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diss 97. 239

**Studies on Mechanisms of Biocompatibility  
Elicited at Blood-Material Interfaces**  
**Effects of Surface Adsorbed Plasma Proteins, Protein-Cell and  
Cell-Cell Interactions at Biomaterial Surfaces**

**Christin Karlsson**

**Department of Anatomy and Cell Biology  
University of Göteborg  
Sweden**



**Göteborg 1997**



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# Studies on Mechanisms of Biocompatibility Elicited at Blood-Material Interfaces

Effects of Surface Adsorbed Plasma Proteins, Protein-Cell and Cell-Cell  
Interactions at Biomaterial Surfaces

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som för avläggande av medicine doktorsexamen vid Göteborgs Universitet kommer att  
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I. KINETICS, SUPRAMOLECULAR STRUCTURE AND EQUILIBRIUM PROPERTIES OF FIBRINOGEN ADSORPTION AT LIQUID -SOLID INTERFACES.  
Nygren, H., Stenberg, M., Karlsson, C. J. Biomed. Mater. Res., 26, 77-91 ( 1992 ).

II. COOPERATIVITY IN THE ADSORPTION OF CELLULOSE ETHERS AND FIBRINOGEN AT LIQUID -SOLID INTERFACES.  
Karlsson, C., Carlsson, A., Stenberg, M., Nygren, H. Colloid Polym. Sci., 270, 377-383 ( 1992 ).

III. PRE-ADSORPTION OF A CELLULOSE ETHER ONTO POLYMER SURFACES: ADSORPTION OF ADHESINS AND PLATELET ACTIVATION.  
Elam, JH., Karlsson, C., Nygren, H. Biomaterials, 14(3), 233-237 ( 1993 ).

IV. PROTEIN-PLATELET AND PLATELET-LEUKOCYTE INTERACTION AT MATERIALS IN CONTACT WITH HUMAN BLOOD.  
Nygren, H., Braide, M., Karlsson, C. J. Vac. Sci. Technol. A, 13, 2613-2618 ( 1994 ).

V. EXPOSURE OF BLOOD TO BIOMATERIAL SURFACES LIBERATES SUBSTANCES THAT ACTIVATE PMNGS.  
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**ABSTRACT**

Biomaterials are used in the construction of medical devices intended to be in intimate contact with blood and tissues of the human body. For many of the biomaterials in direct contact with blood, the dominant mechanism leading to initiation of thrombus formation and inflammation, seems to be the adsorption of adhesive proteins onto the biomaterial surfaces. This adsorbed protein layer may determine the subsequent biological response to the surface, such as cell adhesion and activation of platelets and granulocytes.

Blood exposed to polymer materials, commonly used in medical therapy, and to experimental model surfaces such as hydrophilic and hydrophobic quartz surfaces, with and without surface modifications by cellulose ethers was investigated in the present thesis. The kinetics and supramolecular structure of the adsorbed plasma protein layer, with special emphasis on fibrinogen, was measured by using ellipsometry, ELISA, fluorescence- and electron microscopy.

The subsequent biological response to this spontaneously adsorbed protein layer, such as cell adhesion and activation of platelets and granulocytes, was studied by using ELISA, fluorescence- and electron microscopy, flow cytometry and a new method developed to study the immediate inflammatory response of a blood-biomaterial interaction. In this model, the intracellular calcium level of test granulocytes in suspension reflects the presence of granulocyte activating factors, produced by the previous interactions between human blood and biomaterial. It was further attempted to evaluate granulocyte activation concerning complement activation, integrin expression, respiratory burst and priming.

The results indicate that the wettability of the surfaces significantly affects the initial protein adsorption regarding its kinetics and supramolecular structure. A reduced adsorption of adhesive proteins was observed on the cellulose ether modified surfaces, and the results were in accord with the findings of reduced cell adhesion and activation at these surfaces.

Studies of the initial inflammatory response, show that blood exposition to hydrophilic and hydrophobic surfaces leads to the formation of humoral granulocyte activating factors, which differ between the surfaces. The present data also shows that biomaterials in clinical and laboratory use, differed in respect to the induction of an early inflammatory response during exposure of blood, and that this inflammatory response was dependent on the presence of anticoagulants used during the biomaterial exposure.

In conclusion, the present thesis shows that the properties of a biomaterial surface and the choice of anticoagulants are important determinants of the outcome of the humoral and cellular response to a blood-biomaterial interaction.

**Key words:** Plasmaprotein, bloodcompatibility, biomaterial, platelet, thrombosis, granulocyte, neutrophil, inflammation, calcium, surface-modification.

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by

Christin Karlsson



Göteborg 1997

*To my family*



BIOMEDICINSKA  
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# 1. INTRODUCTION

## 1.1 General remarks

A biomaterial is defined as any material, natural or man-made, that comprises whole or part of a living structure or biomedical device which performs, augments or replaces a natural function. Biomaterials are intended to be in intimate contact with blood and tissues of the body. In order to prevent biological non-self reactions such as unwanted thrombus formation and inflammatory reactions, the materials have to be biocompatible (Silver 1989; Szycher 1983) with the biological host.

The plasma contact activation system is triggered when blood is brought in contact with solid foreign materials. Besides the activation of the coagulation system, contact activation leads to the activation of the fibrinolytic, the kallikrein-kinin and the complement systems (Bouma 1986). The triggering of the coagulation and complement systems cause thrombosis and the production of a number of proteolytic activation products, soluble or surface-bound. These factors have the potential to activate platelets and inflammatory cells, which in turn lead to various cellular responses including the release of active products in all, contributing to the incompatibility cascade.

