteoglycans encompass a heterogeneous group of proteins that are substituted with linear, highly negatively charged glycosaminoglycans (GAGs) polysaccharides. Because their abundant carboxyl and sulfate groups, glycosaminoglycans constitute a major source of macromolecular polyanions that surround almost every cell type in metazoan organisms. Four structurally distinct GAG families exist: heparan sulfate (HS)/heparin, chondroitin (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA). GAGs are linear polysaccharides, comprised of repeating disaccharide units of an amino sugar, either N-acetyl-D-glucosamine (D-GlcNAc) or N-acetyl-D-galactosamine (D-GalNAc), and an uronic acid, either D-glucuronic (D-GlcA) or L-iduronic acid (L-IdoA) except for KS, which consists of a galactose in place of the uronic acid.

Among the sulfated glycosaminoglycans, heparan sulfate, a ubiquitous cell surface component of cells, exhibits the highest structural variability according to the tissue and species of origin [1–3]. These classes of compounds are heteropolysaccharides composed of several distinct disaccharides containing uronic acid and glucosamine with *N*- and 6-*O*-sulfates and *N*-acetyl substitutions.

Heparan sulfate occurs at the cell surface and in extracellular matrix as proteoglycans. Most of cellular heparan sulfate derives from the syndecans and glypicans proteoglycans. The syndecan family are associated with the cell membranes via transmembrane core proteins [4,5], and the glypican family is anchored by glycosilyl phosphatidylinositol anchor core proteins [6]. Also, heparan sulfate proteoglycans are present in basement membranes performing the perlecan family [7], whereas the closely related heparin proteoglycan serglycin is found only inside storage granules in connective-tissue mast cells

Glypicans

The glypicans constitute a family of HSPGs that are linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Their core proteins contain a hydrophobic GPI anchor at the C terminus. Glypicans have attachment sites for the heparan sulfate (HS) chains within the 50 C-terminal amino acids, placing the chains close to the cell membrane. Glypicans also contain a conserved cysteine-rich globular domain at the N-terminal side of the

HS-attachment sites. The glypicans play a crucial role in development and morphogenesis, for example, glypican-3 is mutated in an overgrowth and dysmorphic syndrome (Simpson–Golabi–Behmel syndrome, an X-linked disorder). To date, six members of this family have been described in mammals and two in Drosophila [8].

Syndecans

The syndecan core proteins consist of a family of four distinct genes in vertebrates.The data regarding the sequence of *Drosophila* syndecan as well as chromosomal locations and exon organization, suggest that the gene family arose by gene duplication and divergent evolution from a single ancestral gene, and that syndecan-1 and -3 and syndecan-2 and -4 represent subfamilies. Each gene product is a single type I membrane-spanning protein with an apparently extended extracellular domain of varying size that contains covalently attached heparan sulfate chains distal from the plasma membrane [5] (Figure 1). The syndecans are membrane proteins that do not contain cysteine residues and show few but important structural similarities. Structural variabilities are mainly

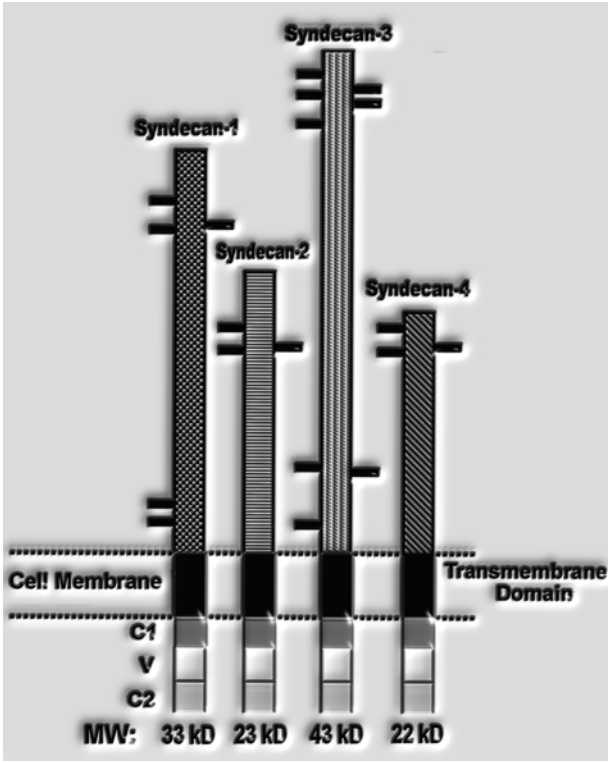

Figure 1. Schematic representation of mammalian syndecans. The proteins are aligned at the signal peptides and the transmembrane domains, leaving gaps in the ectodomains. The glycosaminoglycan attachment sites are indicated as black lines. Syndecans contain a conserved transmembrane domain and a small cytoplasmic domain consisting of one variable (V) and two conserved (C1 and C2) regions. MW indicates molecular weight.

