CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Blood coagulation involves the sequential activation of a series of serine protease, which culminates in the generation of thrombin and subsequently thrombin-catalysed conversion of fibrinogen into insoluble fibrin (Furie and Furie, 1988). Inhibitory modulation of this process of paramount physiological importance is primarily achieved by two principally different mechanisms. The enzymes may be inactivated by serine proteinase inhibitors, known as serpins, which act by formation of stable 1:1 molar complexes with their target enzymes (Travis and Salvesen, 1983). Alternatively, the so-called protein C pathway leads to inactivation of auxiliary coagulation proteins (factor Va and VIIIa) by cleavage at distinct sites (Esmon, 1989; Dahlback, 1991). The prime of regulation is the surface of vascular endothelial cells, which has been known to possess anticoagulant properties (Colburn and Buonassisi, 1982). These properties are particularly conspicuous in the microcirculation, with its high wall surface to blood volume ratio (Busch, 1984).

The ability of certain sulfated polysaccharides, glycosaminoglycans, to interfere with blood coagulation has a long-standing record, as illustrated by the extensive clinical use of heparin as an antithrombotic agent (Roden, 1989). The main effect of heparin (and of its relative heparan sulfate) is to accelerate the inactivation of coagulation enzymes by the serpin antithrombin (Rosenberg 1977; Bjork and Lindahl, 1982). A more complex picture emerged with the finding of an additional, heparin cofactor II, which is activated not only by

heparin, but also by another glycosaminoglycan, dermatan sulfate, and which selectively inactivates thrombin (Tollefsen *et al.*, 1982). Remarkably, also the other major regulatory mechanism, the protein C pathway, involves a glycosaminoglycan-containing molecular species, since the protein C activation cofactor, thrombomodulin, turned out to be a proteoglycan with a functionally important, covalently bound glycosaminoglycan chain (Bourin and Lindahl, 1990).

Heparin, an anticoagulant mucopolysaccharide, plays the most important role in the accelerated inhibition of the thrombin activity together with antithrombin-III (heparin cofactor) (Rosenberg and Damus, 1978). There are many investigations to reveal the relationship between its antithrombogenic activity and the chemical structure. In this field, a couple of major directions are suggested for investigations: firstly, determinations of the role of each functional group of heparin by some chemical treatment to N-sulfate groups or carboxyl groups (Danishelsky and Sishovic, 1976), secondly, approaches by means of model compounds which have similar skeletal structure to that of heparin from some natural polymers such as cellulose, dextran or chitin. Though important information could be obtained by the former method, the latter is one of the useful ways to produce heparin-like compounds. Moreover, it is expected that these artificially-synthesized heparin-like polymers contribute to the development of biomedical materials. Since the usefulness of heparin and its biological activities, a number of polysaccharide sulfates have been synthesized and their biological activities are examined, among them, most extensively, sulfated chitosan.

Chitin, a poly-β(1→4)-N-acetyl-D-glucosamine, is one of the most abundant polysaccharides in nature (Yalpani *et al.*, 1992). It is present in the exoskeleton and appendages of arthropods, and it is also a major constituent of the cell walls of fungi (Parisher *et al.*, 1989). Chitosan, an unbranched (1→4)-linked 2-amino-2-deoxy-β-D-glucan, is prepared by chemical N-deacetylation of chitin. Chitin and its derivatives have been used in a variety of biomedical applications (Yalpani *et al.*, 1992).

As chitin has a similar skeletal structure to heparin, its biomedical properties have received considerable attention. Chitosan has been used to prepare heparin-like derivatives, because it can be chemically modified under heterogeneous conditions in few reaction steps (Nishimura *et al.*, 1986). Several synthetic types of chitin heparinoids have also been reported (Cushing *et al.*, 1954; Wolfrom and Shan Hen, 1958; Whistler and Kosik, 1971; Horton and Just, 1973; Muzzarelli *et al.*, 1984, Hirano *et al.*, 1985). Muzzarelli (1984) have reported the preparation of sulfated N-(carboxymethyl)chitosan as a new type of heparinoid derived from partially deacetylation chitin (degree of deacetylation 0.58) and the main advantage of using this compound instead of sulfated chitosan is the lack of adverse effects on cellular structures rather than their antithrombogenic actions. The introduction of 6-O-carboxymethyl groups to the chitin molecules has been reported to suppress the binding of bovine fibrinogen (Nishimura *et al.*, 1984) which is known as a main factor in the toxicity of heparinoids, especially in the case of dextransulfate. However, the chromium trioxide oxidation of hydroxymethyl groups seems to be much more complicated (Horton and Just, 1973) and some depolymerization may also take place during oxidation. The method of O-carboxymethylation has been improved (Tokura *et al.*,

1983). It was shown to selectively occur at O-6 position of GlcNAc residues owing to its crystalline structure. The biodegradability of chitin has also been enhanced by 6-O-carboxymethylation (Nishimura *et al.*, 1986a). The other approaches such as sulfation of chitin or chitosan with various degree of deacetylation provided different inhibitory effect on thrombin activity. It was found that N-sulfate groups, as well as O-sulfate and O-carboxymethyl groups are required for full inhibitions (Nishimura *et al.*, 1986b).

There are many investigations involving in the biological and biomedical properties of chitosan and chitosan-derived materials (Muzzarelli *et al.*, 1986). In particular, derivatives having N- and/or O-sulfate groups either alone or in conjunction with other substituents have been examined as a potential heparinoids. A variety of sulfating reagents and reaction media have been used for sulfation, however, there were always some weak points of each such as extensive degradation of the polymer chains (Nagasawa *et al.*, 1972). The other factors influenced in the sulfation including time and temperature of the reaction. Sulfation reactions of multi-functional polysaccharides are inevitably followed by the appearance of structural heterogeneity in the polymer chains. When chitosan is sulfated, a structural variety of products were obtained. This may be related to the various reactivities of the three functional groups of the parent polymer, leading to different degrees of completion in the individual groups (Gamzazade *et al.*, 1997). This gives rise to uncertainty but on the other hand some structures that emerge from random distribution of the modified groups along the chain can reveal new features of biological functions. In general, only the influence of molecular weight and/or substitution degree of sulfated polysaccharides on their biological activities is considered in a majority of works involving in the anticoagulant or antiviral properties of these substances (Horton and Usui, 1978). The

solubility properties of chitosan and its derivatives, and their use in biomedical applications, entail some problems in common.

Thus, taken together, synthesis of the heparin-mimetic compound from a starting material, chitosan derived from the shells of marine crabs in a semi-heterogeneous and mild conditions appears to be a challenge approach. This synthetic sulfated chitosan will be studied for its anticoagulant activities. In addition, its involvement in the immune response *in vitro* will be observed.

1.2 Objectives

The main objective of this study was to synthesize the heparin-mimetic compound, chitosan polysulfate. The raw material was a commercial chitosan which was derived from the shell of marine crabs. The feasibility of the synthesis in a semi-heterogeneous phase with some modifications for the mild conditions was performed. The resulting product was separated into different average molecular weights and purified. The chitosan polysulfate was analyzed for their chemical properties and determined for their anticoagulant activities. Finally, they were studied *in vitro* in order to evaluate whether they would effect on the human immune response.

1.3 Scope of the study

This study was divided into 5 steps including:

1. Preparation of chitosan polysulfate by random substitution
2. Isolation and purification for a particular molecular weight
3. Chemical and structural analysis by spectroscopic and physical methods
4. Investigation of its anticoagulant activities
5. Investigation of its involvement in the immune response *in vitro*

1.4 Literature reviews

1.4.1 Chitin and chitosan

Chitin is a high molecular weight linear polymer of N-acetyl-D-glucosamine (N-acetyl-2-amino-2-deoxy-D-glucopyranose) units linked by β–D(1⟶4) bonds. It is a highly insoluble material assembling cellulose in its solubility and low chemical reactivity. It may be regarded as a cellulose with hydroxyl at position C-2 replaced by an acetamido group. Like cellulose, it naturally functions as a structural polysaccharide (Figure 7). It is most abundant in crustaceans, insects and fungi (Yalpani *et al.*, 1992). Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. Chitosan is the N-deacetylated derivative of chitin, though this N-deacetylation is almost never complete (Yalpani *et al.*, 1992). A sharp nomenclature border has not been defined between chitin and chitosan based on the degree of N-deacetylation. Chitin and chitosan are commercial interesting due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulose (1.25%) (Muzzarelli *et al.*, 1986). This

makes chitin a useful chelating agent. As most of the polymers are synthetic material, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, chitin, chitosan and their derivatives. However, these natural abundant materials also exhibit a limitation in their reactivity and processability. In this respect, chitin and chitosan are recommended as suitable resource materials, because the natural polymers have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption property and so on.

Nowadays, much attention has been paid to chitosan as a potential polysaccharide resource. Although, several efforts have been reported to prepare functional derivatives of chitosan by chemical modifications, only few example attained solubility in general organic solvents (Pariser and Lombordi, 1980). Chitosan in soluble aqueous solutions of some acids, some selective N-alkylidenations, and N-acylations have also been attempted. Though, several water-soluble or highly swelling derivatives were obtained, the development of solubility in common organic solvents was found to be difficult by these methods. Chemically modified chitin and chitosan structure which resulting in the improved solubility in general organic solvents has been reported by many workers (Pariser and Lombordi, 1980).

1.4.1.1 Processing of chitin and chitosan

Chitin is easily obtained from crab or shrimp shells and fungal mycelia (Yalpani *et al.*, 1992). In the first case, production is associated with food industries such as shrimp canning. In the second case, the production of chitosan–glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from *Aspergillus niger*, *Mucor rouxii*, and *Streptomyces*, which involves alkali treatment yielding chitosan–glucan complexes (Muzzarelli *et al.*, 1986). The alkali removes the protein and deacetylates chitin simultaneously. Depending on the alkali concentration some soluble glycan are removed (Hackman, 1954). The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate that is present in crab shells at high concentrations. The resulting chitin is deacetylated in 40% sodium hydroxide at 120 °C for 1-3 hrs (Hackman, 1954). This treatment produces 70% deacetylated chitosan.

1.4.1.2 Economic aspects of chitin and chitosan (Ravi Kumar, 1999)

The production of chitin and chitosan is currently based on crab and shrimp shells discarded by the canning industries in Oregon, Washington, Verginia and Japan and by various fishing fleets in the Antarctic. Several countries possess large unexploited crustacean resources e.g. Norway, Mexico, Chile and Thailand. The production of chitosan from crustacean shells obtained, as a food industry waste is economically feasible, especially if it includes the recovery of carotenoids. The shells contain considerable quantities of astaxanthin, a carotinoid that has so far not been synthesized, and which is marketed as a fish food additive in aquaculture, especially for Salmon.

To produce 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required in addition to nitrogen, processed water (0.5 ton) and cooling water (0.9 ton). Important items in estimating the production cost include transportation, which varies depending on labor and location. The worldwide price of chitosan is US $ 7.5 / 10 gm. (Sigma-Aldrich price list). In India, Central Institute of Fisheries Technology, Kerala, has initiated the research on chitin and chitosan. From their investigation, they found that dry prawn waste contained 23% and dry squilla contained 15% chitin. They have also reported that chitinous solid waste fraction of average Indian landing of shell fish ranged from 60,000 to 80,000 tons. Chitin and chitosan are now produced commercially in India, Japan, Poland and Australia.

1.4.1.3 Properties of chitin and chitosan

Most of the natural occurring polysaccharides e.g. cellulose, dextran, pectin, alginic acid, agar, agarose, carrageenans are neutral or acidic in nature, whereas, chitin and chitosan are examples of highly basic form of polysaccharides. Their unique properties include polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics (Muzzarelli, 1973).

Like cellulose, it naturally functions as a structural polysaccharides, but differs from cellulose in the properties. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugations with aqueous solutions of mineral acids and dimethyl acetamide containing 5% lithium chloride (Muzzarelli, 1977). Chitosan, the deacetylated product of chitin is soluble in very dilute acids like acetic acid, formic acid etc. Recently, gel forming ability of

chitosan in N-methylmorpholine-N-oxide and its application in controlled drug release formulations has been reported (Muzzarelli, 1977). Hydrolysis of chitin with concentrated acid under drastic conditions produces relatively pure amino sugars, D-glucosamine (Muzzarelli, 1977).

The nitrogen content in chitin are varies from 5% to 8% depending on the extent of deacetylation (Muzzarelli, 1977) whereas, the nitrogen in chitosan is mostly in the form of primary aliphatic amino groups. Chitosan, therefore, undergoes the typical reactions to form amines, of which N-acetylation and Schiff reaction are the most important. Chitosan derivatives are easily obtained under mild conditions and can be considered as substituted glucans. N-acylation with acid anhydrides or acyl halides introduces amino groups at the chitosan nitrogen. Acetic anhydride affords fully acetylated chitins. Linear aliphatic N-acyl groups above propionyl, permit rapid acetylation of hydroxyl groups. High benzoylated chitin is soluble in benzyl alcohol, dimethyl sulfoxide, formic acid and dichloroacetic acid. The N-hexanol, N-decanoyl and N-dodecanoyl derivatives have been obtained in methanesulfonic acid.