For many of the biomaterials in direct contact with blood such as polymer materials, used in catheters and other biomedical applications, the dominant mechanism leading to initiation of thrombus formation and inflammation, seems to be the adsorption of adhesive proteins onto the biomaterial surfaces (Brash 1987 ; Schmaier 1983 ; Vroman 1964 ; Vroman 1969 ; Vroman 1971 ; Vroman 1977 ; Vroman 1980 ; Vroman 1986 ; Wojcieszowski 1986). The initial adsorption of plasma proteins and the resulting adsorbed layer structure and composition initiates complex processes which determine the subsequent biological response to the surface, such as cell adhesion and activation of platelets (Ginsberg 1988 ; Preissner 1991; Zwaal 1986 ) and leukocytes (Bonfield 1989 ; McEver 1991; Vroman 1977 ). Polymer material surfaces in direct contact with human blood was investigated in the present thesis, together with experimental model surfaces such as hydrophilic and hydrophobic quartz surfaces.

A brief introduction is given below concerning events further discussed in detail in the subsequent individual papers.

## 1.2 Surface adsorption from single plasma protein systems.

The initial adsorption of water, small ions and plasma proteins onto artificial surfaces in contact with blood is believed to trigger subsequent cellular responses. Therefore, it is important to understand the role that human plasma proteins play for the complicated interactions taking place at interfaces (Brash 1987; Leonard 1987). Proteins are rather sticky molecules that will be adsorbed to almost any solid surface, and the process of protein adsorption to solid surfaces is an important step in the physiological function of plasma proteins. One of the plasma protein of special interest is fibrinogen, which is the most abundant plasma protein at material surfaces in contact with blood (Nygren 1995), this protein also acts as an adhesive protein for platelets (Ginsberg 1988) and participates in surface-induced thrombosis (Packham 1969; Zucker 1969). Surface adsorption of human fibrinogen is discussed in several papers in the present thesis.

### **1.2.1 Fibrinogen**

#### *Molecular structure*

Plasma fibrinogen, also known as coagulation factor I, is a glycoprotein present in blood in concentrations of 3-4 g/l and is synthesised in liver cells. Fibrinogen present in the platelet  $\alpha$ -granules is mainly synthesised by bone marrow megakaryocytes. The fibrinogen molecule is a 9 nm x 45 nm trinodular rod (Bachman 1975; Fowler 1979; Hall 1959; Krakow 1972; Siegel 1953) with a molecular weight of 340,000 (Caspary 1957) and is represented as a symmetrical arrangement of two identical sets of polypeptide chains. Each set consists of three independent subunit chains linked together with disulphide bonds. The three chains are designated  $A\alpha$ ,  $B\beta$  and  $\gamma$ . The fibrinogen molecule is therefore, assigned the general formula  $(A\alpha, B\beta, \gamma)_2$  (Blombäck 1972). Heterogeneity of normal fibrinogen with molecular weight variations in the plasma fibrinogen population, is due to enzymatic degradation of the  $A\alpha$ -chain protuberance from the carboxy-terminal end (Mosesson 1967; Mosesson 1974). These  $A\alpha$ -chain variations give rise to three main fibrinogen fractions: High molecular weight fibrinogen (HMW; MW 340,000), low molecular weight fibrinogen (LMW; MW 300,000) and a further degraded low molecular weight fibrinogen (LMW'; MW 280,000). These fractions constitute approximately 70%, 26% and 4%, respectively, of the normal plasma fibrinogen (Blombäck 1957).

#### *Supramolecular structure*

Surface adsorbed fibrinogen shows a divergence in the supramolecular structure, i.e. the structure of the adsorbed protein layer, at material surfaces with different surface energy, such as hydrophilic and hydrophobic quartz surfaces (Nygren 1988b). Individual molecules appeared at both hydrophilic and hydrophobic surfaces, after



adsorption from low bulk concentrations of fibrinogen. Adsorption from higher concentrations of fibrinogen in solution resulted in adsorbed spherical structures at the hydrophobic surface, and in end-to-end dimers at the hydrophilic quartz surface (Nygren 1988b). An ordered formation of a polymeric network (aggregates) of fibrinogen molecules, spontaneously occurring on hydrophobic silicone dioxide surfaces, has been described in another study (Wigren 1991), suggesting that intermolecular interactions takes place at interfaces. Supramolecular packing patterns of adsorbed fibrinogen have also been reported by others (Brynda 1986 ; Eberhart 1977) using electron microscopic techniques, showing ridges and walls on the surface. The amount and configuration of the fibrinogen deposits and the more extensive local ordering, suggest that protein-protein interactions are an important determinant of adsorption.

### *Kinetics*

The adsorption of plasma proteins onto a clean surface is a very rapid process and mass transport may well become the rate limiting step. This means that a material surface introduced into blood will be covered with a monolayer of albumin within approximately  $4 \times 10^{-4}$  seconds. Protein adsorption is generally considered to be irreversible in buffer solution (MacRitchie 1972 ; Norde 1980 ; Soderquist 1980), but desorption may occur in exchange reactions with other proteins (Brash 1987; Vroman 1980 ).

Measurements of the kinetics of fibrinogen adsorption have shown that the initial adsorption at a solid-liquid interface is rapid, often becomes diffusion-rate limited (Gorman 1971 ; Wojcieszowski 1986) and is followed by a slow association rate process (Cuypers 1987). A later study (Nygren 1988b) confirmed that the initial adsorption of fibrinogen at low bulk concentration was diffusion- rate limited, and stable plateau levels of adsorbed amounts were seen at a surface concentration as low as  $0.1 \text{ pmol/cm}^2$  which is below that of a monolayer of fibrinogen adsorbed side-on. However, the dissociation rate seems slow enough to make the binding of fibrinogen to the solid surface practically irreversible (Brynda 1986 ; Wojcieszowski 1986).