found in the extracellular domains (ectodomains) that are among the most rapidly diverging vertebrate proteins with the exception of their regions for GAG attachment, cell interaction, proteolytic cleavage site, and oligomerization. These domains contain sequences of Ser-Gly-X-Gly preceded by an acidic amino acid, being consensus sequences for the glycosylation of heparan sulfate chains. Also, protease cleavage sites composed of basic amino acids are found close to the transmembrane portion of the protein core and are related to shedding of the proteoglycan from the cell surface (Figure 1).

The transmembrane domains are relatively stable evolutionarily, since only a few amino acids differ among the vertebrate sequences. These domains contain regions for interactions with other membrane proteins and for localization to distinct membrane compartments. The cytoplasmic domains contain two invariant regions, a membrane proximal common region (C1) containing a serine and a tyrosine and a C-terminal common region (C2), separated by a region (V) of variable length and composition (Figures 1 and 2). The C2 region shows an EFYA sequence at the C-terminus that can bind to the

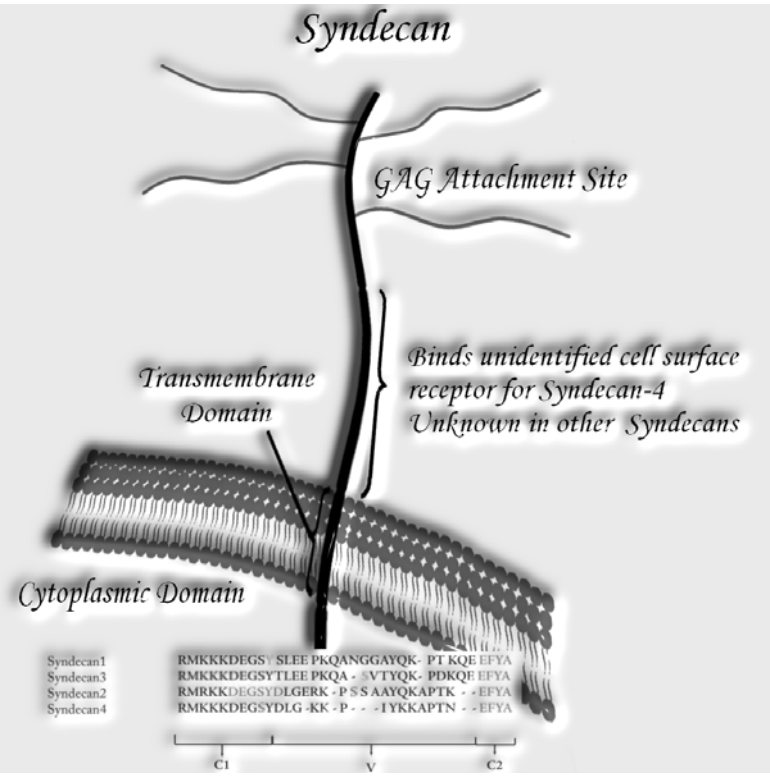

Figure 2. Syndecan core protein structure. The extracellular domains of the four vertebrate syndecans contain several sites for heparan sulfate chain attachment. The transmembrana domain is highly conserved among the family members. The cytoplasmic domains contain regions (C1 and C2) that are exactly conserved in each of the four syndecans (the exception being a conservative substitution of arginine for lysine in syndecan-2). These flank a central variable (V) region that is distinct for each family member.

PDZ domain present in specific proteins. PDZ domains, named for PSD-95, Discs-large, and Zonula occludens-1 proteins, bind specific C-terminal sequences and organize and assemble protein complexes on the inner surface of the plasma membrane and are thought to link membrane components to the underlying actin-containing cytoskeleton (Figure 2).