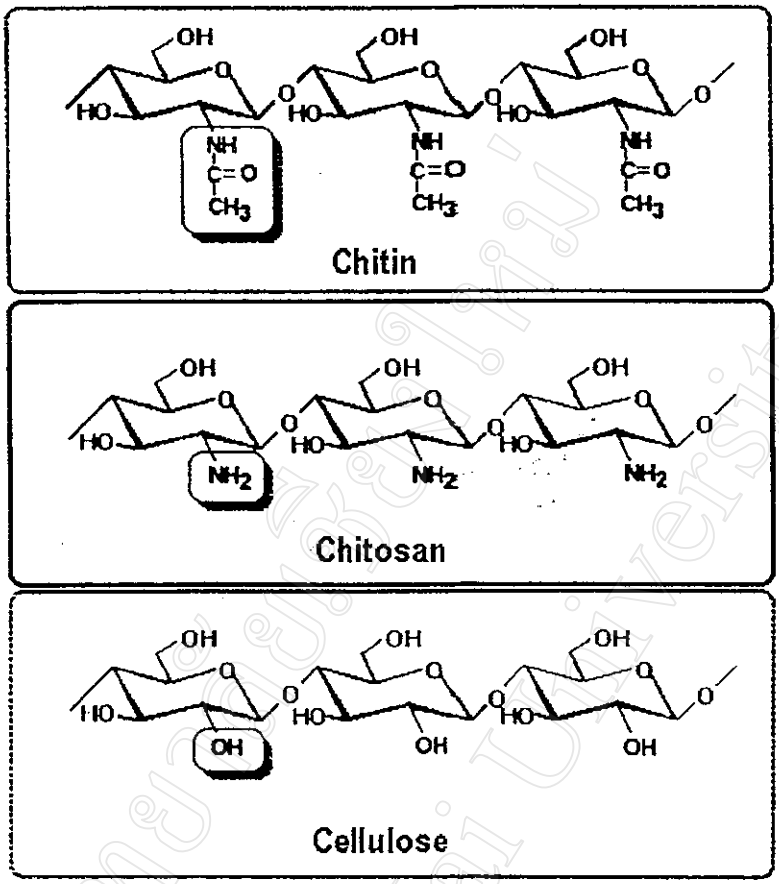

Figure 1 Structural formula of chitin, chitosan and cellulose (Yalpani, 1992)

At room temperature, chitosan forms aldimines and ketimines with aldehydes and ketones, respectively (Yalpani *et al.*, 1992). Reactions with ketoacids followed by reaction with sodium borohydride produces glucans carrying proteic acid and non-proteic amino groups. N-carboxymethyl chitosan is obtained from glyoxylic acid (Trujillo, 1968). Examples of non-proteic amine acids glucans derived from chitosan are the N-carboxybenzyl chitosans obtained from *o*- and *p*-phthalaldehydic acids (Muzzarelli, 1977). Chitosan and simple aldehydes produce N-alkyl chitosan upon hydrogenation (Nud'ga *et al.*, 1973). The presence of the more or less bulky substituent can weaken the hydrogen

bonds of chitosan, therefore, N-alkyl chitosan swell inspite of the hydrophobicity of alkyl chains. They retain the film forming property of chitosan.

1.4.1.4 Physical and chemical characterization

Cellulose is a homopolymer, while chitin and chitosan are heteropolymers. Neither random nor block orientation is meant to be implied for chitin and chitosan. The properties of chitin and chitosan like origin of material, degree of N-deacetylation, molecular weight, solvent and solution properties are stated in brief.

Degree of N-deacetylation

An important parameter to closely examine is the degree of N-acetylation in chitin, i.e., the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose structural units. This ratio has striking the effect on chitin solubility and solution properties. Chitosan, the universally accepted non-toxic N-deactetylated derivative of chitin (Muzzarelli, 1977), where chitin is N-deacetylated to such an extent, that it becomes soluble in dilute aqueous acetic acid and formic acids. To define this ratio, attempts have been made with IR spectroscopy (Shigemasa *et al.*, 1996), pyrolysis gas chromatography (Sato *et al.*, 1998), gel permeation chromatography (Aiba, 1987), uv spectrophotometry (Tan *et al.*, 1998), ^{1}H-NMR spectroscopy (Aiba, 1987; Tan *et al.*, 1998), ^{13}C solid state NMR (Duarte *et al.*, 2001) and various titration schemes (Prochazkova *et al.*, 1999).

Molecular weight

The average molecular weight (Mw) of chitin and chitosan has been determined by light scattering measurement. Viscometry is a simple and rapid method of the determination of Mw, the constant and K in the Mark-Houwink equation have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution (Roberts and Domszy, 1982). The intrinsic viscosity is expressed as:

$$[\eta] = km\alpha = 1.81 \times 10^{-3} M^{0.93}$$

To the charged nature of chitosan in acid solvents and chitosan's propensity, to form the aggregation complexes requires care when applying these constants. Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The average molecular weight of chitin in 1.03×10^6 to 2.5×10^6 kDa, but the N-deacetylation reaction reduces this to 1×10^5 to 5×10^5 kDa (Yalpani *et al.*, 1992).

Solvent and solution properties

Both cellulose and chitin are highly crystalline, intractable materials and only a limited number of solvents are known (Muzzarelli, 1977), which are applicable as reaction solvents. Chitin and chitosan can degrade before melting (Yalpani, 1992), which is a typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve chitin and chitosan in an appropriate solvent system to impact functionality. For each solvent system, polymer concentration, pH, counterion concentration and temperature

effects on the solution viscosity must be known. The comparative data from solvent to solvent are not available. As a general rule, researchers dissolve the maximum amount of polymer in a given solvent that still retained homogeneity and then regenerated in the required form. A coagulant is required for polymer regeneration of solidification. The nature of coagulant is also highly dependent on the solvent and solution properties as well as the polymer used (Muzzarelli, 1977).

1.4.1.5 Applications of chitin and chitosan

The interest in chitin originates from the study of behavior and chemical characteristics of lysozymes, an enzyme present in the human body fluids. It dissolves certain bacteria by cleaving the chitinous material of the cell walls (Uchida and Izume, 1989). A wide variety of medical applications for chitin and chitosan derivatives have been reported over the last three decades. It has been suggested that chitosan, may be used to inhibit fibroplasias in wound healing and to promote tissue growth and differentiation in tissue culture (Shigemasa and Minami, 1996).

The poor solubility of chitin is the major of limiting factor in its utilization and investigation properties and structure. Despite these limitations, various applications of chtitin and modified chitins have been reported in the literature (Yalpani, 1992), e.g. as raw material for man-made fibers. Fibers made of chitin and chitosan have been useful as absorbable sutures and wound-dressing materials. The chitin sutures resist attack in bile, urine and pancreatic juice, which are problem areas with other absorbable sutures. It has been claimed that wound dressing made of chitin and chitosan fibers have potential applications in waste-water treatment, where the removal of heavy metal ions by chitosan

through chelation has received much attention. Their use in the apparel industry, with a much larger scope, could be a long-term possibility.

Many applications of chitosan (Muzzarelli, 1973; Muzzarelli, 1977; Yalpani,1992) are summarized in Table 1.

Table 1 Applications of chitosan

Fields	Applications
Wastewater treatment	-Removal of metal ions
	-Flocculant/coagulant: protein, dye, amino acids
Food industry	-Removal of dye, suspended solids etc.
	-Preservation
	-Colour stabilization
	-Animal feed additive
Medical	-Bandages
	-Blood cholesterol control
	-Controlled release of drugs
	-Skin burns
	-Contact lens etc.
Biotechnology	-Enzyme immobilization
	-Protein separation
	-Cell recovery
	-Chromatography
	-Cell immobilization
Agriculture	-Seed coating
	-Fertilizer
	-Controlled agrochemical release
Cosmetics	-Moisturizer
	-Face, hand and body creams
	-Bath lotion etc.
Pulp and paper	-Surface treatment
	-Photographic paper
Membrane	-Permeability control
	-Reverse osmosis

1.4.1.6 Chitin, chitosan and its derivatives

Chitosan, and its parent biopolymer chitin, are objects of substantial research into their utilization by industry and biotechnology (Muzarelli *et al.*, 1986; Muzzarelli, 1977). This interest has given rise to the preparation of chitosan-based materials in a variety of N-, O-, and N, O-substituted forms, having substituents that include alkyl, acyl, and imino groups (Muzzarelli, 1985). In general, hydrophilic adducts such as carbohydrate branches (Hall and Holme, 1986), or carboxyalkyl substituents (Muzzarelli, 1981; Muzzarelli *et al.*, 1982; Hayes, 1986), render the chitosan soluble. Alternatively, some derivatives are of interest less for their solubility than their ability to form membranes (Muzzarelli, 1973) or films (Hayes, 1986; Muzzarelli *et al.*, 1986), or act as metal-chelating agents (Muzzarelli *et al.*, 1982; Muzzarelli *et al.*, 1984). Furthermore, there has been a growth in research activity into the biological and biomedical properties of chitosan and chitosan-derived materials (Muzzarelli *et al.*, 1986). Chitin and chitosan have been modified into a variety of derivatives in order to make it more soluble in organic solvents or even water. The ultimate aim was its applications in many fields of interests. These modifications includes cyanoethylation (Nud'ga *et al.*, 1975), carboxymethylation (Okimasu, 1958; Miyazaki and Matsushima, 1968; Hayashi *et al.*, 1968; Trujillo, 1968), hydroxyethylation (Senju and Okimasu, 1950), N-alkylation (Nud'ga *et al.*, 1973), xanthation (Noguchi *et al.*, 1973), glycosylation (Neely, 1964). However, there was no reported about the application of these chitin and chitosan derivatives. In particular, derivatives (Warner *et al.*, 1958; Horton and Just, 1973; Nishimura *et al.*, 1986; Muzzarelli *et al.*, 1984; Hirano *et al.*, 1985) having N- and/or O-sulfate groups either alone or in conjunction with other substituents have been examined as potential heparinoids. Another approach for the preparation of heparinoid

polymers involves the attachment of heparin oligosaccharides to chitosan (Hall and Holme, 1986; Casu *et al.*, 1986; Shively and Conrad, 1976).

Sulfation of chitosan

As chitin has a similar skeletal structure to heparin, the biomedical properties of chitin have received considerable attention. Chitosan has been used to prepare heparin-like derivatives, by sulfate modification, because it can be chemically modified under homogeneous conditions in few reaction steps (Warner and Coleman, 1958; Wolfrom and Shen Han, 1959). In general only the influence of molecular weight and/or substitution degree of sulfated polysaccharides on their biological activity is considered in the majority of works involving the anticoagulant or antiviral properties of these substances (Horton and Usui, 1978).

Chitin and chitosan exist in the solid state under various forms such as films, yarns, flakes, powder, microparticles, and lyophilisates. Most of them are issued of a regeneration from a solution or a gel of these polymers. The properties of chitin and chitosan in the solid state depend on several parameters which can be classified in three categories corresponding to the chemical structure, the molecular weight and the crystalline organization.

1. Role of crystallinity and morphology

Chitin and chitosan are semi-crystalline polymers and as such, most of their properties in the solid state depend essentially on the percentage of the crystalline part and sometimes of the crystalline form. As already discussed, most of chitin in the native state exists essentially under two polymorphs termed α and β chitin (Roberts, 1992). Although the second polymorph allows a denser system, some hydrogen bondings are more

elongated than in the first case (Roberts, 1992) which, for some reactives would explain a better accessibility to the crystalline parts. If we consider the deacetylation of chitin, it depends both on the accessibility of the crystalline domain and the brittleness of the chains in the amorphous parts. Then the difference of accessibility between α and β chitin is such that it is more difficult to deacetylate chitin from arthropod cuticles. As a consequence, more drastic conditions are necessary leading to products having a lower molecular weight and a higher coloration than in the other case.

Chitosan is also partly crystalline and whatever the origin or degree of deacetylation, regenerated or raw, the crystallinity of chitosan samples in the free amino form seems to be always between 30-35%. Nevertheless, it can play a very important role on the properties of chitosan in the solid state. As a consequence, according to the application, it can be interesting to try to modify this parameter. Chitosan in the solid state is partially hydrated and for normal conditions of temperature and pressure, this water corresponds to approximately 2 molecules per monomer unit (Belamic *et al.*, 1997). In the amorphous part, this water is sufficient to dissolve gaseous HCl and then, after heating to allow the partial hydrolysis of these domains. After elimination of the oligomers produced by this reaction without any dissolution of the other molecules, the remaining product contains polymer chains which the degree of acetylation can reach 75% (Muzzarelli, 1973). Conversely, it is possible to reduce considerably the crystallinity of a given sample of chitosan by a factor 3 if we convert it in the salt form after dissolution in a stoiechiometric amount of HCl followed by freeze-drying. The role of crystallinity on chitosan can be easily shown on studying the adsorption properties of chitosan in a dispersed state in a water solution of uranyl ions. For the same degree of deacetylation, the adsorption of metal ions

decreases when increasing the percentage of crystallinity and tends toward zero at 100% crystallinity confirming of the inaccessibility of the ordered domains.

Next to the crystallinity, the morphology in the solid state is also very important. It is interesting to notice that crude chitosan is in a state of molecular organization which is not so far from the situation in which chitin is in the cuticle. The material is very dense (Piron and Domard, 1997) and the accessibility to chitosan chains is relatively difficult. The consequence is that hydration of such a material is very long compared to a regenerated chitosan in a lyophilized state (Piron and Domard, 1997).