In spite of the apparently irreversible adsorption of fibrinogen to solid surfaces, the surface concentration of adsorbed protein correlates to the bulk concentration of protein, and a measurable exchange of fibrinogen between surface and solution is seen when these are in contact (Chan 1981). Adsorbed fibrinogen can also be replaced by other plasma proteins (Vroman 1980). These findings indicate dynamic equilibrium properties of fibrinogen adsorption at adequate surface concentrations and bulk concentrations of protein.

### 1.2.2 Surface modifications by cellulose ethers

Non-ionic water-soluble cellulose ethers in particular ethyl(hydroxyethyl)cellulose (EHEC), have been examined with regards to the possibility of a competitive exchange of fibrinogen to EHEC at a material surface, or to pre-adsorb EHEC to the surface in order to reduce protein adsorption and a subsequent cell adhesion/activation.

Cellulose ethers are manufactured from cellulose by substitution. Each anhydroglucose unit in cellulose contains three hydroxyl groups and the cellulose molecule could be considered as a polyalcohol. It undergoes the normal reactions for alcohols and is readily converted to ethers and esters (Hercules Incorporated 1982).

A general flow chart of the steps in the synthesis of cellulose ethers is given below (Felcht 1985);

---

Milling of cellulose ---- Activation with aqueous NaOH---- Heterogeneous reaction with etherifying reagents (ethyl chloride and ethylene oxide) ---- Neutralisation with acids---- Isolation of crude cellulose ether ---- Purification by extraction of salts and other by-products ---- Compounding, cross-linking and drying----Milling and sifting

---

EHEC display many features typical of non-ionic colloids and has found a broad technical applicability in colloid chemistry. Thus, besides biomaterials applications in the biotechnology field, EHEC is applied in a number of fields like industrial adhesives, building and painting- industry, in food products, in pharmaceutical-, fat- and cosmetics products.

EHEC belongs to a class of non-ionic polymers and surfactants which displays a rather complex phase behaviour in water and organic solvent. Above the lower critical solution temperature a process known as "clouding" occurs. The term "clouding" arises from the fact that the turbidity of the solution increases rapidly at the phase separation temperature and is thus easy to detect by eye. If the system is allowed to stand above the cloud point (CP) for some minutes, a separation into two macroscopic phases occurs, which in the case of EHEC, one liquid-like polymer-depleted phase and one solid-like polymer-rich phase is obtained. The process is reversible, on cooling the separated phases remix and the system retains its original phase behaviour. The cloud point increases strongly with an increasing substitution of hydrophilic groups (hydroxyethyl) and decreases with increasing fraction of the hydrophobic (ethyl) groups.

The hydroxyethylcellulose polymer (HEC) is a hydrophilic polymer (i.e. has substitution with polar groups, such as hydroxyethyl groups) and dissolves quickly in water and has a cloud point  $> 100^{\circ}\text{C}$ . The solubility properties of the cellulose ether is thereby altered by introducing ethyl groups in the polymer. The ethyl(hydroxyethyl)cellulose (EHEC) is a hydrophobic polymer (i.e. is substituted with



non-polar groups consisting of high ratio of ethyl to oxyethylene groups), soluble in water and organic solvents, and has a cloud point in water slightly above room temperature.

The possibility of increasing the blood compatibility of biomaterials, was examined by using surface modifications with cellulose ethers. The questions of whether adsorbed fibrinogen on material surfaces was exchanged by the presence of cellulose ethers in the analyte solution, or if pre-adsorption of cellulose ethers may affect the adsorption of plasma proteins and subsequent cell adhesion/activation, were investigated in the present thesis.

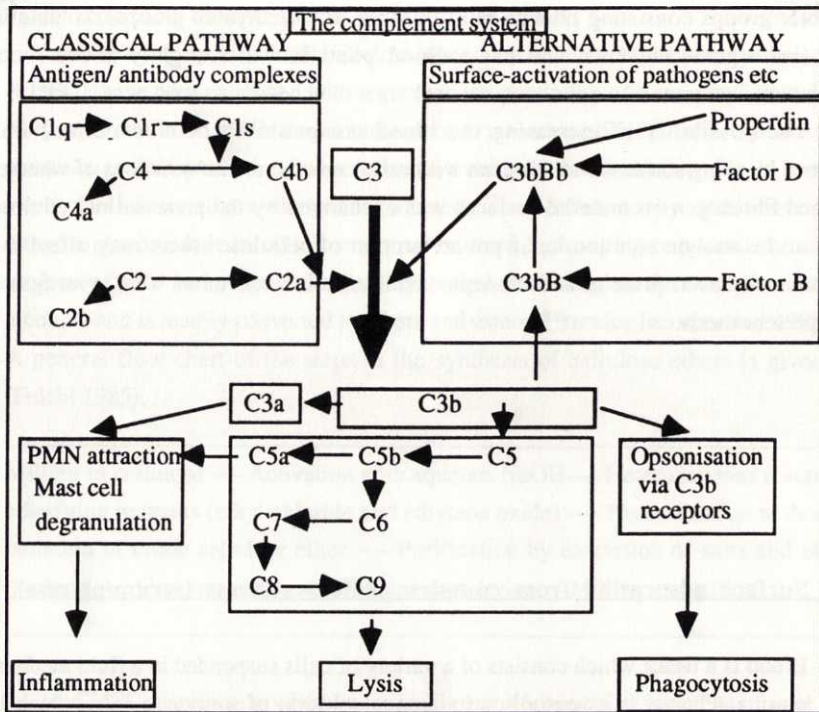
### **1.3 Surface adsorption from complex protein systems (serum/plasma).**