All mammalian syndecan cytoplasmic domains contain a conserved EFYA sequence at the C-terminus, which can bind PDZ domain-containing scaffold proteins (CASK and syntenin) and coordinate clustering of receptors and connection to the actin cytoskeleton (Figure 2). *In vitro* studies indicate that all syndecans can interact with CASK, and may form similar complexes in different contexts. CASK forms a ternary complex with syndecan-2 and protein 4.1, possibly linking the extracellular membrane and the cytoskeleton [9].

The variable (V) region is distinct for each of the 4 family members, but its syndecan specific identity is conserved across species (5). The function of this domain is largely unknown except for syndecan- 4, where it is responsible for the assembly of syndecan-4 tetramers with phosphatidylinositol 4,5 bisphosphate (PIP2) and activated protein kinase C-α(PKC-α) in focal adhesions [10,11]. Dramatic changes in syndecan expression occur during development and are associated with morphological transitions, cell differentiation, or changes in tissue organization [12,13].

Serglycin

The serglycin proteoglycans are present in cytoplasmic granules in a wide range of haematopoietic cells. While the core protein may be the same in the PGs in many secretory granules, the type of GAG attached may differ between cells types [14]. Human mast cells have also been shown to contain both oversulphated chondroitin sulfate and heparin [15,16]. Macrophages, basophils and mucosal mast also show highly sulfated chondroitin sulfate units [17,18]. The chondroitin 4-sulfate (CS-A) glycosaminoglycan is the dominant form in haematopoietic cells [19], but platelets synthesize chondroitin 6-sulfate also (CS-C) [19,20]. Basophils and mucosal mast cells have been shown to contain oversulfated dermatan sulfate (CS-diB) [21].

In haematopoietic cells, the intracellular serglycin proteoglycans are crucial for the storage of various secretory granule enzymes. Serglycin proteoglycans bind tightly to lysozyme [22], lactoferrin [23], granzyme B [24], elastase [25], myeloperoxidase [26], mast cell tryptase [27] and mast cell chymase [28,29]. The generation of granules, granule homoeostasis, protection and regulation of granular proteins, and regulation of secretion are depending on intracellular proteoglycans [14]. It has been shown that intracellular PGs interact with granular proteins to organize and concentrate their contents in granules, and also to protect these from inactivation or

destruction [30]. The gene for NDST-2 is specifically designed for the biosynthesis of heparin, targeting of the gene for NDST-2 resulted in a drastic reduction in the various proteases that are normally found in the secretory granules of connective-tissue mast cells, accompanied by greatly distorted morphology of the secretory granule [31,32]. Also, the storage of histamine was markedly reduced [31,32].

2. Endocytosis mediated by heparan sulfate proteoglycans

Heparan sulfates proteoglycans are correlated to the cellular internalization of viruses, basic peptides and polycation–nucleic acid complexes and thus, possibly, they have important implications for gene transfer and protein delivery to mammalian cells [33]. The heparan sulfate proteoglycans-mediated, lipid raft-dependent macropinocytosis provides a common route of entry for proteins and peptides [34]. Also, it has been shown that Arf6 mediates syndecan recycling through endosomal compartments by a process controlled by syntenin PDZ domain-PIP_2 interaction. Syndecans that cannot recycle via this pathway become trapped intracellularly and inhibit cell spreading. This syntenin-mediated syndecan recycling pathway may regulate the surface availability of a number of cell adhesion and signaling molecules [35].

Lipid rafts are phospholipid- and cholesterol-rich plasma membrane domains that provide fluid platforms to segregate membrane components and dynamically compartmentalize membranes [36]. These domains contain specific sets of proteins that include GPI-anchored proteins, doubly acetylated proteins (Src family tyrosine kinases), Gα subunit of heterotrimeric G-proteins, and palmitylated proteins including endothelial NO synthase [37,38]. Caveolae are a subset of lipid rafts. Caveolins are the principal building blocks of caveolae and are usually accepted as its marker. Caveolin-1 oligomeric complex creates a stable membrane structure that transiently interacts with plasma membrane and endosomes [39]. Caveolae are flask-shaped invaginations of the plasma membrane [40] that play a role in regulation of certain endocytic pathways and regulation of enzymes such as endothelial NO synthase. A variety of protein- and lipid-signaling molecules are concentrated in caveolae. These include PKC, Rho GTPases, and nonreceptor tyrosine kinases such as c-Src, Yes, Fyn, phosphatidylinositol 3-kinase, and phosphatidylinositol kinases, among others [40-42].