2. Role of degree of acetylation (DA)

The role of DA has already discussed. In the solid state, it plays a similar role, in particular on the solvation by water or the adsorption of metal ions. In the latter case, when a true complex is formed, it involves free amine groups and then, when degree of deacetylation increases, it was observed that there was a decrease of the metal adsorption which tends toward zero at 100% acetylation (Piron and Domard, 1997). The DA has also an important role on the permeation and adsorption properties of chitosan membranes in the presence of oxygen, CO_2 and water vapor. In another hand it was shown that the mechanical properties of chitosan film depends on this parameter.

3. Role of the molecular weight

As for all polymer material, the molecular weight should have an important influence on the mechanical properties of chitin and chitosan but not many information is available in the literature, probably in relation with the fact that the problem of the determination of the molecular weights of both chitin and chitosan is far to be solved in particular in the former case.

1.4.1.7 Biomedical applications of chitin, chitosan and its derivatives

So many chemical modifications have been applied to prepare soluble chitin and chitosan in general organic solvent including water (Kurita, 1995). Since the smaller molecule is the better to apply as biomaterial on the aspect of immunogenicity, there is almost maximum size of introduced group to negotiate the toxicity on the administration into animal body. Carboxymethyl (CM)-chitin, one of water soluble chitin derivatives, was found to be insensitive to stimulate the immune system in animal body, although CM-chitin activated macrophages in peritoneal of mouse similar as that of chitin (Nishimura *et al.*, 1986) CM-chitin has been found to be delivered and stood for several days to spleen for the oral administration of mouse and to bone marrow on the intravenous administration (Tokura *et al.*, 1996). A hapten pendanted CM-chitin was applied to induce hapten specific antibody in the presence of strong immunoadjuvant (Tokura *et al.*, 1987). CM-chitin was also found to be an excellent carrier of drug to achieve controlled release of drug in the absence of immunoadjuvant (Tokura *et al.*, 1990).

The sulfation site of chitin was found to be important factor to regulate biomedical activities such as anticoagulant activity, antimetastasis and etc. (Tokura *et al.*, 1994). Phosphorylation of chitin has also be achieved by applying mild condition to prevent the oligomerization of chitin and phosphorylated chitin (P-chitin) was found to work as metal ion adsorbent (Yalpani, 1992). Biological functions of sulfated CM-chitin (SCM-chitin) were investigated in comparison to heparin (Tokura and Tamura, 1998). It was demonstrated that SCM-chitin showed high antimetastatic activity against melanoma cells which were confirmed as binary inhibitor of heparanase and type IV collagenase which were secreted from melanoma cells to invade through venous wall as listed in Table 5.

Table 2 Biological activities of SCM-chitin and heparin (Tokura and Tamura, 1998)

Carbohydrate composition	SCM-chitin	Heparin
	N-acetylglucosamine	Glucosamine + Uronic acid
Tumor metastasis in mice		
Experimental:	↓	↓
Spontaneous:	↓	↓
Tumor cell arrest in lungs	↓	↓
Tumor invasion	↓	↓
Tumor migration (heptotaxis)	↓	↓
Tumor cell adhesion		
To endothelium:	—	±
To extracellular matrix:	↓	↓
Enzymatic degradation of extracellular matrix		
Heparanase:	↓	↓
Type IV collagenase:	↓	—
Tumor angiogenesis	↓	—
Coagulation/platelet aggregation	±	↓
Tumor cell growth	—	—

↓ : inhibition, ± : partial inhibition, — : no effect

The solubility properties of chitosan and its derivatives, and their use in biomedical applications, entail some problem in common, particularly for use as a polymer support or carrier material. Many researches aimed to prepare soluble materials that might have interesting rheological properties. Another concern was the solution compatibility of these derivatives with anionic polymers, because chitosan and some of its derivatives form

insoluble complexes with such important polymers as O-(carboxymethyl)cellulose, xanthan gum, alginate, and heparin (Kikuchi and Noda, 1976; Holme and Hall, 1987). Both purposes were achieved by converting amino groups of chitosan preparations of low N-acetyl content into anionic centers, through selectively N-sulfation. The products, designed to vary in their levels of derivatization, were evaluated as to their rheological characteristics, and their solution compatibility with other anionic polymers (Holme and Perlin, 1997). The N-sulfation under a variety of reaction conditions of chitosan with degree of acetylation (DA) 0.04, 0.10 and 0.22, yielded a range in degree of sulfation (DS) from 0.4-0.86. All products were soluble in water, and the rheological properties of their solution varied markedly with DA and DS. Both ionic strength and pH had an effect on their solubility properties, and also on interactions that they exhibited with O-(carboxymethyl)cellulose, xanthan gum, and heparin. Being compatible with other polyelectrolytes, the chitosan derivatives may be useful in some aqueous formulations.

In this connection, direct synthesis of similar heterogeneous structures is of great interest and, in any case, calls for attentive study of the preparation conditions of these compounds and correct identification of their structures. Earlier works of investigating for the structural features of chitosan sulfates dealt with the consideration of the zwitterionic structure of chitosan 6-O-monosulfate relative to the pH-dependence of NMR signals in this substance (Naggi *et al.*, 1991). These authors indicated the presence of molecular and structural heterogeneity in the chitosan sulfate obtained and connected this with a high content of acetyl groups or with an after effect of polymer degradation. In another work (Hirano *et al.*, 1991), three sulfated derivatives of chitosan were analyzed by ^{13}C NMR spectroscopy and the structures of chitosan disulfates were confirmed. Preparation of

sulfated chitosan in various conditions under homogeneous, pseudo-homogeneous, and semi- heterogeneous state of chitosan, Gamzazade and his colleagues (1997) demonstrated that sulfation conditions of chitosan essentially affect the position and degree of substitution with sulfate in the derivatives of chitosan. Sulfated products obtained under homogeneous conditions are characterized by more heterogeneity and they have to be considered as copolymers of chitosan 6-O-monosulfate and 3,6-disulfate, whereas those produced by semi-heterogeneous synthesis may be considered preferentially as chitosan 3,6-disulfate.

1.4.2 Blood coagulation

1.4.2.1 Factors involving in the blood coagulation

The coagulation of blood is resulted from the formation of fibrin by the interaction of more than a dozen of proteins in a cascade of proteolytic reactions as shown in Figure 1. At each step, a clotting factor undergoes limited proteolysis and becomes an active protease. This process continues until an ultimate insoluble fibrin clot is formed. The soluble precursor of fibrin circulates in the blood as fibrinogen. The fibrinogen is a substrate of enzyme thrombin (factor IIa), a protease which is formed during the coagulation process by the activation of circulating prothrombin (factor II). The prothrombin is activated by factor Xa (prothrombin activator) in the presence of factor V, calcium (factor IV) and phospholipid. The Stuart-Prower factor (factor X) is activated by the extrinsic or intrinsic mechanism as described later. The activated factor X (Xa) interacts with calcium and factor V, adsorbed on the surface of platelet phospholipids, will transform prothrombin (II) into thrombin (IIa).

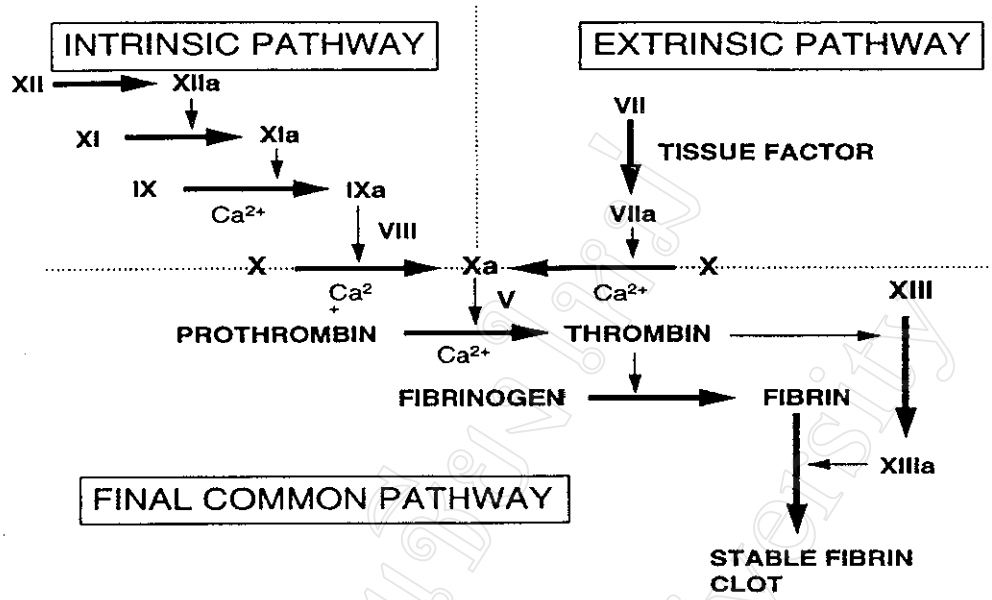

Figure 2 Classic "waterfall" hypothesis for coagulation with the intrinsic, extrinsic, and final common pathways. (Pasi, 1999)

Two separate pathways lead to the formation of activated factor X. In the intrinsic pathway (contact pathway), all protein factors necessary for coagulation are present in the circulating blood. This process requires in minutes for activation of factorX. This pathway is initiated by a physical disturbance through the contact with artificial surfaces (e.g. glass) of certain blood components in the absence of extravascular tissue factors. Basically, Hageman factor (XII) undergoes contact activation and becomes bound to artificial surfaces. This surface-bound factor XII undergoes proteolytic activation by kallikrein in the presence of high molecular weight kininogen (HMWK). Factor XIIa, in the presence of HMWK also activates factor XI. Factor XIa in the presence of calcium activates factor IX to IXa. The factor VIII, factor IXa, calcium and phospholipid micelles from the platelets form a lipoprotein complex with factor X which results in activated factor X (Xa). Factors V and

Xa, calcium and phospholipids form complex with prothrombin (factor II) and activate it to thrombin (IIa). Within seconds, thrombin split two small pairs of peptides of fibrinogen (factor I) to produce soluble fibrin monomers. Factor XIII which is also activated by thrombin to XIIIa cross-links adjacent fibrin monomers covalently to form the insoluble fibrin clot.

In the extrinsic pathway (*in vivo* pathway), coagulation can be initiated by the interaction of blood elements with the substances liberated from injured tissues. Factor VII undergoes the activation by factors XIIa, XIa and kallikrein. Factor VIIa, calcium, tissue thromboplastins (factor III) and factor X form a lipoprotein complex that activates factor X. The process is then the same as the intrinsic system. However, the extrinsic system is faster than the intrinsic system since it takes less time in the reaction.

Thrombin (factor IIa) and factor Xa are the principal factors which can be inhibited by heparin. To be active, factors II, VII, IX and X must bind to calcium. This capability is conferred to post-translational γ carboxylation of a series of N-terminal glutamic acid residues. This carboxylation process requires the reduced vitamin K as a cofactor. It is important to note that except for the first two steps in the intrinsic pathway, calcium ions are required for the promotion of all reactions. Therefore, in the absence of calcium, blood clotting will not occur.

1.4.2.2 Factors involving in the dissolvation of blood clot

There is an euglobulin in blood called plasminogen which is usually incorporated in large amounts in blood clots. When activated by tissue plasminogen activator (t-PA, plasminogen becomes plasmin which digests not only the fibrin threads but also fibrinogen, thrombin, factor V and VIII as well as other proteins. This leads to the dissolution of clot or fibrin degradation products (FDPs). The activity of plasmin is controlled by antiplasmin. Activity of the activated plasmin is restricted to the clot because activators are effective mainly on plasminogen absorbed on fibrin. Any plasmin which escapes into the circulation is inactivated. The lysis of blood clots allows slow clearing (over a period of several days) of extraneous blood from the tissues and in a number of instances even allows reopening of clotted vessels. The overall pathway is demonstrated in Figure 3.