Blood is a tissue which consists of a variety of cells suspended in a fluid medium called plasma. Plasma is essentially an aqueous solution of inorganic salts which is constantly exchanged with the extra cellular fluid of all body tissues. Plasma also contains proteins, the plasma proteins, of three main types; albumins, globulins and fibrinogen. Thus, plasma consists of a complex mixture of different proteins together with other compounds such as lipids, polysaccharides, hormones etc. Some of the plasma proteins are involved in chain reactions, so called protease cascades, with specific tasks in the coagulation and inflammation systems of the body. The contact of blood with an artificial material surface can lead to the activation of one or more of the protease cascades (Bouma 1986) such as the coagulation and kallikrein-kinin system, the complement system and the fibrinolysis system, which may trigger the subsequent adhesion and activation of blood cells, primarily platelets and granulocytes (Zwaal 1986)

#### **1.3.1 The complement system.**

The complement system is a part of the non-specific immune system; it is involved in the eradication of foreign cells and immune complexes, as well as inflammation and immunoregulation. The complement system comprises a series of at least 20 serum glycoproteins, that are activated in a cascade sequence, with proenzymes that undergo sequential proteolytic cleavage (Law 1988) in two pathways of activation presented below in general:

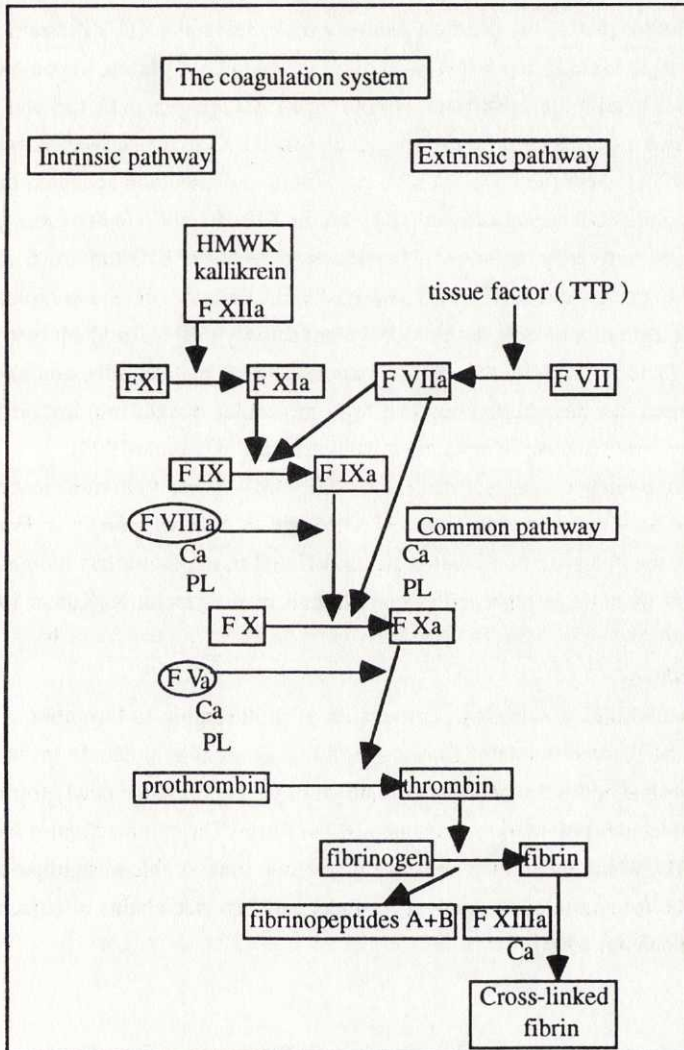




Complement activation does not occur in the fluid phase, but is localised on the surface of artificial materials, foreign cells or immune complexes that triggered the reaction. Activation of the classical pathway is calcium and magnesium dependent and occurs by the binding of C1q with IgM or IgG-containing antigen-antibody immune complexes. The alternative pathway can be triggered by numerous foreign surfaces such as artificial surfaces, microbial surfaces etc., and the components of this pathway are factor B, factor D and properdin. The pathways converge in the activation of C3, both forming individual C3 convertases. This leads into the final common pathway with the assembly of C5-C9 into the membrane attack complex (MAC), which forms a transmembrane channel leading to cell lysis by osmotic shock (Souhami 1994).

### 1.3.2 The coagulation system.

Coagulation involves a series of enzymatic reactions leading to the conversion of soluble plasma fibrinogen to a fibrin clot. The coagulation factors are either enzyme precursors (factor XII, XI, X, IX and thrombin) or co-factors (V and VIII), except for fibrinogen which is the subunit of fibrin. The enzymes apart from factor XIII are serine proteases and hydrolyse peptide bonds. The system is conventionally divided into intrinsic and extrinsic pathways, converging on a common pathway (Kumar 1994).



### *Extrinsic pathway.*

The extrinsic system is triggered by the release of a tissue factor, the tissue thromboplastin (TTP), a protein-phospholipid mixture that activates factor VII. The tissue thromboplastin and factor VII activate factors IX and X. In the presence of PL (platelet phospholipid),  $Ca^{2+}$  and factor V, activated factor X catalyses the conversion of prothrombin to thrombin, which is part of the common pathway (Souhami 1994).