It has been shown that GPI anchored heparan sulfate proteoglycan glypican-1 undergoes rapid endocytosis [43]. Most of the intracellular glypican-1 carries truncated heparan sulphate chains with more N-unsubstituted glucosamine residue than the cell-surface variant. The presence of N-unsubstituted glucosamine residue in glypican-1 occurs by an autocleavage

mechanism dependent on nitric oxide and divalent copper ions [44]. The NO-dependent cleavage takes place in caveolin-1-containing endosomes [45], after an initial degradation step catalysed by an endoheparanase [46]. Also, syndecan-4 is predominantly present in the nonraft compartments in unstimulated cells, whereas clustering with FGF2 or anti–syndecan-4 antibody induces its shift to lipid rafts [47]. The shift of syndecan-4 to lipid rafts seems to be necessary for the initiation of FGF2 endocytosis because its blockade by cholesterol depletion from the plasma cell membrane completely blocks FGF2 uptake [48]. FGF receptor accompanies syndecan along the syntenin-mediated recycling pathway, in a heparan sulfate- and FGF-dependent manner. [35]. Similarly, low-density lipoprotein (LDL) causes clustering and clathrin-independent uptake of syndecan-1, syndecan-2, and syndecan-4 [49]. LDL clustering also induced a shift of syndecan-1 into the lipid rafts [49].

Macropinocytosis seems to provide a more efficient route for the cytosolic entry of cell penetrating-peptides and basic proteins since macropinosomes have an intrinsic leakiness compared with the other types of endosomes [50]. Also, macropinosomes do not necessarily fuse into lysosomes, a circumstance that prevents the degradation of their contents [51]. Internalization of a number of basic peptides requires uptake by endocytosis, initiated by binding to anionic cell surface heparan sulfate proteoglycans, followed by escape from endosomes. Syndecan-4 with caveolin-1–positive vesicles seems to be related to a fast delivery of syndecan-4 pinosomal cargo to intracellular destinations [52,53].The endosomal escape is governed by the concentration of the endocyted molecules and by the transmembrane pH gradient (inside acidic) across phospholipid bilayers [54].

Once internalized, FGF-2 has been observed in the cell nucleus of proliferative cells. Controlled nuclear entry of exogenous FGF-2 was observed around the G1 restriction point of the cell cycle and seemed to depend on cell proliferation [55]. As mentioned above, the internalization of FGF-2 is consequence of syndecan-4 endocytosis [48]. Heparan sulfate proteoglycans have also been detected in nuclear compartments [56]. Syndecans are translocated to the nucleus in a time-dependent manner. The mitotic spindle shows more syndecans than that found in interphase cells [55]. Glypican also has the putative nuclear localizations motifs. C6 cells entering the G1 phase acquire prominent foci of nuclear glypican immunoreactivity, suggesting that this nuclear localization may be related to the cell proliferation [57]. Interestingly, it has been observed that heparan sulfate proteoglycans is mainly synthesized by proliferative cells during G1 phase of the cell cycle [58,59]. So, according to this scenario, these data strongly suggest that FGF-2 can penetrate into the proliferative cells via heparan sulfate proteoglycans synthesized during G1 phase of the cell cycle.

3. Caveolin-integrin-proteinases-syndecan cooperation governs cell adhesion and cell migration

Cell adhesion to fibronectin (FN) triggers the formation and maturation of adhesion complexes by modulating the activity of the Rho family of GTPases. Cells plated onto a ligand of integrin alpha5beta1 spread but fail to form focal adhesions or fully organize actin into bundled stress fibres unless co-stimulated with a ligand of syndecan 4. Engagement of syndecan 4 in such pre-spread cells recapitulates the Rac1 and RhoA activation profiles observed during spreading on whole FN. Furthermore, since adhesion to a ligand of alpha5beta1 alone does not activate Rac1, engagement of syndecan 4 appears to be an absolute requirement. Thus, integrin-syndecan cooperation governs the assembly of signaling complexes during cell spreading [60]. Downregulation of caveolin-1 results in inhibition of fibronectin internalization and degradation [61]. Previous studies have been shown that downregulation of caveolin-1 also suppressed the localization of β1-integrin to caveolae HCT 116 human colorectal carcinoma cell. These data suggest also the participation of fibronectin receptors such as integrins or syndecans in caveolae-dependent uptake of fibronectin.