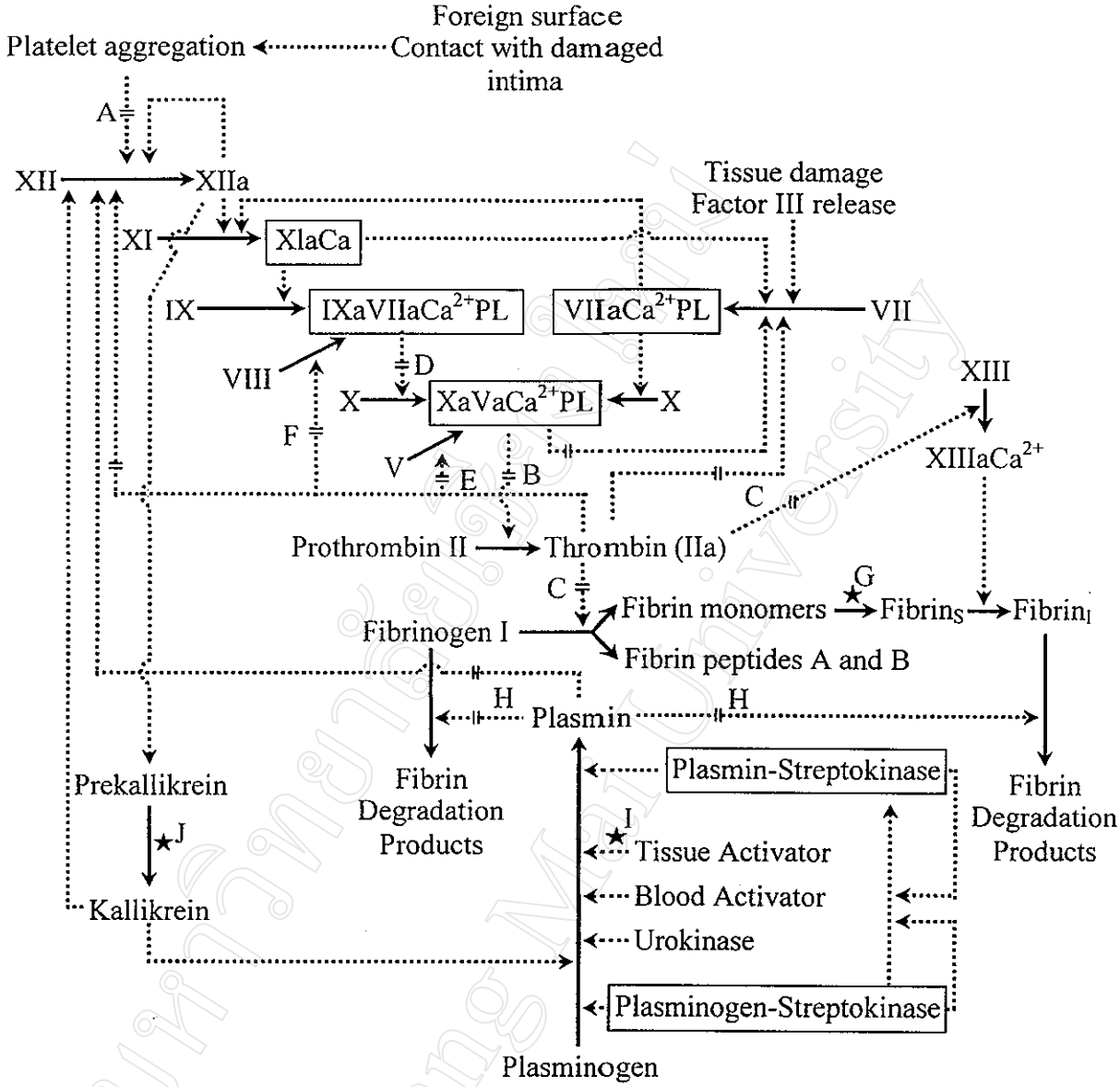

Figure 3 Schematic diagram demonstrating the overall hemostasis pathway. Solid arrows indicate conversions; dotted arrows indicate catalytic activities. Complexes are blocks. Stars indicate potentiation by pentosan polysulfate; solid bars cross arrows indicate inhibition by drug. A) Platelet aggregation, B) Inhibition of factor Xa, C) Inhibition of thrombin, D) Inhibition of factor IXa, E) Inhibition of the activation of factor V, F) Inhibition of factor VIII activation, G) Stimulation of fibrin polymerization, H) Inhibition of plasmin, I) Stimulation of fibrinolysis. $Fibrin_s$ and $Fibrin_l$ refer soluble and insoluble fibrin respectively.

1.4.3 Anticoagulants

Natural, physiological anticoagulants fall into two broad categories, 1, serine protease inhibitors (SERPINs) and 2, those that neutralize specific activated coagulation factors (Protein C system). These systems are the major physiological significance. They are active from the very outset of the coagulation process and often brought fully into play before fibrin deposition has occurred.

1.4.3.1 Serine protease inhibitors (SERPINs)

SERPINs includes many of the key inhibitors of coagulation, such as antithrombin, heparin cofactor II, protein C inhibitor, plasminogen inactivators, and α_2-antiplasmin. Of these, antithrombin is perhaps the most important (Perry, 1994). AT is a single-chain glycoprotein (MW 58 kDa) and inhibits all the coagulation serines protease (II, VII, IX, X, XI, XII), but its antithrombin and anti-Xa activity are physiologically important. AT activity/inhibition is increased 5 - to 10000 – fold in the presence of heparin and other sulfated glycosaminoglycans. Heparin is not normally found in the circulation, and physiologically, antithrombin probably binds to heparan sulfate on the vascular endothelial cells.

1.4.3.2 Protein C system

Factor Va and VIIIa are powerful cofactors in coagulation-enhancing activity of serine proteases. Both Va and VIIIa are specifically inactivated by components of the protein C pathway. Protein C is the key inactivating enzyme (Dadhlback, 1995). It is a

single-chain vitamin K-dependent protein synthesized by the liver. Together with its cofactor, protein S, it activates factors Va and VIIIa.

Thrombin generated during coagulation binds to thrombomodulin (Tm) on the surface of vascular endothelial cells. Thrombin/Tm complex is a potent activator of protein C. Tm accelerates protein C activation of approximately 20000-fold. Protein C is activated by cleavage at Arg_{169}-Leu_{170}. The activated protein C is inhibited by the specific inhibitor, protein C inhibitor (PCI) and since it is a serine protease, it is also inhibited by antithrombin.

Protein S, the cofactor for protein C, is vitamin K-dependent. It circulates in plasma as a single-chain glycoprotein (MW 60 kDa) and is synthesized by liver, endothelial cells, and megakaryocytes. Approximately 60% of protein S is complexed to C4b binding protein. Only the unbound or free protein S is physiologically active.

All factors that involve in the overall hemostasis and coagulation pathway are summarized in Table 3-4 (Pasi, 1999).

Table 3 Plasma coagulation factors

Factor	Synonyms	Half-life	Plasma concentration	Function
I	Fibrinogen	72-120 h.	200-400 mg/dl	Changed by thrombin to fibrin monomer. α–chain enhances t-PA activation of plasminogen on β–chain.
II	Prothrombin	48-96 h.	100-150 µg/ml	Zymogen of serine protease activated by prothrombinase complex (Xa, Va, PL, Ca^{2+}) to IIa. Vitamin K-dependent
III	Tissue factor			Transmembrane protein +PL complex. TF + VII to activate X and IX
IV	Calcium			Essential for coagulation. Binds vitamin K-dependent factor via γ–carboxyglutamic acid residues to PL
V	Proaccelerin	12-36 h.	10-15 µg/ml	Activated by IIa, cofactor for Xa in prothrombinase complex
VI	No factor			
VII	Proconvertin	4-6 h.	0.13-1.0 µg/ml	Zymogen of serine protease VIIa (Vitamin K dependent). Complexd with TF activates factors X & IX.
VIII	Antihemophilic factor (AHF)	8-12 h.	<100 ng/mL	Activated by IIa. Cofactor for Tenase complex (IXa, VIIIa, PL, Ca^{2+})
vWF	von Willebrand factor	18-20 h.	5-10 µg/mL	Carrier for VIII. Essential for platelet adhesion.
IX	Christmas factor	18-30 h.	3-5 µg/mL	Zymogen of serine protease IXa. Part of Tenase complex. Vitamin K-dependent.
X	Stuart/Prower factor	40-60 h.	5-10 µg/mL	Zymogen of serine protease Xa. Part of prothrombinase complex. Vitamin K-dependent.
XI	PTA-plasma thromboplastin antecedent	45-54 h.	5 µg/mL	Zymogen of XIa activated by IIa & XIIa. Zymogen is carried by HMK in the blood.
XII	Hageman factor	48-96 h.	30-36 µg/mL	Zymogen of XIIa. Activates XI, PK, HMK.
XIII	FSF-fibrin stabilizing factor	3-5 days	4-10 µg/mL	Zymogen of transglutaminase (fibrinoligase) which functions to cross-link fibrin polymers. Actiavted by IIa, Xa & requires Ca^{2+}
PK	Prekallikrein	48-52 h.	50 µg/mL	Zymogen of kallikrein which activates XII with HMK & produces Kinin.
HMK	High MW kininogen	6.5 days	70-90 µg/mL	Non-enzymatic cofactor for PK and XII. XI carrier.

Table 4 Inhibitors of coagulation factor pathways

Component	Description & function
Antithrombin III (ATIII)	ATIII inhibits serine proteases, primarily factor IIa & Xa. Also factors IXa, XIa, XIIa, plasmin & kallikrein.
Protein C	Inactivates factors Va & VIIIa, enhances fibrinolysis. Vitamin K-dependent.
Protein S	Cofactor for protein C. Binds to complement component C4-binding protein. Vitamin K-dependent.
Tissue factor pathway inhibitor (TFPI)	TFPI is a complex for factor Xa and lipropotein which inhibits tissue factor VIIa activation for factor X & IX
Thrombomodulin (TM)	TM is an endothelial cell protein which when complexed with IIa uniquely activates protein C.
Activated protein C inhibitor (aPCI)	aPCI inhibits activated protein C. It inhibits IIa, Xa, trypsin and chymotrypsin. Heparin accelerates the inhibition.
α-2 antiplasmin (α-2AP)	Main inhibitor of plasmin. Minor degrees of inhibition of Xa, Kallikrein, XIa & IIa.

1.4.4 Glycosaminoglycans and the regulation of blood coagulation

The proteoglycans comprise a heterogeneous groups of macromolecular glycoconjugates that are composed of sulfated glycosaminoglycan chains covalently linked to a protein core. They are widely distributed in animal tissues and appear to be synthesized by virtually all types of cells. All glycosaminoglycan identified, except the non sulfated polysaccharide hyaluronan, which occurs as free glycosaminoglycan chains, are synthesized as proteoglycan form. The large (an growing) number of core proteins identified, the variable extent of substitution with glycosaminoglycan chains and the

variability in glycosaminoglycan structure contribute to the overall structural diversity of the proteoglycans.

Classification of glycosaminoglycans takes note of the basic structure of the glycan backbone, which may be composed of (1) [HexA-GalN]n, (2) [HexA-GlcN]n, or (3) [Gal-GlcN]n type of disaccharide units. The type-3 disaccharide unit occurs in keratan sulfate only, which has so far not been implicated with blood coagulation. Types 1 and 2 may be further subdivided, type 1 into condroitin sulfate and dermatan sulfate, type 2 into heparan sulfate and heparin (Figure 4). The type-2 saccharides also include hyaluronan, which differs from the heparin/heparan sulfate family with regard to the position of glycosidic linkages and by lacking sulfate substituents; again, this glycosaminoglycan species does not seem to be directly involved in blood coagulation.

The definition of subspecies within each class of glucosamino- or galactosamino-glycans is complicated due to the extensive microheterogeneity of the glycan structures, which is best understood through a short discourse of glycosaminoglycan biosynthesis.

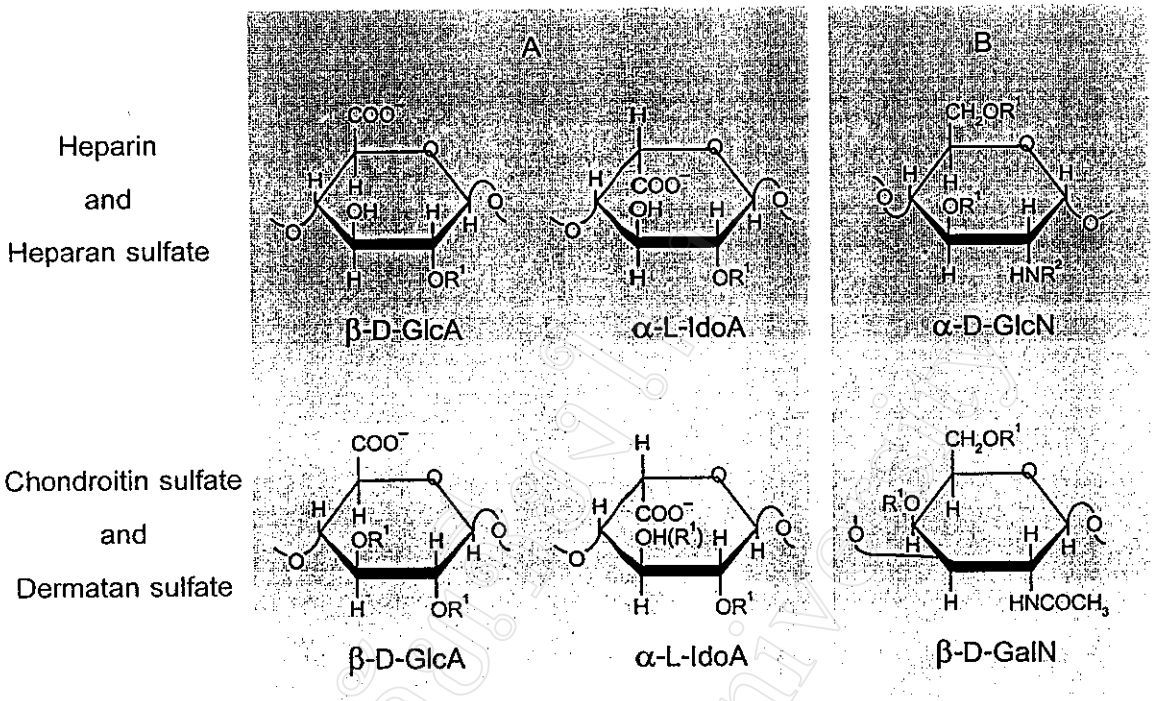

Figure 4 Structure of glycosaminoglycans. A; HexA residue (GlcA or its C-5 epimer, IdoA), B; GlcN (in the glucosaminoglycans, heparin and heparan sulfate) or GalN (in the galactosaminoglycans, chondroitin sulfate and dermatan sulfate). R^1 = -H or $-SO_3^-$, R^2 = $-SO_3^-$ or $-COCH_3$.