### *Intrinsic pathway.*

The upper part of the intrinsic pathway includes factor XII, kallikrein and high molecular weight kinogen (HMWK). Exposure of blood and plasma to non-endothelial surfaces, leads to an initial adsorption of both factor XII (Hageman factor) and HMWK. HMWK carries prekallikrein and the coagulation factor XI to the surface (Mandle 1976 ; Thomson 1977), where they interact with the initially adhered and activated factor XII, with concomitant activation of factor XI by factor XIIa. Factor XIIa activates factor IX, which in turn activates factor X. This reaction requires calcium ions, a platelet phospholipid surface and factor VIII as a co-factor. Factor VIII is a complex protein consisting of a small molecule with procoagulant activity (VIII:C) and a larger part, von Willebrand factor (VIII:vWF), which is associated with platelet adhesion and readily forms multimers in the circulation. The high molecular weight multimeric forms of VIII:vWF are very effective in producing platelet adhesion (Kumar 1994).

Recent evidence suggests that factor XII, HMWK and kallikrein reactions, are probably the only relevant ones in the *in vitro* and *in vivo* situations. For *in vivo* haemostasis, the complex of activated factor VII and tissue factor has its main role to activate factor IX in the intrinsic pathway, besides activating factor X (Kumar 1994).

### *Common pathway*

Activated factor X leads to conversion of prothrombin to thrombin. Thrombin hydrolyses the peptide bonds of fibrinogen, a highly soluble molecule in the plasma, releasing fibrinopeptides A and B. After removal of the peptides, the newly formed fibrin monomer molecules polymerise spontaneously to fibrin. Thrombin activates factor XIII to factor XIIIa, which then in the presence of calcium ions, is able to stabilise the fibrin strands by the formation of covalent cross-links between side chains of adjacent fibrin molecules (Souhami 1994).



### *Anticoagulant drugs*

The following substances are used in the present thesis :

- EDTA ( ethylenediaminetetraacetic acid ) : EDTA is a metal ion chelator which complex binds calcium and magnesium, thereby interfering with several steps in the coagulation cascade (Gemmel 1995).
- Hirudin : Hirudin is a specific thrombin inhibitor, the use of which offers anti-coagulation at normal  $Ca^{2+}$  concentrations (Nygren 1988a).
- Heparin : Heparin has an anticoagulatory effect principally mediated through a potentiation of antithrombin III, which is the major inhibitor of thrombin. It also inhibits factors IXa, Xa, XIa and XIII (Jordan 1987 ; Kestin 1993). In addition, heparin has numerous other effects such as blocking platelet activation by thrombin (Kestin 1993), preventing platelet aggregation, preventing serotonin release and thromboxane B2 production (Renesto 1991). Heparin oligosaccharides are effective L- and P-selectin inhibitors in vitro and have anti-inflammatory activity in vivo (Nelson 1993).

### **1.3.3. Exchange reactions between proteins in complex protein systems.**

Exchange reactions of protein molecules at solid surfaces have mainly been studied for the plasma proteins, since the nature of the protein film adsorbed from blood on biomaterials seems to play an important role for the biocompatibility of the material. When blood or plasma is exposed to a foreign solid surface, the initially formed protein layer shows varying stability upon prolonged exposure to blood. Protein exchange reactions on solid surfaces seem to occur in sequence, with kinetics dependent on the properties of the surface. Earlier studies have shown that fibrinogen initially adsorbed onto hydrophilic glass and crystalline surfaces is replaced by high molecular weight kininogen (HMWK) (Schmaier 1983, Wojcieszowski, 1986 #67 ; Vroman 1980 ; Vroman 1986 ), a phenomenon known as the " Vroman effect ".

The kinetics of the exchange reactions differ between surfaces (Nygren 1988a; Vroman 1980 ), but the general rule is that plasma proteins adsorb on hydrophilic surfaces in the sequence :

albumin - IgG - fibronectin - fibrinogen - high molecular weight ( HMWK )

meaning that large and less soluble proteins will be enriched on the surface over time.

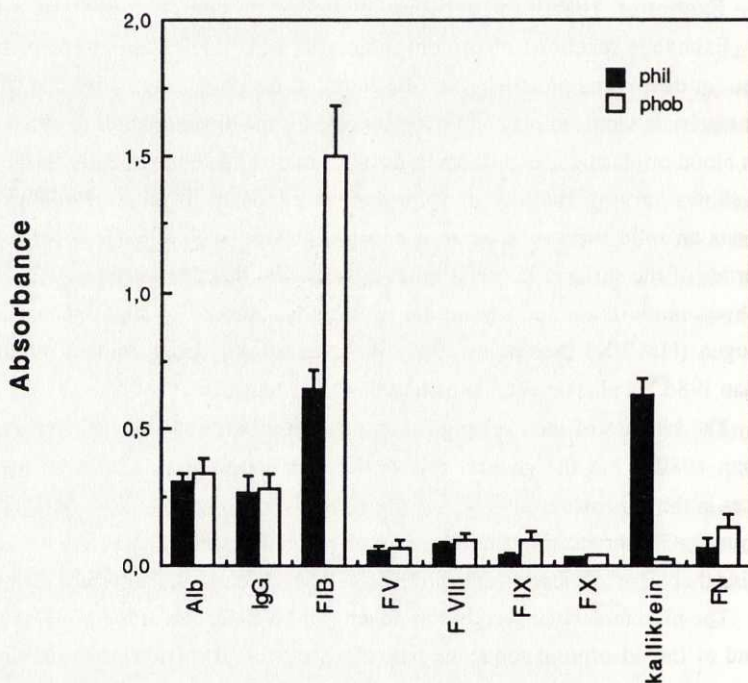
The high molecular weight kininogen (HMWK) is one of the proteins that form the end of the adsorption sequence onto the surfaces. It carries prekallikrein and the coagulation factor XI, (Mandle 1976 ; Thomson 1977) to the surface , where they interact with initially adhered and activated factor XII, initiating the coagulation cascade. Other protease cascades are initiated on the surface in similar ways.