It has been hypothesized that caveolae/caveolin-1 serve as organizing centers for cell-surface proteolysis in order to modulate proteolytic events that lead to ECM degradation and invasion of HCT 116 human colorectal carcinoma cell. Caveolin-1 is implicated in both the intracellular and the pericellular degradation of type-IV collagen by HCT 116 cells, probably through regulation of caveolae-associated proteases. Cathepsin B, pro-urokinase plasminogen activator and p11 colocalize into caveolae of HCT 116 human colorectal carcinoma cells, an association mediated by active K-RAS [62]. Up regulation of proteases and their trafficking to tumor cell surfaces have been shown to correlate with increased degradation of ECM proteins during tumor invasion. Translocation of cathepsin B from the apical region of the cells to the basal plasma membranes parallels malignant progression of colon cancer, occurring in late adenomas and early carcinomas [63]. Many of the cell-surface proteases that degrade ECM have been localized to caveolae of cancer cells [64]. Colocalization of cathepsin B and p11 to caveolae of colon cancer cells is important because cathepsin B activates soluble and membrane-bound pro-uPA [65,66] and p11, the light chain of annexin II tetramer, is involved in the binding and activation of plasminogen [67], events associated with cell-surface proteolytic events during tumor invasion. uPA is involved in tumor-cell invasion by converting plasminogen to plasmin, which can either directly degrade ECM components or activate MMPs. Several MMPs, including MMP-2 and MT1-MMP, have been localized to caveolae of glioblastomas, fibrosarcomas and endothelial cells [68-70], consistent with the

membrane region facilitating local invasion. Caveolae have also been identified as a novel pathway for the trafficking of MT1-MMP (MMP-14) during angiogenesis, regulating the localization, activity and function of the enzyme in migrating endothelial cells [69].

During cell migration, pools of syndecan-1, syndecan-4, and caveolin-1 are directed into the same retracting region of the moving cell [71-73]. The caveolin property of inhibiting kinase activity of a variety of proteins involved in leading edge formation was speculated to play a key role in cell migration [74]. In contrast to the deactivating role of caveolin, syndecan-4 is needed for activation of several kinases, including focal adhesion kinase (FAK), which results in increased turnover of focal adhesions [75-77]. Silencing of caveolin-1 gene expression or introduction of a dominant-negative mutant of syndecan-4 leads to slow migration and impaired tube formation of endothelial cells on Matrigel [71, 78]. Syndecan-4 and caveolin-1 knockout mice demonstrate reduced postnatal angiogenesis that may be related to the impairment of endothelial cell migration [74,79]. Whereas syndecan-4 does not colocalize with caveolin-1 at the cell surface, syndecan-4– and caveolin-1–containing vesicles frequently move in tandem, presumably along microtubules [47]. Remarkably, internalization of caveolae depends on Src and PKC_{α} activation [80]. It is well established that syndecan-4 can activate PKC_{α} and syndecan-3 can also activate Src [81]. Interestingly, it had been shown that heparan sulfate proteoglycans can bind both cathepsin B [82] and annexin II tetramer. More, the secretion of macrophage urokinase plasminogen activator and MMP-9 is dependent on proteoglycans [14]. Take together, these data show that syndecan and glypican may be responsible for modulation of several functions of caveolae, mainly those involved in the control of endocytosis, cell adhesion and cell migration [83,84].

4. Glycosaminoglycans modulate proteolytic enzymes in inflammatory response

The interaction of glycosaminoglycans with proteins regulates a broad spectrum of biological processes. These proteins fall into quite diverse groups, such as proteins involved in hemostasis, proteins of extracellular matrix, growth factors, proteins of lipid metabolism, oxidative stress, inflammatory response and others [85]. Many of these biological processes are regulated by proteolytic enzymes.