Heparin is a naturally occurring proteoglycan with a molecular of about 1000 kD. Heparin has been isolated from many animal species (Hovingh, *et al.*, 1986, Pejler *et al.*, 1987, Linhardt *et al.*, 1992). Heparin is produced by mast cells and basophils and consists of approximately 11 linear polysaccharide chains (molecular weight about 100 kD each) attached to a serine-glycine rich linear protein core (Comper, 1981). Heparin chains are highly sulfated and consist of repeating 14 linked glucosamine and hexuronic acid residues (Casu, 1985) making it the most acidic polymer in the body (Casu, 1985).

The biosynthesis of heparin/heparan sulfate (Lindahl, 1989; Lindahl and Kjellén, 1991) is initiated by formation of a polysaccharide chain with the structure (-GlcAβ1,4-GlcNAcα1,4-)n. This polymer is N-deacetylated/ N-sulfated and subsequently undergoes, in the order as mentioned, C-5 epimerization of glucuronic acid to Iduronic acid (IdoA) units, 2-O-sulfation of IdoA, and 6-O-sulfation of GlcN units. Additional O-sulfate substituents may be incorporated at C-3 of GlcN units (Kusche *et al.*, 1988) and at C-2 (or C-3) of GlcA units (Bienkowski and Conrad, 1985; Kusche and Lindahl, 1990). Due to the stepwise nature of the process, and the substrate specificities of the enzymes involved, the product of any given reaction will be the substrate for the subsequent reaction. Polymer modification is incomplete in the sense that the enzymes generally act upon only a fraction of the potential substrate residues; hence, the structural complexity and heterogeneity of the polysaccharide chain under modification increase through out the process.

Following the initial N-deacetylation/N-sulfation reaction all subsequent modifications will depend on N-sulfate groups for substrate recognition. Therefore, the iduronic acid units and O-sulfate groups of the final products are accumulated in the N-sulfated regions of the glycosaminoglycan chains, while the N-acetylated sequences retain GlcA units and remain largely nonsulfated. Heparin is extensively N-sulfated, and therefore rich in iduronic acid and O-sulfate groups, whereas heparan sulfate contains more N-acetylated, unmodified, regions (Lindahl and Kjellén, 1991). However, mixed type, "irregular" regions may occur in both heparin and heparan sulfate and, furthermore, may be of functional importance, as illustrated by the antithrombin-binding region (Figure 5). This pentasaccharide sequence is composed of three GlcN units, one of which is preferentially N-acetylated, one GlcA unit, and one IdoA unit, with O-sulfate groups in various positions.

The galactosaminoglycans, chondroitin sulfate and dermatan sulfate, are generated by principally similar modifications of an initial polymerization products, which has the structure (GlcA β1, 3-GalNAc β1,4-)n (Rodén, 1980). The structural diversity is less pronounced than for the heparin-related glycosaminoglycans, since the GalNAc residues remain exclusively N-acetylated. Nevertheless, owing to the variable location of O-sulfate groups and the presence of GlcA as well as IdoA units, as many as nine different HexA-GalNAc disaccharide units have been identified (Seldin *et al.*, 1984). By definition, chondroitin sulfate contains GlcA as the only HexA component, whereas any galactosaminoglycan with detectable amount of IdoA will be referred to as a dermatan sulfate. The amount of suifate content is ~1/disaccharide unit, thus less variable than in the heparin/heparan sulfate family, although "oversulfated" species have been described.

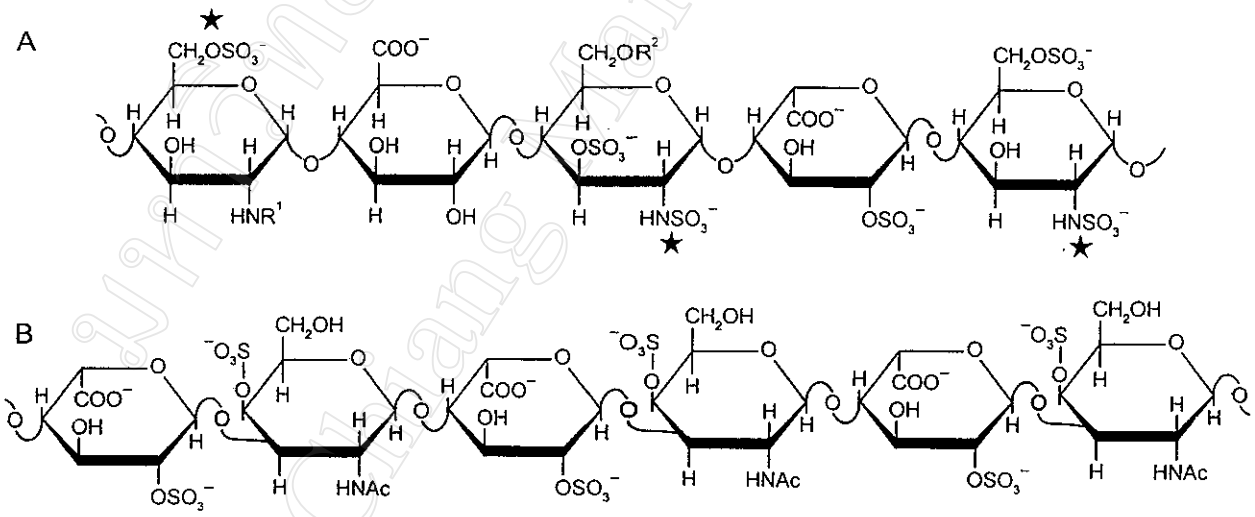

Figure 5 SERPINs binding regions in glycosaminoglycans. A; the antithrombin-binding region in heparin (heparan sulfate), B; the heparin cofactor II-binding region in dermatan sulfate, R^1; $-SO_3$ or $-COCH_3$, R^2; -H or $-SO_3$. The three sulfate groups marked (★) are also highly important to this interaction at antithrombin III.

Most of the biological activities known to be associated with glycosaminoglycans are due to interactions between the negatively charged glycosaminoglycan chains and various proteins (Kjellén and Lindahl, 1991). In general, IdoA-containing glycosaminoglycans interact more avidly than those containing GlcA only (Casu *et al.*, 1988). With regard to the proteins involved in blood coagulation and its regulation the interactions with glycosaminoglycan vary from specific, "lock-and-key" type binding to the relatively nonspecific, co-operative electrostatic association.

1.4.4.1 Effects of the glycosaminoglycans on the protease inhibitors

The major inhibitors of serine proteases involved in blood coagulation are antithrombin and heparin cofactor II. The mechanisms of action are profoundly influenced by glycosaminoglycans, which accelerate the rates of inhibition by binding to inhibitors and, albeit with some exceptions, to their target enzymes. Other inhibitors, such as protein C inhibitor, protease nexin-1, and tissue factor pathway inhibitor (TFPI) are also affected the glycosaminoglycans; however, these interactions have not been elucidated to the same extent and will therefore be considered of less detail.

1.4.4.2 Effects of the glycosaminoglycans on antithrombin

Antithrombin is an α_2-glycoprotein of Mr ~58,000 that synthesized in the liver and present in human blood at ~2.7 μM concentration. Antithrombin is the major inhibitor of thrombin in plasma, but also inactivates the other serine proteases of the intrinsic pathway, factors IXa, Xa, XIa and XIIa. Antithrombin inhibits serine proteases by forming tightly, equimolar complexes through interaction between a specific reactive bond of the inhibitor

and the active site of the enzyme (Olson and Bjork, 1992). The stability of these complexes has suggested that they represent acyl-intermediates formed during the cleavage of the reactive bond as in reaction with a normal substrate. The protease may cleave the reactive bond in the inhibitor instead of forming a stable complex. This is indicated by the observation that small amounts of free inhibitor, cleaved at the reactive site, are produced during the reaction of antithrombin with proteases (Olson, 1985). The reactive bond of antithrombin has been identified as the Arg_{393}-Ser_{394} bond near the C-terminus of the inhibitor. A peptide sequence of 8-12 amino acid residues N-terminal to the reactive bond (designated residues P8-P12) appears to be of critical importance for antithrombin to function as an inhibitor of proteases. Natural antithrombin variants in which amino acids within this region are mutated, were shown to be inactive as usual inhibitors, but are instead excellent substrates of their target enzymes, which efficiently cleave antithrombin at the reactive bond (Casu *et al.*, 1991). Moreover, Asakura *et al.*, (1990) showed that a monoclonal antibody that binds to the P8-P12 region also transforms antithrombin from an inhibitor to a substrate of thrombin. A more detail of understanding of this inhibitor-substrate transition has emerged from X-ray crystallographic studies of homologous SERPINs (Mouray *et al.*, 1990), including the noninhibitory SERPINs, ovalbumin (Stein *et al.*, 1990), and from molecular dynamic simulations. Cleavage of the reactive bond induces a drastic conformational change of the P1-P16 region, an exposed peptide loop in the native state, such that it may insert into the major β-sheet of the protein. These findings suggest that a partial insertion of the P1-P16 loop might be involved in trapping a proteinase in a stable complex (Skriver *et al.*, 1991). Mutations in the P8-P12 region of the loop would interfere with such insertion and thereby allow the

exposed reactive bond to be cleaved as a normal substrate. In support of this hypothesis, addition of a synthetic, competing P1-P14 peptide, blocking the insertion site for the reactive loop, resulted in a loss of the ability of antithrombin to inhibit thrombin, and in concomitant cleavage by the enzyme of the reactive bond of the inhibitor (Bjork *et al.*, 1992). Recent X-ray diffraction studies on human plasminogen activator inhibitor-1 support the general concept of a conformationally flexible reactive loop adjacent to the scissile bond in SERPINs (Mottonen *et al.*, 1992).

Rosenberg and Damus (1973) suggested that heparin binds to antithrombin and effects a conformational change which results in a greatly accelerated reaction with thrombin. Following complex formation with thrombin, antithrombin loses its high affinity for heparin, which will be released and ready to "activate" another antithrombin molecule. Heparin thus acts as a catalyst.

The accelerating effect of heparin on antithrombin-protease reactions depends on the presence of a unique antithrombin-binding pentasaccharide sequence in the glycosaminoglycan chains (Atha *et al.*, 1985). This region is composed of one GlcA unit, one IdoA unit and three GlcN units, two of which are invariably N-sulfated whereas the remaining one may be either N-acetylated or N-sulfated (Figure 5). Both N-sulfate groups, the non-reducing-terminal 6-O-sulfate group and the 3-O-sulfate group on the internal GlcN unit are essential for the biological activity. The latter residue is a distinguishing structural feature of the antithrombin-binding sequence, and thus, by and large, serves to indicate anticoagulant activity, although it has also been detected in other regions of heparin and heparan sulfate chains (Kojima *et al.*, 1992). Heparin as well as heparan sulfate

preparations from various sources vary with regard to the proportion of molecules having high affinity for antithrombin (Lindblom *et al.*, 1991).

There is now ample evidence that the interaction between antithrombin and heparin chains that contains the specific antithrombin-binding pentasaccharide sequence is accompanied by a conformational change in the inhibitor (Olson and Bjork, 1992). In a comparative study, Shore *et al.*, (1989) found that the full-length, high-affinity heparin and synthetic antithrombin-binding pentasaccharide induced highly similar conformational changes in antithrombin, as evidenced by spectroscopic methods. From rapid kinetic experiments showed that of both full-length heparin and the pentasaccharide bind antithrombin in a two-step process, comprised by an initial weak interaction that is essentially identical for the two saccharides, followed by a conformational change that is responsible for generating the high-affinity binding (Peterson and Blackburn, 1987).

The mechanism behind the heparin-induced acceleration of antithrombin-protease reactions has been an issue of controversy. The conformational change of the antithrombin molecule, largely induced by the specific antithrombin-binding pentasaccharide sequence was assumed to be an important contributor to the rate enhancement (Carrel *et al.*, 1987), presumably by causing the reactive bond of the inhibitor to be more accessible to the active site of the proteases (Figure 6b). This conclusion appeared to be supported by the findings that the reaction of antithrombin with proteases such as factor Xa, factor XIIa and plasma kallikrein were potentiated in approximately similar fashion by full-length heparin and by the antithrombin-binding pentasaccharide, or small oligosaccharides containing this specific sequence. On the other hand, the reactions of antithrombin with other proteases such as thrombin, factor IXa and factor XIa were

negligibly influenced by small-sized, high-affinity oligosaccharides; instead, a minimum chain length of ~18 saccharides was found to be required to significantly enhance the rates of inhibition (Shore *et al.*, 1989). These findings were better explained by an alternative mechanism, which predicted that heparin was acting as a surface or bridge to approximate antithrombinn and the enzyme by the binding of both proteins to the same heparin chain (Figure 6c) (Holmer *et al.*, 1979). This proposal was supported by chemical modification studies in which the rate-enhancing effect of heparin could be selectively abolished by modification of basic residues of the protease assumed to be involved in heparin binding (Pomerantz and Owen, 1978). Moreover, kinetic experiments showed inhibition of the rate of enhancement at high heparin concentrations which correlated with the binding of inhibitor and protease to separate heparin chains (Hoylaerts *et al.*, 1984). Finally, the binding studies indicated that the smallest heparin fragment capable of significantly accelerating the antithrombin-thrombin reaction corresponded to the smallest saccharide unit that could bind both antithrombin and the active-site already blocked protease (Danielsson *et al.*, 1986).