On hydrophobic surfaces, however, the initial layer of fibrinogen is stable (Brash 1987; Horbett 1982 ; Vroman 1969 ; Vroman 1971 ), and no exchange reactions occur. Fibrinogen is an adhesive protein for cell-interactions (Ginsberg 1988) and has a tri-

peptide, Arg-Gly-Asp (RGD-sequence) present in the amino-sequence of the protein, acting as a cell-attachment site. This leads to the adhesion and subsequent activation of platelets via specific membrane-located receptors (GP IIb / IIIa) (Ginsberg 1988 ; Zwaal 1986).

Thus, both hydrophilic and hydrophobic surfaces finally cause thrombus formation, although via different parts of the initiating pathway of the coagulation process. The hydrophilic surfaces mainly induce plasma coagulation and the hydrophobic surfaces initiate the cellular pathway of blood coagulation by platelet activation.

As an illustration of the two pathways for thrombus formation, some model experiments were performed in the present study and the results are shown below. Human whole blood was anti-coagulated with hirudin and incubated for 10 minutes in hydrophilic and hydrophobic glass tubes, rinsed, and the adsorption of plasma proteins was then measured by an enzyme-linked immunosorbent assay.





#### 1.3.4 Arg-Gly-Asp (RGD)-sequence, the cell attachment site.

The site in adhesive proteins (adhesive proteins, incorrectly called adhesins in the third paper in the present thesis) that promotes cell attachment contains a tripeptide, the amino-sequence Arg-Gly-Asp (RGD), which is recognised by cell surface receptors (Ruoslahti 1986). Fibrinogen is an adhesive protein together with other adhering proteins such as fibronectin (a multifunctionell extracellular matrix and plasma protein), vitronectin (the active component of serum spreading factor and the S-protein of the complement membrane attack complex) and von Willebrand factor (F VIII : vWF). The RGD sequence is also the cell recognition site of a number of other extracellular matrix and platelet adhesion proteins (Ruoslahti 1988).

Platelets express various membrane receptors, some of which belong to the integrin family. The integrin GP IIb / IIIa on platelets binds irreversibly to the RGD-sequence of surface-bound fibrinogen and promotes adhesion of platelets to fibrinogen coated surfaces (Savage 1992). As an effect of fibrinogen binding, an extra pool of GP IIb / IIIa , located inside the platelets, is translocated to the cell surface (Wenchel-Drake 1993). The translocation induces an activation of GPIIb / IIIa, necessary for the binding to the ligands. Besides fibrinogen, also vWF, vitronectin and fibronectin mediate platelet aggregation (Goto 1995; Ruoslahti 1988 ). Fibrinogen adsorbed to solid surfaces has been shown to promote platelet adhesion-aggregation and participate in surface-induced thrombosis (Elam 1992; Packham 1969 ; Park 1991 ; Zucker 1969 ) and may lead to an inflammatory reaction in the surrounding tissue in contact with biomaterials (Elam 1993; Tang 1993 ).



#### **1.4. Interactions within complex cell systems (whole blood).**

Blood is a tissue which consists of a variety of cells suspended in plasma (Junqueira 1992). The cells of blood are of three major functional classes: erythrocytes, leukocytes and platelets.

Cell	Size	Number / $\mu$ l
Erythrocytes	6-8 $\mu$ m	4-6,000,000
Leukocytes :		6-10,000
Gr. Neutrophil	12-15 $\mu$ m	6-7,000
Gr. Eosinophil	12-15 $\mu$ m	2-400
Gr. Basophil	12-15 $\mu$ m	0-100
Lymphocyte	6-18 $\mu$ m	2-3,000
Monocyte	12-20 $\mu$ m	3-800
Platelets	2-4 $\mu$ m	2-400,000

The blood cells in a sessile drop of whole blood on material surfaces, make contact with the surface in accordance with the rules of mass transport. The flux of blood cells towards the material surface, is governed by parameters similar to those for proteins, namely size and concentration. Erythrocytes and platelets are the blood cells that collide most frequently with a material surface introduced into blood, where adhering platelets are the first reactive cells in the blood-biocompatibility relation. Since leukocytes are larger than the other blood cells and only represent about 0.1 % of the total flux of blood cells towards a blood-material interface, their cell adhesion will occur at a later time.

##### **1.4.1 Platelets**

The main function of blood platelets *in vivo* is to participate in the interactions with coagulation factors and vessel wall that constitute the haemostatic process. The major component of haemostasis is the formation of a platelet plug, followed by cross-binding of fibrin. The haemostatic system *in vivo* is feed-back controlled by physiological inhibitors in order to control and limit excessive or inappropriate activation and thus maintain vascular normal status. Blood is normally separated from the activators of haemostasis by the endothelial cell. Exposure of platelets to collagen and subendothelial basement membranes in injured vessels *in vivo*, or to artificial material surfaces *ex vivo* and *in vitro*, sets in motion a series of events in which the platelets may undergo adhesion, release and aggregation (Souhami 1994). This process also involves the expression of integrins and other membrane receptors.