Indeed, it has been shown that heparin can modify the activities of some serine proteinases and its natural inhibitors *in vitro* [7,86,87] and also that heparan sulfate proteoglycans, syndecan-1 ectodomain, and syndecan-4 ectodomain are shed into acute inflammatory wound fluids [88]. The purified syndecan-1 ectodomain protects cathepsin G from inhibition by α1-antichymotrypsin and

squamous cell carcinoma antigen 2, and it protects elastase from inhibition by α1-proteinase inhibitor. Moreover, the degradation of endogenous heparan sulfate from wound fluids reduces proteolytic activities in the fluid. These results strongly suggest that syndecan-1 and syndecan-4 maintain the proteolytic balance in acute wound fluid [89]. Syndecans, via their heparan sulfate chain, bind many of the factors that orchestrate the inflammatory response to tissue injury, as well as a variety of extracellular matrix components [88,89].

Mast cells are well known for their harmful effects during inflammatory conditions such as asthma and allergy, but they are also important for our innate defense against bacterial infections and parasites [90-92]. Mast cell is activated and thereby undergoes degranulation by antigen-mediated cross-linking of IgE molecules bound to their high-affinity receptor FcεRI. Various preformed inflammatory mediators are released from mast-cell secretory granule upon degranulation, including histamine, cytokines, and a range of proteases, tryptases, chymases, cathepsin E and carboxypeptidase A. These inflammatory mediators are tightly associated with heparin proteoglycan within the mast-cell. The association of cathepsin E and carboxypeptidase A with heparin proteoglycan seems to be functionally important by bringing cathepsin E into close contact with pro-CPA, thus facilitating the proteolytic action of cathepsin E on pro-CPA [93]. Similarly, it has been shown that the heparin part of the heparin-chymase complex attracts heparin-binding substrate proteins and thus presents them to the chymase for efficient proteolysis [94].

Glycosaminoglycans regulate cysteine cathepsins proteinases activities

Papain is considered to be the archetype of cysteine proteinases. The papain-like cysteine proteinases are the most abundant among the cysteine proteinases. This family consists of papain and related plant proteinases, such as chymopapain, caricain, bromelain, actinidin, ficin, and aleurain, and the lysosomal cathepsins B, H, L, S, C, K, and X [95,96]. The lysosomal cysteine proteinases cathepsins B and L have been implicated in a variety of pathological conditions, especially in diseases involving tissue remodeling states, such as tumor metastasis [97,98], parasite infection [99,100], arthritis [101], and other types of inflammatory injury [102].

Cathepsins B and L can participate in metastasis formation by degradation of several extracellular matrix components [103-105]. Confocal microscopy image analysis indicated that cathepsin B was associated with the external basal cell surface in the murine B16 amelanotic melanoma cells [106] has been shown that membrane-bound forms of cathepsin display modified properties, *e.g.* resistance to inactivation at alkaline pH [107]. Previous results in the

literature have shown that papain and cathepsin B are able to bind to laminin of basement membrane [108]. These results are consistent with the proposed role of cysteine proteinases in degradation of extracellular matrix components. Therefore, the interaction of cysteine proteinases of the papain superfamily with glycosaminoglycans is of significant interest for the understanding about the biological role of cysteine proteinases in tissue remodeling states. In addition, cathepsin X, other lysosomal cysteine proteinase, is capable to bind at cell surface heparan sulfate proteoglycan. This interaction can regulate the cellular trafficking and the enzymatic activity of cathepsin X [96].

The lysosomal cysteine proteinases are synthesized as an inactive zymogen, and further converted into its active form after removal of the proregion, via an autocatalytic mechanism at acidic pH of lysosomal vesicles. Glycosaminoglycans and dextran sulfate have been known to be able to accelerate the conversion of zymogen forms of cysteine cathepsins into its mature forms, this process was observed to the activation of cathepsin B [109], cathepsin L [110], cathepsin S [111] and congopain [112]. The location of glycosaminoglycans in lysosomes as the result of proteoglycan recycling, in the extracellular matrix, at the cell surface and at basement membranes supports the proposal that GAGs are involved in in vivo processing of cysteine cathepsins both inside and outside lysosomes. Interestingly, procongopain [112], a parasitic cysteine protease from Trypanosoma congolense, and the mammalian procathepsin S [111], were found to be capable of autocatalytic activation not only at the acidic pH of lysosomes but also at neutral pH in the presence of dextran sulfate and related glycosaminoglycans [113].