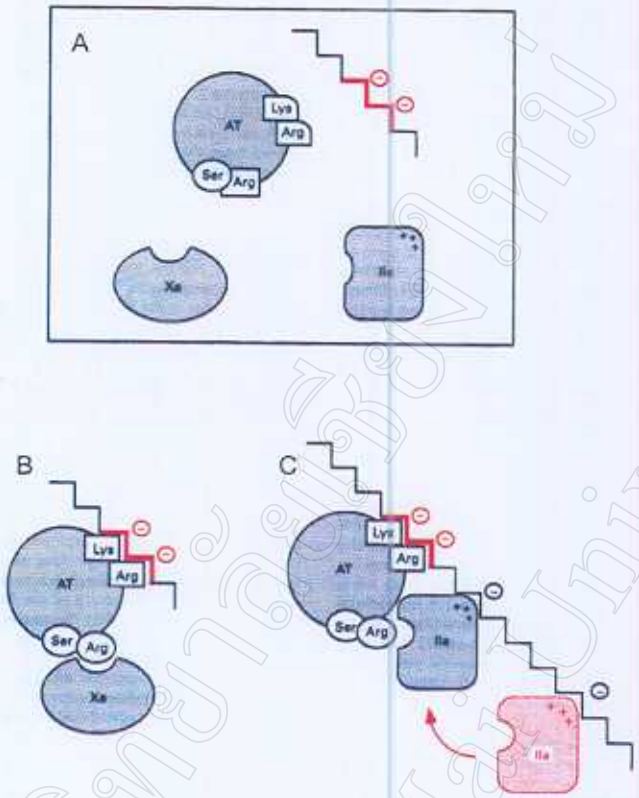

Figure 6 Effects of heparin on the reactions between antithrombin and coagulation enzymes. A; Schematic display of the interacting species, B; Binding to heparin induces the conformational change in the AT that will facilitate its reaction with FXa, C; The effect of heparin on the reaction between AT and thrombin (factor IIa) involves binding of both the enzyme and the inhibitor to the heparin chain, with thus needs to contain a saccharide segment of certain length in addition to the AT-binding region. (Heuck *et al.*, 1985; Boreslord *et al.*, 1990).

1.4.4.3 Effects of the glycosaminoglycans on heparin cofactor II

Heparin cofactor II is another SERPINs, a single polypeptide chain that is identical or closely related to Leuserpin 2 (Ragg and Preibisch, 1988). Heparin cofactor II inactivates thrombin by formation of a stable 1:1 complex, but does not react with Fxa (Parker and Tollefsen, 1985). It functions as a pseudosubstrate for thrombin, The reactive site peptide bond containing a leucine rather than the more typical arginine (Griffith *et al.*, 1985). Because of leucine at the reactive site, the inhibition of thrombin is very slow in the absence of glycosaminoglycan. Both dermatan sulfate and heparin increase the rate of inhibition of thrombin by heparin cofactor II more than 1000-fold, whereas chondroitin 4- or 6-sulfate have no effect except chondroitin 4,6-disulfate that has some ability to promote the heparin cofactor II (Tollefsen *et al.*, 1983).

The effect of heparin on the inhibition of thrombin by heparin cofactor II appears to be governed mainly by overall charge (Hurst *et al.*, 1983), without requirement for any specific oligosaccharide sequence akin to that involved in antithrombin binding. Moreover, the catalytic efficiency of heparin saccharides increased continuously with the molecular size of the chain, up to > 20 monosaccharide residues (Tollefsen *et al.*, 1990).

More detailed information regarding the role of glycosaminoglycans in heparin cofactor II-mediated thrombin inhibitions was obtained through analysis of recombinant inhibitor mutants. The N-terminal region contains two repeated acidic domains that are homologous to thrombin-binding domains in the C-terminal portion of hirudin and have been implicated as a site of interaction between heparin cofactor II and thrombin (Hortin *et al.*, 1989). Deletion of this region did not affect the rate of inactivation of thrombin by heparin cofactor II in the absence of glycosaminoglycan but dramatically impeded the

increase in reaction rate normally obtained in the presence of heparin or dermatan sulfate. This and other observations have been interpreted in terms of the model illustrated in Figure 6 which shows the effect of glycosaminoglycans on the interactions of native or truncated heparin cofactor II with thrombin. In the absence of glycosaminoglycan, N-terminal, hirudin-like acidic domain of heparin cofactor II binds intramolecularly to a glycosaminoglycan-binding site, and covalent complex formation with thrombin occurs at the basal rate (Figure 7a). Glycosaminoglycans displace the acidic region from the internal binding site, thereby enabling it to interact with the hirudin-binding site of thrombin (anion-binding exosite, Fenton, 1986); simultaneous binding of the glycosaminoglycan chain also to a glycosaminoglycan-binding site of thrombin results in maximal acceleration of the rate of thrombin inhibition (Figure 7b). Heparin cofactor II lacking the N-terminal region is unstable to interact with hirudin-binding site of thrombin, even in the presence of glycosaminoglycan, the resulting modest increase in reaction rate presumably being due to approximation of the two proteins bound to the glycosaminoglycan chain (Figure 7c). Oligosaccharides which are too short to simultaneously accommodate both proteins may nevertheless promote heparin cofactor II-thrombin complex formation (van Deerlin and Toffelsen, 1991) by displacing the acidic N-terminal region, which may then interact with thrombin (Figure 7d). Accordingly, deletion of the acidic region reduces thrombin inhibition to the basal rate in the presence of small oligosaccharides. Results in accord with these conclusions were obtained in studies on the inhibition of proteolytically modified thrombin by heparin cofactor II in the presence of heparin (Rogers *et al.*, 1992). Ultimate confirmation of the model will require crystallization of heparin cofactor II and its complexes with saccharides and thrombin.

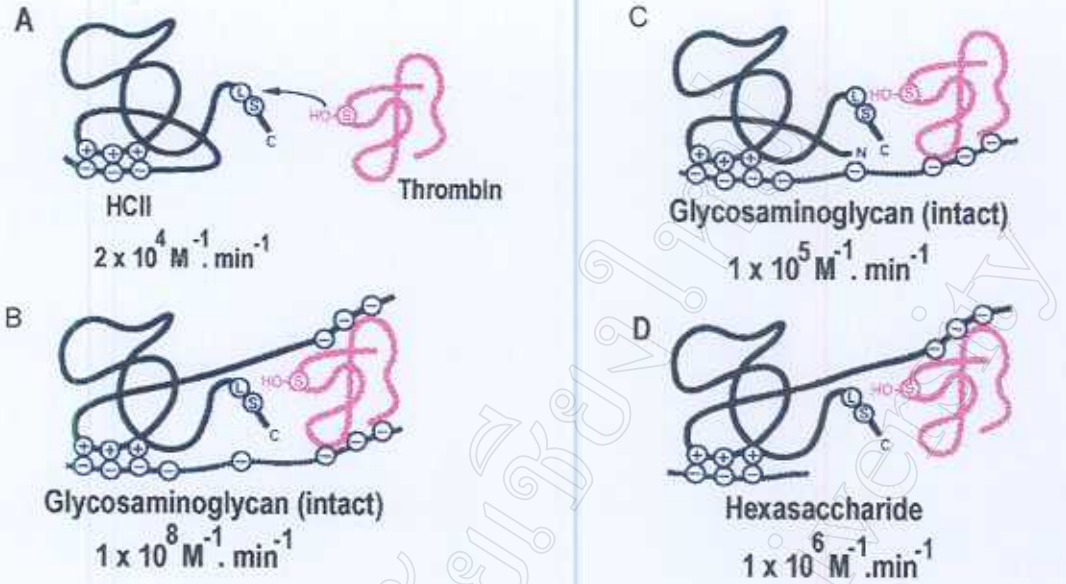

Figure 7 Model for the effects of glycosaminoglycans on the inhibition of thrombin by heparin cofactor II. A; native heparin cofactor II and thrombin (red) in the absence of glycosaminoglycan, B; native heparin cofactor II and thrombin in the presence of full-sized glycosaminoglycan chain, C; heparin cofactor II, lacking the N-terminal domain, and thrombin in the presence of full-sized glycosaminoglycan chain, D; native heparin cofactor II and thrombin in the presence of dermatan sulfate hexasaccharide (van Deerlin and Tollefsen, 1991).

1.4.4.4 Effects of the glycosaminoglycans on protein C inhibitor

Activated protein C is a serine protease with anticoagulant properties, not inhibitable by antithrombin. The rather low rate of protein C inactivation by this inhibitor was found to be increased ~ 30-fold in the presence of heparin (Suzuki *et al.*, 1984). A non-heparin dependent inhibitor of protein C was identified as α1-antitrypsin (Heeb and Griffin, 1988). A novel heparin-dependent inhibitor of protein C was isolated from human urine and found

to be immunologically distinct from other known protease inhibitors, including antithrombin and plasminogen activator inhibitor-1 (Geiger *et al.*, 1988). This inhibitor was considered to be the urinary counterpart of the plasma protein C inhibitor and to be functionally related to urinary urokinase inhibitor (Stump *et al.*, 1986). In the absence of heparin, the urinary inhibitor inhibits both protein C and urokinase at similar rates, whereas in the presence of heparin the inhibitory activity is preferentially increased toward protein C (Geiger *et al.*, 1988). The heparin-dependent plasma and urinary protein C inhibitors were immunologically identical and, moreover, closely related to the plasminogen activator inhibitor-3 (Heeb *et al.*, 1987).

The heparin-dependent protein C inhibitor/plasminogen activator inhibitor-3 belongs to the SERPINs family and shows some sequence similarity to other members of the family (Suzuki *et al.*, 1987). The effect of heparin is strongly dependent on charge interactions and can be mimicked by dextran sulfate, the inhibitory activity increasing with both the molecular size and degree of sulfation of the polymer (Suzuki, 1985). These observations are in accord with a "template" model by which binding of inhibitor and target enzyme to the same glycan chain are required for efficient protein C inhibition. Recent findings by Pratt and Church (1992) suggest that the ability of glycosaminoglycans to accelerate protease inhibition by protein C inhibitor depends on the formation of ternary inhibitor-glycosaminoglycan-enzyme complexs.

1.4.4.5 Sulfate derivatives of chitosan inhibits the coagulation pathway

Sulfated derivatives of chitosan possess anticoagulant activity (Doczi *et al.*, 1953; Wolfrom *et al.*, 1953). Whereas O-sulfated chitin has ~25% of the activity of heparin (Roth *et al.*, 1954), N-sulfated chitosan has no activity (Warner and Coleman, 1958). Conversion of position 6 into a carboxyl group in N-sulfated chitosan gives a product with ~23% of the activity of heparin (Horton and Just, 1973), and its O-sulfated derivative exhibits ~45% activity (Whistler and Kosik, 1971). O-sulfated N-carboxymethyl chitosan exhibits ~45% activity (Muzzarelli *et al.*, 1984). N, O-sulfated chitosan has a low toxicity in mice (Doczi *et al.*, 1953). When position 6 of N-sulfated chitosan is converted into carboxyl, the product is much more toxic (Horton and Just, 1973).

Hirano ad his coworkers (1985) found that a 6-sulfate group in the hexosaminyl moiety is a main active site, and there is no activity when it is absent (Warner and Coleman, 1958), the activity is maintained more or less when position 6 is CO_2H or $CH_2OCH_2CO_2H$. A 3-sulfate group in the hexosaminyl moiety promotes the activity when position 6 is an acidic group, but it is not essential. The important contribution of the N-sulfate group to the activity has been pointed out (Doczi *et al.*, 1953; Wolfrom *et al.*, 1953), but its presence is not a prerequisite for the activity, as demonstrated in his study with respect to aPTT. In fact, dextran sulfate which has no hexosaminyl moiety, shows anticoagulant activity, and the N-acetyl group is a minor component of heparin (Lindahl and Hook, 1978). It is also clear that molecular weight is an important factor for the anticoagulant activity, as reflected by the sequence of activities O-sulfated N-acetylchitosan (26 kDa) > heparin > N, O-sulfated chitosan (12 kDa) > sulfated O-carboxymethylchitosan

(540 kDa) with respect to activated partial thromboplastin time (aPTT), thromboplastin time (TT) and antithrombin activity (AA).

Nishimura and his colleagues (1986b) reported the anticoagulant activities of sulfated chitin and sulfated O-carboxymethylchitins together with their preparations. In their investigation, the cooperative effect of O- carboxymethyl and O-sulfate groups introduced was suggested to be an important factor in the inhibitory action against thrombin activity in the absence of antithrombin III (ATIII), as SCM-chitin tended to bind with thrombin rather than ATIII and the highest degree of inhibition was observed on the SCM-chitin (DS for $-SO_3Na$ = 0.60 and for $-CH_2COONa$ = 0.56). Although the extent of the inhibition of fibrinogen-fibrin interconversion was almost comparable with that of heparin in the absence of ATIII, only 50% acceleration has been observed when compared to that of heparin in the presence of ATIII.