### **Platelet cell surface receptors and integrins.**

The many cell membrane and cytoplasmic proteins that characterise haemopoietic cells have been given names in an international system, which relates to proteins (antigens) expressed at different phases of differentiation (Barclay 1994). These proteins are called CD molecules (Cluster of Differentiation). Antibodies to CD molecules are widely in use to identify specific types and activity stages of blood cells.

The integrins, making up one of the families of cell surface receptors, are heterodimers of two protein subunits, known as the  $\alpha$  and the  $\beta$  subunit (Ginsberg 1993). The integrins possess an extracellular domain that binds to the plasma protein RGD-sequence, a transmembrane domain, and a cytosolic domain binding to actin. Platelets have at least five different integrins, composed of five different  $\alpha$  subunits and two different  $\beta$  subunits (Wenche-Drake 1993).

Non activated platelets express CD 61, which is an integrin  $\beta$  chain expressed as a heterodimer, noncovalently associated with the integrin  $\alpha$  chain; CD41 forming the GPIIb/IIIa complex, or with CD51 forming the vitronectin receptor (Barclay 1994). GPIIb/IIIa binds to extracellular matrix proteins in a  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  dependent way (Ginsberg 1993).

CD62 also named P-selectin, GMP-140 or PADGEM, is present on activated platelets. CD62 is a polypeptide and a member of the selectin family of cellular adhesion molecules, containing lectin domains that recognise carbohydrate ligands. It is localised in the membrane of secretory granules of platelets and is translocated to the plasma membrane upon activation. A soluble form of CD62 has also been identified. CD62 has marked structural homology with E-selectin (ELAM-1) and L-selectin (LAM-1). CD62 mediates the interaction of activated platelets with neutrophils and monocytes (Nagata 1993) and is also responsible for the rolling attachment of neutrophils to activated endothelium at physiological shear stress in vivo (Barclay 1994).

### **Adhesion of platelets**

Circulating platelets adhere to non-endothelial structures, aided by the plasma proteins fibrinogen, fibronectin and high molecular weight multimers of factor VIII complex (FVIII : vWF). The RGD -sequence of those plasma proteins are recognised by the receptor protein, for example the integrins, leading to cell adhesion. The integrin GP IIb/IIIa binds directly to fibrinogen, fibronectin and F VIII:vWF (Savage 1992 ; Stewart 1993). Thus, GP IIb/IIIa on non activated platelets will bind to surface adsorbed fibrinogen and promote adhesion of platelets to fibrinogen coated surfaces (Nygren 1988a ; Savage 1992). Platelet adhesion to collagen in vivo is dependent on the platelet membrane receptors GP Ia, which binds directly to collagen, and GP Ib which binds to von Willebrand factor (VIII : vWF) that adheres to collagen (Kumar 1994; Savage 1992).



## Release

Following the adhesion of a single layer, platelets undergo a shape change from the normal disc form to a sphere, and they spread and form pseudopods and may release the contents of their cytoplasmic granules (degranulation). The platelet granules include the dense bodies (containing ADP, ATP, serotonin etc.) and the  $\alpha$ -granules (containing  $\beta$ -thromboglobulin, fibrinogen, F VIII : vWF, factor V, fibronectin, P-selectin, platelet-derived growth factor, platelet factor 4, thrombospondin etc.) and lysosomal granules (containing lysosomal enzymes) (Souhami 1994).

## Aggregation

Release of ADP leads to the exposure of an extra pool of GP IIb/IIIa, translocated from the inside of the adhering platelets to the membranes (Wenchel-Drake 1993). Fibrinogen binds to the exposed GP IIb/IIIa, forming bridges between the platelets which form activated aggregates (platelet reversible aggregation) (Vander 1994). Platelet aggregation requires adequate  $Ca^{2+}$  -levels in the surrounding medium, and it is induced via a large range of factors which react with receptors on the platelet surface. Many of these factors such as ADP, serotonin and thromboxane  $A_2$ , are themselves released by activated platelets, producing a positive feedback loop (Kumar 1994). The process of platelet activation and aggregation is mediated by at least two separate pathways (Kumar 1994).

Ligands such as collagen, bind to their specific receptors on platelets. This leads to activation of membrane phospholipases with the release of arachidonic acid from the platelet membrane phospholipids. A proportion of arachidonic acid is then converted to cyclic prostaglandin endoperoxides, and then to thromboxane  $A_2$  (TXA $_2$ ). TXA $_2$  is biologically highly active, and mediates a rise in intracellular  $Ca^{2+}$  and platelet granule release of ADP which then promotes further platelet aggregation (Souhami 1994).

Other activating factors such as thrombin, bring about a rise in intracellular  $Ca^{2+}$  and degranulation by a mechanism distinct from that of arachidonic acid metabolism and TXA $_2$  generation. Thrombin, released by the activation of the common pathway in the coagulation cascade, stimulates the formation of a platelet plug. Factor VIII: vWF, factor V and fibrinogen are stored in the  $\alpha$ -granules and released by the activated platelets, which in addition, expose phospholipids on the membrane surface that facilitate the activation of factors VIII and V. In this way, activated platelets trigger the coagulation cascade by activating factors IX and X which converts prothrombin to thrombin. The presence of thrombin encourages fusion of platelets, and fibrin formation reinforces the stability of the platelet plug (irreversible aggregation) (Kumar 1994).