The expression of cathepsins S and K has been described in smooth muscle cells and macrophages, both cell types are critical in the pathophysiology of the arterial vascular system [114,115]. Elastin is an integral component of the intima and responsible for the elasticity of the blood vessels. Degradation of the elastin matrix leads to a destabilization of the vessel structure eventually causing aneurysms or blood vessel ruptures. Weiss and co-workers [116,117] have suggested that thiol-dependent cathepsins and matrilysin, are the major elastolytic enzymes in macrophages. Moreover, cathepsin-mediated elastolytic activities in atherogenic plaques have been implicated in plaque formation and plaque rupture [118]. Human cathepsin K, followed by cathepsins S and L, displayed the highest activity against elastin at pH of 5.5. However, it has been demonstrated that cathepsin V has the most potent elastase activity in mammalian cells yet described. Cathepsin V is only detectable in activated macrophages within intimal plaque specimens [119]. Interestingly, chondroitin sulfate specifically inhibits the elastolytic activities of cathepsins V and K. In contrast, cathepsin S, which does not form complexes with chondroitin sulfate, is not inhibited; thus suggesting a specific regulation of elastolytic activities of cathepsins by GAGs. In contrast to

cathepsin S, cathepsins K and V are not constitutively expressed in monocytes and are induced only during maturation into macrophages. The inhibition of those two enzymes by GAGs may constitute an additional protective mechanism against excessive elastin degradation during inflammatory processes whereas cathepsin S may have a house keeping function in elastin turnover. During atherogenesis, however, a depletion of the GAG content in blood vessel walls has been observed [120] which in turn may lead to an increase of the elastolytic activities of inducible cathepsins K and V and thus to an exaggeration of the blood vessel wall erosion.

Also, the degradation of collagen is observed in bone remodeling, wound healing, angiogenesis, and during organ development [121-123] and in several diseases, such as osteoporosis [124], arthritis [125], aneurysms of blood vessels [126] and metastasis [127]. The triple helical collagens, in particular type I and II collagens, are highly resistant to general proteolysis and require specific proteases for their degradation. The mammalian enzymes with collagenolytic activities include members of the matrix metalloprotease family such as MMP-1, -2, -8, -13, and -14 [128], the serine protease, human neutrophil elastase [129], and thiol-dependent cathepsins [121]. It has been shown that cathepsins B and L cleave in the non-helical telopeptide extensions of collagens [130,131]. However, cathepsin K cleaves the collagen at the triple helical region [132-135]. The biological relevance of the collagenolytic activity of cathepsin K was underlined by the finding that deficiency in this protease causes the bone-sclerosing dysplasia, pycnodysostosis, in man [136] and an osteopetrotic phenotype in mice [137]. Interestingly, the collagenolytic activity of cathepsin K is critically dependent on the interaction with chondroitin sulfate present in bone matrix, whereas collagenolytic activities of cathepsins L and S are potently inhibited by glycosaminoglycans [138].

The interaction of heparin and other glycosaminoglycans with papain may be representative of many mammalian cysteine proteinase-glycosaminoglycan interactions that can regulate the function of this class of proteinases in vivo [82]. The binding of heparin to the papain perturbs its catalytic activity upon substrates. Also, heparin shifts 0.5 unit of the papain pH activity profile to the right, allowing papain to be active at alkaline pH. In addition, the presence of heparin reduces the loss of papain α-helix content induced by alkaline pH. Heparin increases the stability of papain at alkaline pH, which is a reflex of the higher helix amount observed for the papain-heparin complex compared with that for the papain alone. Although a heparin-binding domain in papain has not yet been demonstrated, the papain sequence 188–191 (RIKR) is a putative heparin-binding site, as previously suggested [139]. The putative heparin-binding site in the 188–191 stretch of the papain displays β-strand structure [140]. This cationic sequence is located adjacent to the residues Asn175 and Trp177. In papain, it is known that Asn175 [141] and Trp177 [124] contribute

to the electrostatic field, and the residues influence the formation of the catalytically active thiolate-imidazolium ion pair, whereas residue Trp177 is also involved in enzyme substrate interactions [142]. Binding of heparin or heparan sulfate to papain may change the relative orientation of the surface structures, forcing a conformational change in the protein. This conformational change could be communicated to the rest of the protein via tertiary structure or disulfide bonds [143]. Both Arg and Lys residues are found in the established heparin-binding domains various proteins. Also, the heparin binding may stabilize the papain helices by eliminating detrimental electrostatic interactions, and the negatively charged sulfate and carboxylate groups of heparin may neutralize positive charges that might otherwise contribute to helix destabilization [144].

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