Since Nagasawa and Uchiyama (1984) has demonstrated that the ability of heparin to form a complex with ATIII was regulated by the number of N-sulfate and N-acetyl groups. In addition to the O-sulfate group, it has been shown that whale heparin (DS for N-sulfate = 0.70 and for N-acetyl = 0.25) has the greatest ability to bind with ATIII from among several heparinoids from other species. Partially N-deacetylated chitin (45%, 70% and 95% of deacetylations) have been used to balance N-sulfate and N-acetyl groups. The optimal ratio of N-sulfate to N-acetyl groups was demonstrated to be 7 : 3 so far. Thus N- and O-sulfations of partially N-deacetylated 6-O-carboxymethylchitin were carried out stepwise to obtain the final substance. The anticoagulant activity of chitin heparinoids is discussed in comparison with that of heparin through their inhibition constants (K_i) both in the presence and absence of ATIII. It was found that antithrombin activity was achieved by

the sulfation of partially N-deacetylated O-carboxymethylchitin among various modified chitin derivatives. It was also suggested that the distribution of N-sulfate and N-acetyl groups on the C-2 position might be essential to the selective binding by ATIII to inhibit thrombin activity. Kinetic evaluations demonstrated the non-competitive inhibition on direct interaction with thrombin (K_i = 9.26 x 10^{-8} M) and the competitive inhibition with ATIII (K_i = 3.33 x 10^{-8} M) as well as heparin (Nishimura and Tokura, 1986).

Although there have been many attempts (Horton and Just, 1973; Muzzarelli *et al.*, 1984) to prepare chitosan heparinoids, the role of each functional group has not been clarified satisfactorily. Use of chitin as a precursor of heparinoids allows the role of ether-linked functional groups to be demonstrated independently of influence by N-sulfate groups. Carboxymethylation of chitin was employed as carboxymethyl groups has been reported to augment the anticoagulant activity of dextran sulfate. Carboxymethylation of chitin to DS 0.8 under mild conditions (Tokura *et al.*, 1982) causes mainly 6-O-substitution. O-(carboxymethyl)-chitin (CM-chitin) has been reported to absorb bovine blood proteins, with a positive contribution by Ca^{2+}, and the extent of adsorption is regulated by the degree of substitution (Nishimura *et al.*, 1984). Nishimura *et al.* (1986) reported the preparation of various chitin derivatives by sulfation of chitin and CM-chitin under mild conditions, and studied of the inhibition of thrombin activity, using bovine fibrinogen. It was demonstrated that the inhibition was increased upon introduction of carboxymethyl groups in the sulfated chitin, as also observed for dextran sulfate. The sulfated CM-chitin showed activity comparable to that of heparin, even at low concentration in the absence of ATIII. As there was little inhibition by chitin heparinoids in the presence of ATIII, especially at low

concentration, it appeared that N-sulfate groups, as well as O-sulfate and O-CM groups are required for totally inhibition.

In addition, much recent interests has been directed towards the specific modulating effects of a variety of glycosaminoglycans (GAGs) on those extracellular biological process dependent on protein-carbohydrate interactions (Bourin and Lindahl, 1993). GAGs and chemically designed sulfated polysaccharides have been regarded as a new class of potential inhibitors of some effector molecules in biochemical and medicinal fields concerned with cellular surfaces and extracellular matrices (ECM) (Hay, 1991). The interactions of GAGs or related polyanionic compounds with the major envelope glycoprotein (gp120) of HIV-1 also is one of the most important factors in connection with regulation of the initial stage of HIV-1 infection in human T lymphocytes (Mbemba *et al.*, 1992; Rider *et al.*, 1994). In addition to basic studies on the interaction of GAGs with envelope glycoprotein, a variety of sulfated polysaccharides have been reported to show potent inhibitory effects on the replication of AIDS virus *in vitro*. Yoshida and his coworkers found that curdlan sulfate, with a linear β–(1→3)-linked glucopyranose backbone, strongly inhibits AIDS virus infection *in vitro* at drug concentrations as low as 3.3 μg/ml and it was not cytotoxic *in vitro* (Yoshida *et al.*, 1990). At present, this sulfated polysaccharide is regarded as one of the most potent and practical antiretroviral polysaccharide reagents. N-carboxymethylchitosan with N,O-sulfate, a heparin-like polysaccharide derived from N-carboxymethylchitosan by random sulfation reaction (Muzzarelli *et al.*, 1984) was also shown to inhibit HIV-1 replication and viral binding with CD4 (Sosa *et al.*, 1991). However, it should be noted that much attention must be paid in the design of novel sulfated

polysaccharides with specific anti-AIDS activity while lacking unfavorable anticoagulant activity or other side effects.

Thus, the interest is focused on the regioselective synthesis of sulfated polysaccharides and structural-activity studies seeking for the potent antiretrovirus activity with little anticoagulant and low cytotoxicity. Nishimura and his colleagues (1998) reported a novel and convenient method for the regioselective syntheses of sulfated analogs of chitin and chitosan in relation to studies on structure-biological activity. Fully protected, soluble derivatives of chitosan were found to be useful intermediates for the syntheses of a novel class of sulfated polysaccharides, 2-acetamido-2-deoxy-3-O-sulfo-(1→4)-β-D-glucopyranan (3-sulfate), and (1→4)-2-deoxy-2-sulfoamido-3-O-sulfo-(1→4)-β-D-glucopyranan (2,3-disulfate). These compounds were tested for their activities in the inhibition of HIV-1 replication *in vitro* and inhibition of anticoagulation. The results revealed that the selective sulfation at O-2 and O-3 afforded potent antiretroviral agents showing a much higher inhibitory effect on the infection of AIDS virus *in vitro* than that by the known 6-O-sulfated derivative (6-sulfate). Moreover, the 2, 3-disulfate derivative can completely inhibit the infection of AIDS virus to T lymphocytes at concentrations as low as 0.28 μg/ml without significant cytotoxicity. The regioselective introduction of sulfate group(s) at O-2 and/or O-3 had little effect on generating anticoagulant activity, whereas 6-O-sulfated chitin strongly inhibited blood coagulation. These results suggested that the specific interaction of these new types of chitin sulfates with gp120 of the AIDS virus depended significantly on the sites of sulfation rather than on the total degree of substitution on sugar residues.

1.4.5 The immune system

The immune system is much like the other body systems in that it is composed of a number of different cell types, tissues and organs. Many of these cells are organized into separate lymphoid organs or glands. Since attack from microbes can come be upon different sites of the body, the immune system has a mobile force of cells in the blood stream which are ready to attack the invading microbe wherever it enters the body. Cells of the immune system maintain communication among theselves through cell contact and molecules secreted by them. For this reason the immune system is similar to the nervous system. Like the other body systems, the immune system is only apparent when it goes wrong. This can lead to severe, sometimes overwhelming infections and even death. One form of dysfunction is immunodeficiency which can result from infection with the human immunodeficiency virus (HIV) causing AIDS. On the other hand, the immune system can be "hypersensitive" to a microbe (or even to a substance such as pollen) and this itself can cause severe tissue damage sometimes leading to death. Thus, the immune system must strike a balance between producing a life-saving response and tissue-damaging reactions. This regulation is maintained both within the immune system and through nonimmune cells, tissues and their products.

Having penetrated the external defenses, microbe comes into contact with immune cells and their products and a battle commences. A number of cell types and defense molecules are usually present at the site of invasion or migrate to the site. These constitute the "first line of defense". This is called "innate immune system" since it is presence at birth and changes little throughout the life of the individual. The cells and molecules of this innate system are mainly responsible for the first stages of expulsion of

the microbe and may give rise to inflammation. Phagocytes and dendritic cells are important cells in the innate immune system since they ingest and kill microbes.

The second line of defense is "adaptive immune system". This is brought into action while the innate immune system is dealing with the microbe especially if it is unable to remove the invading microbe. The key difference between the two systems is that the adaptive system shows far more specificity and can remember that a particular microbe has previously invaded the body. This leads to a more rapid expulsion of the microbe on its second and third time of entry. Cells, molecules and characteristics of innate and adaptive immune system are shown in Table 5.

Table 5 The Innate and adaptive immune systems (Lydyard *et al.*, 2000)

Characteristics	Cells	Molecules
Natural immunity		
Responds rapidly	Phagocytes (PMNs, macrophages),	Cytokines
Has some specificity	Natural killer cells, Mast cells,	Complement
No memory	Dendritic cells	Acute phase proteins
Adaptive immunity		
Slow to start	T and B cells	Antibodies
Highly-specific		Cytokines
Memory		

Although, innate and adaptive immunity are often considered separately for convenience and to facilitate their understanding, it is important to recognize that they frequently work together. For instance, macrophages are phagocytic cells but produce

important cytokines that help to induce the adaptive immune response while complement components of the innate immune system are activated by antibody, molecules of the adaptive system. Various cells of both systems work together through direct contact with each other and through interactions with chemical mediators, the cytokines and chemokines. These chemical mediators can either be cell bound or released as localized hormones, acting over short distances. Cells of both systems have a large number of surface receptors: some are involved in adhesion of the cells to blood endothelial walls (e.g. leukocyte function antigens LFA-1), some recognize chemicals released by cells (e.g. complement, cytokine and chemokine) and others trigger the function of the cell such as activation of the phagocytic process.

The lymphocytes selected for clonal expansion are of two major types, B cells and T cells, each giving rise to a different form of immunity. T lymphocytes mature under the influence of the thymus and, on stimulation by antigen, give rise to cellular immunity. The B lymphocyte population matures under the influence of bone marrow and/or gut-associated tissues and gives rise to lymphoid populations which, on contact with antigen, proliferate and differentiate into plasma cells. These plasma cells make a humoral factor (i.e. antibody or immunoglobulin) which is specific for the antigen and able to neutralize and/or eliminate it.

The development of the immune response to an antigen also requires cell co-operation. T and B cell populations, as well as macrophages, interact in the development of specific immunity. In particular, subpopulations of T cells regulate (e.g. help) humoral and cellular immune responses. Although immune responses to most antigens (especially

proteins) require cell co-operation, some antigens (T-independent) are able to initiate an immune response in the absence of T lymphocytes.

1.4.5.1 Antigen processing and presentation (Germain and Margulies, 1993)

Antigen processing is the act of breaking large proteins into small peptide antigens and the antigen presentation is the act of expressing MHC molecules associated with peptide antigens on the surface of antigen presenting cells. The cells that process antigen in this way may be either specialized antigen-presenting cells (APCs), which are capable of stimulating T-cell division, or may be virally infected cells within the body which then become a target for Tc cells. There are two antigen processing pathways: cytosolic and endocytic pathway. Cytosolic pathway processes endogenous antigen in the cell cytoplasm and the proteins made in the cell associates with class I molecules. Endocytic pathway processes exogenous antigen that has been phogocytosed and associates with class II molecules.

In order to initiate a specific immune response to an infectious agent, the immune system must be able to wade through the sea of molecules that are associated with pathogenic invasion and isolate particular protein products that will hone the efforts of host defense. Implicit to this model of counteraction is the processing of an immunogenic peptide epitope and its presentation on the surface of a team of cells. The result of these actions is the induction of a T-cell response that recruits and engages the other molecular participants of the immune response.

At the core of this immune system element is the Major Histocompatibility Complex (MHC). MHC is highly polymorphic set of genes located on human chromosome 6 that

encode for molecules essential to self/non-self discrimination and antigen processing and presentation. The power of this multigenic complex lies in its polymorphism, which enables different allelic class I and class II products to bind almost infinite array of peptides. The nature of MHC suggests the now fundamental concept of self-MHC restriction, $CD4^+$ Th cells are activated only by antigen presenting cells that shares class II MHC alleles with them. That is antigen recognition by $CD4^+$ Th cells is class II MHC restricted. Antigen recognition by $CD8^+$ Tc cells, on the other hand, is class I restricted. Such division of labor is a response to the endogenous and exogenous origin of pathogenic proteins and a consequence of a design paradigm that allows for routing of the immune response.

1.4.5.2 Antigen presenting cells

The main phagocytic cells in mammals are dendritic cells, macrophages and B cells (Gordon, 1999). These cells have been termed "professional antigen presenting cells" by virtue of their ability to ingest a variety of particles, and they are largely responsible for the recognition and control of invading microbes. Other cells, such as fibroblasts, epithelial cells, endothelial cells, Glial cells and pancreatic beta cells are termed "nonprofessional antigen presenting cells". These cells may function as antigen presenting cells but only do so under stress such as inflammatory response. The ability to induce phagocytosis in these "nonprofessional" phagocytes is exploited by a variety of intracellular pathogens to escape the microbicidal responses of the professional phagocytes.

1.4.5.3 Structure and assembly of MHC molecule/peptide complexes

Class I and Class II molecules are membrane bound glycoproteins that are members of Ig superfamily. MHC Class I molecules are made up of 2 chains that form a heterodimer. These chains are called the α chain and β2 microglobulin. The α chain has 3 external domains called α1, α2, α3. α1 and α2 form the peptide-binding cleft. α3 contains amino acid sequences that interact with CD8 molecule on the T cell. The structure of the peptide-binding cleft is 8 anti-parallel beta strands that form the floor of the cleft. These are spanned by 2 α helices that interact with both peptide and T cell receptor (TCR). This peptide-binding cleft of the MHC Class I molecule can bind a peptide of 8-11 amino acids in length. β2 microglobulin interacts with mostly the α3 domain of the Class I molecule. It also has some interaction with amino acids in the α1 and α2 domain. β2 microglobulin and peptide are absolutely necessary in order to get proper stability and folding of the MHC Class I molecule which leads to surface expression (Maffei, 1997).

Like MHC Class I, MHC Class II molecule is also a two chain heterodimer. This molecule is made up of an α and β chain. Each chain has two external domains called α 1, α2, and β1, β2. The α1 and β1 domains of the MHC Class II molecule form the peptide-binding cleft. The β2 domain interacts with CD4 on the T cell (Castellino, 1997).

The peptide-binding site on an MHC molecules has a variety of pocket, clefts, ridges, intrusions and depression. Its precise topology depends partly on the nature of the amino acids within the groove, and thus varies from one haplotype to the next. Peptide binding depends on the nature of the side chains of that peptide and their complementarity with the MHC molecule's binding groove. Some amino acid side chains of the peptide are oriented out of the groove and are available to contact the TCR.

Peptide/MHC association occurs in specific intracellular organelles and interaction of peptides with Class I and Class II antigens occurs at different sites within the cells.

1.4.5.4 Class I molecules and endogenous antigen processing

In cells, protein levels are controlled by continuous breakdown and synthesis of proteins. Proteins can be broken down in the cell's cytoplasm into peptides by proteolytic degradation. Many times, proteins that are to be broken down, whether they are normal cell proteins, viral proteins or tumor proteins, are targeted for proteolytic degradation by the addition of a small molecule called ubiquitin. The addition of ubiquitin to a protein is a signal for that protein to be processed into small peptides by the proteosome. The proteosome is a large, cylindrical complex made up of 4 rings subunits that form a central channel. Ubiquitin bound proteins are broken down in the center of the channel. This avoids having other proteins in the cell cytoplasm being processed by the proteosome. Once the peptides are made by the proteosome, they are transported into rough endoplasmic reticulum (RER) and associate with MHC Class I molecules.

Peptide transport into RER is facilitated by transporters associated with antigen processing (TAP) proteins. TAP is a membrane-spanning molecules made of two proteins TAP1 and TAP2. MHC Class I folding requires peptide and takes several steps involving the help of proteins called molecular chaperones. These molecular chaperones help to stabilize the Class I molecule until it interacts with β2 microglobulin and peptides so that it can be expressed on the cell surface. The first protein of the molecular chaperones to associate with Class I α1 chain is calnexin. Calnexin promotes proper folding of the Class I α1 chain. When β2 microglobulin binds to α chain, calnexin is released. Along with β2

microglobulin binding, two new chaperones bind, calreticulin and tapasin. Tapasin brings TAP and class I together to allow the peptide to be loaded into the peptide binding cleft. When the peptide binds, calreticulin and tapasin are released. Once the peptide is bound, the Class I molecule is stable enough to move to the golgi and then out to the surface of the cell *via* cellular vesicles and antigen is presented to $CD8^+$ Tc cells. (Bochtler *et al.*, 1999, Bonifacino and Weissman, 1998).

1.4.5.5 Class II molecules and exogenous antigen processing

Exogenous antigens are internalized by antigen-presenting cells (APCs). APCs can phagocytose and/or endocytose antigen, endosomally process it, and present it in association with Class II molecules.

Internalized antigen is processed within three increasingly acidic endosomal environments: early endosomes (pH 6.0-6.5), late endosomes (pH 5.0-6.0) and lysosomes (pH 4.5-5.0). Acid-dependent hydrolytic enzymes degrade antigen into peptides consisting if 13-18 amino acids. Class II α and β chains associate within the RER, the site of interaction between endogenous antigens and MHC Class I molecules of the cytosolic pathway. A protein known as the invariant chain (Ii) binds to the peptide-binding cleft of the MHC Class I molecule and thereby prevents its binding with endogenous antigens. The Ii chain seems to provide other important functions for Class II MHC molecule, too. It is involved in the folding of α and β chains, the exit of the complex from the ER, and its targeting to the endocytic compartments.

The Class II MHC-invariant chain complex is transported to early endosomes. As proteolysis increases in the successive endosomal compartments, the invariant chain is

degraded and the MHC molecule takes on an open conformation. A fragment of the invariant chain known as CLIP (Class II-associated invariant chain peptide), however, remains bound to the peptide-binding cleft to prevent premature interaction with partially-processed antigen. In the lysosome, HLA-DM, a Class II MHC-like molecule, mediates the removal of CLIP and the binding of antigen. The MHC-antigen complex then moves to the plasma membrane where the neutral environment stabilizes it. Here, at the cell surface, antigen is presented to $CD4^+$ Th cells (Castellino, 1997).

1.4.5.6 CD1 molecules and non-peptide antigen presentation

Some MHC Class I-like genes map outside the MHC region. One family of such genes, called CD1, also functions in antigen presentation to T cells although it does not present peptide antigens.

CD1 molecules were identified in the late 1970s. It was encoded by genes located outside of the major histocompatibility complex. CD1 molecules exhibit characteristics of the MHC Class I and Class II molecules. CD1 is structurally related to MHC Class I (Porcelli *et al.*, 1998). The five identified CD1 genes fall into two classes; CD1d is found in many mammals, including rats and mice, and CD1a, CD1b, CD1c, and CD1e are found only in human. Human CD1 molecules expressed on the surface of professional antigen-presenting cells including dendritic cells, Langerhan's cells, B cells and activated monocytes (Ulanova *et al.*, 1999). In the presence of monoclonal antibody to CD1a, CD1b and CD1c, proliferation of human PBMCs stimulated with purified protein derivatives or tetanus toxoid was inhibited. This suggests that it play a role in activation of cell mediated immunity. It was found that CD1 molecules could be detected on the CD14-positive

population of some healthy donors (Gregory *et al.*, 2000). The CD1 expression on monocytes is correlated with an activation state of the donors as demonstrated by the increased expression of the CD25, CD38, CD45RO and MHC class II molecules on their lymphocytes. It was also demonstrated that intracellular calcium flux was induced in the monocytes following CD1a engagement, and this effect was partially inhibited by preincubation of these cells with superantigen (Gregory *et al.*, 2000). By functional studies, the ability of CD1 proteins to restrict the antigen-specific responses of T cells in human was demonstrated. Identification of naturally-occurred antigens presented by CD1 has revealed that they are predominantly foreign lipids and glycolipids, found prominently in the cell walls and membranes of pathogenic mycobacteria (Porcelli and Modlin, 1999). Structural, biochemical, and biophysical studies support the view that CD1 proteins bind hydrophobic alkyl portions of these antigens directly and position the polar or hydrophilic groups of bound lipids and glycolipids for highly specific interactions with antigen receptors on $\alpha\beta$T, $\alpha\delta$T, and NK T cells. Presentation of antigens by CD1 proteins requires uptake and intracellular processing by antigen presenting cells, and evidence exists for cellular pathways leading to the presentation of both exogenous and endogenous lipid antigens.

1.4.5.7 Microbial recognition

Since most microbes can proliferate far more quickly than the ability of host to mount a specific antimicrobial response, the innate immune system must be able to recognize quickly and control an invader while the adaptive immune response is initiated. This has been achieved by the evolution of a set of germ line-encoded proteins, termed Pattern Recognition Receptors (PRRs), which are either free in the plasma or membrane

bound and are capable of recognizing conserved microbial molecules, termed pathogen associated microbial patterns (Medzhitov and Janeway, 1997). These microbial molecules include lipoteichoic acid of gram-positive bacteria and LPS of gram-negative bacteria. By recognizing the pathogen-associated microbial patterns, the PRRs enable the host to recognize a wide variety of microbes with a limited set of molecules. The PRRs determined the mechanism of uptake (phagocytosis) and influence the maturation pathway of the resultant phagosome and the inflammatory responses mediated by the phagocyte. PRRs also provide a means of entry for intracellular pathogens.

The plasma-derived PRRs include the collectins, pentraxins, and complement, which coat the microbe (opsonization), allowing recognition and binding by opsonic receptors on host phagocytes (Medzhitov and Janeway, 1997). After the induction of acquired immunity, opsonic recognition is enhanced by the production of specific antimicrobe antibodies. The best-characterized opsonic receptors on phagocytes are the complement and antibody receptors. The direct or non-opsonic recognition and binding of microbes is mediated by a variety of membrane-bound PRRs on phagocytes, including C-type lectins (macrophage mannose receptor), leucine-rich proteins (CD14), scavenger receptors (scavenger receptor A type I) and integrins (CR3) (Aderem and Underhill, 1999).

PRRs act as receptors for the binding and entry of many intracellular pathogens. *Mycobacterium tuberculosis*, for example, utilizes complement receptors (CRs), the mannose receptor, CD14 receptor and the surfactant protein A (collectin) receptor to bind to host macrophages (Ehlers and Daffe, 1998). Since the various receptors can generate different host cell responses, the intracellular survival of pathogens may depend on which receptor they utilize to gain entry into the host cells. *Toxoplasma gondii*, for example, is

killed by the antimicrobial respiratory burst after entry through the Fc receptor but survives after entry through CR3, which does not lead to a respiratory burst (Gordon, 1999). Although less well characterized, receptors other than the PRRs are also used by pathogen to bind to and/or enter host cells; an example is provided by Dr-fimbriated *E. coli*, which binds to epithelial cells via decay-accelerating factor (CD55) (Selvarangan *et al.*, 2000). Extracellular pathogens, on the other hand, have evolved a number of mechanism to avoid recognition, such as the capsules of staphylococcal and streptococcal pathogens, which prevent complement deposition on the bacterial surface and contact with host phagocytes (Whitnack *et al.*, 1981).

1.4.5.8 Production of soluble mediators

In conjunction with their ability to recognize, take up, and kill microbes, phagocytes must rapidly signal and recruit other cells to the sites of infection. Furthermore, the adaptive immune system must be primed and a specific immune response must be initiated to remove the invader. The signaling and recruitment of cells are achieved through the release of soluble mediators, such as cytokines and chemokines, which are generated after recognition and/or uptake of the microbial invader. These mediators, along with antigen presentation to lymphocytes, help direct the generation of the adaptive immune response. The development of the final immune response is complex and depends greatly on the types of soluble mediators produced and the interplay of these factors among the various immune cells.

The binding of microbes or their products to PRRs results, in most cases, in the production and/or release of proinflammatory mediators such as reactive oxygen

intermediates and arachidonic acid metabolites, as well as a number of proinflammatory cytokines. Although not all the signaling pathways leading to the NFkB-dependent expression of these cytokines are completely understood, the recently identified Toll-like receptors (TLR) are important components (Underhill *et al.*, 1999). The TLRs, originally identified in *Drosophila*, are a family of receptors with homology to the IL-1 receptor. They are recruited to the phagosome and can distinguish between various pathogens; TLR2 recognizes fungi and gram-positive bacteria, while TLR4 recognizes LPS and gram-negative bacteria. Similar to the IL-1 receptor, signaling through the TLRs occurs via MyD88 and IRAK to activate NFkB. A number of other TLRs have been identified and, although not yet characterized, may also be involved in microbial discrimination and cytokine release.

Cytokines are central molecules of the immune response, forming a complex network that mediates and regulates both innate and adaptive immunity (Pretolani, 1999). Cytokines bind to their cognate cell surface receptors and signal through a variety of pathways, although may signal through the Janus family kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins. Il-12, IL-18, and IFN-γ are central in macrophage activation and the generation of a Th1-type response. Il-12 released by macrophage stimulates NK cells and T cells to produce IFN-γ, which intern augments IL-12 secretion, generating a positive feedback loop. Activation by IFN-γ stimulates phagocytosis and induces phagosome-lysosome fusion, the respiratory burst, and the production of NO intermediates. Macrophage can also be activated by IFN-α/β stimulating antiviral and antimicrobial mechanism. Il-4 and IL-13, on the other hand, stimulate an alternative form of macrophage activation, while IL-10 leads to macrophage deactivation. The formation of

granulomas, organized lesions of T cells and macrophages at the site of infection and often critical in restricting microbial dissemination, depends on cytokines such as TNF-α and IFN-γ and various chemotactic cytokines (chemokines). Cytokines such as TGF-β are also critical in the suppression and resolution of the immune response.

Chemokines signal cells through seven transmembrane G-protein-coupled receptors, and they function to recruit neutrophils, monocytes, immature DC, and activated T cells to the sites of infection (Thomson, 1998). Chemokines are divided into four families based on the number and the position of conserved cysteines: C, CC, CXC, and the CX3C chemokines. In general, CXC chemokines, such as IL-8, attract neutrophils, while CC and C chemokines, such as monocyte chemotactic protein 1 (MCP-1) and lymphotactin, attract monocytes and lymphocytes. Chemokines can also induce the release of proinflammatory mediators and stimulate the respiratory burst.

Pathogens modulate cytokine networks to favor their survival either by inhibiting the production of proinflammatory cytokines or by stimulating the production of anti-inflammatory cytokines. Examples include the poxvirus and herpesvirus, which produce virokines, mimics of anti-inflammatory cytokines, and viroceptors, soluble mimics of proinflammatory cytokine receptor (Mosser and Karp, 1999). Other example include mycobacterial deactivation of macrophages, making these phagocyte refractory to activating cytokine and stimulating them to produce TGF-β, and the ability of *Yersinia* to inhibit NFkB activation, thereby preventing the expression of proinflammatory cytokines (Fitzpatrick and Bielefeldt-Ohmann, 1999).