### **Formation of platelet-derived microparticles.**

Platelet-derived microparticles are formed in response to activation of platelets by a variety of platelet activators such as thrombin and collagen (Gilbert 1991), the complement proteins C5b-9 (Gilbert 1991; Sims 1988), immune complex (Larsson 1994), artificial material contact (Gemmel 1995) etc. Release of (shedding of) platelet-derived microparticles from the platelet surface takes place in parallel with secretory fusion of the  $\alpha$ -granule membrane with the plasma membrane (Sims 1988). The platelet-derived microparticles is a heterogeneous population, ranging in size from 0.1 to 0.8  $\mu\text{m}$  (Gemmel 1995) and contain the plasma membrane glycoproteins GP Ib and GP IIb/IIIa as well as the  $\alpha$ -granule membrane protein P-selectin (CD 62P) (Sims 1988). The fusion of the microparticle membrane with the cell membrane has been shown to provide a catalytic phospholipid surface consisting of phosphatidylserine, for assembly of factors Va, VIIIa, Xa. This stable procoagulant surface has the possibility to accelerate the blood coagulation with generation of thrombin, leading to fibrin formation and ultimately causing artificial materials failure (Gilbert 1991; Jy 1995; Sims 1988).

### **Platelet markers of artificial material-induced activation.**

Several markers of artificial material-induced platelet activation are used in the present thesis to register activation events, such as ; fibrinogen receptor expression and up-regulation (activated GP IIb/IIIa, CD 61 expression), analysis of  $\alpha$ -granule release of  $\beta$ -thromboglobulin (a platelet specific protein),  $\alpha$ -granule release with P-selectin expression (CD 62P expression), platelet derived microparticle formation, registration of the occurrence of activated platelets binding to granulocytes (platelet-granulocyte aggregates) and the activation state of the platelet in any such aggregates (Rinder 1992).

### 1.4.2. Granulocytes

The neutrophil granulocytes (neutrophils, polymorphonuclear leukocytes or PMNs) are the dominating phagocytic cell-type during the acute phase of an inflammation *in vivo*. The neutrophils have the possibilities to recognise, phagocytose and kill bacteria, and have been called the body's first line of defence against bacterial infections (Ganong 1995). They leave the circulation at sites of inflammation in order to localise and neutralise invading bacteria through phagocytosis, followed by the secretion of cytotoxic agents. The two main types of cytotoxic secretion products are the proteolytic enzymes, stored in the intracellular granules of the neutrophils, and the oxygen radical species, produced by a chemical reaction known as the respiratory burst. At sites of prolonged inflammation, the short lived neutrophils are followed by other phagocytic cell-types, such as monocytes and macrophages (Gallin 1992). Leukocyte recruitment to sites of inflammation and tissue injury is mediated by multiple adhesion molecules, which are required for the attachment of the cells to the blood vessel endothelium, their subsequent extravasation into the surrounding tissue, their migration and phagocytosis. Leukocyte adhesion receptors are grouped into three main families: the integrin family, the selectin family and the super-immunoglobulin family.

The granulocyte is the second type of cell that will interact with an artificial surface (after the platelets). It is well established that contact between blood and, for instance artificial surfaces of extracorporeal bypass circuits, results in activation of neutrophils and the complement, kinin, fibrinolytic and coagulation cascades (Kirklín 1989; Lew 1985; Westaby 1987). This generalised inflammatory response has been associated with cardiac, pulmonary, and renal dysfunction (Kirklín 1989).

#### **Adhesion**

Adhesion between neutrophils and endothelial cells is a complicated process *in vivo*, occurring in at least two steps:

- *Rolling* The first step, known as "rolling", is constituted by a recurrent transient adhesion between the leukocytes and the endothelial cells, leading to a rolling motion of the leukocytes along the vessel wall. The rolling adhesion, often seen early during an initial inflammatory process, involves members of the selectin family. The P-selectin (CD 62) is localised in the membrane of secretory granules of endothelial cells (and platelets) and is rapidly translocated to the plasma membrane upon activation (McEver 1989). The P-selectin is responsible for the rolling attachment of neutrophils to activated endothelium at physiological shear stress (CD 62 on platelets mediates the interaction of activated platelets with neutrophils) (Lawrence 1991) where CD 62 binds to the carbohydrate structure sialyl-Lewis X on neutrophils. Because CD62 can be expressed and rapidly



mobilised, CD62 may be in position to catch previously unstimulated neutrophils at very early stages (Polley 1991).

Endothelial cells adjacent to a site of inflammation are stimulated by cytokines which provide an up-regulation of E-selectin (ELAM-1) on the endothelium. ELAM-1 would then serve to sustain the phenomenon of rolling and mediate the initial interaction between the stimulated endothelium and unactivated neutrophils (Gallin 1992).

• *Sticking* The next step is constituted by "sticking" or firm, stationary adhesion (Ley 1992). Stationary adhesion is associated with obvious activation of the neutrophil. The cells change shape, becoming ruffled, flattened, and bipolar. The mechanisms that account for these events are not well-defined, though locally generated chemotactic factors, and the adhesive mechanisms that initially catch the neutrophils may also contribute (Lo 1991 ; Lorant 1991). This stage accomplishes an important transition for the neutrophil, not only from an unactivated to an active cell, but from a primarily selectin-family dependent adhesion to predominantly integrin-mediated adhesion by the CD18-family molecules expressed in the plasma membrane of the neutrophils. Downregulation/shedding of the selectin-family molecules and up-regulation of neutrophil CD11b/CD18 (Mac-1, CR3) occur during this transition. The integrins CD11b/CD18 and to CD11a/CD18 (LFA-1) bind to the CD54 (ICAM-1) receptor on activated endothelial cells (Barclay 1994; Harlan 1992 ). CD11b/CD18 also mediates adhesion to and phagocytosis of particles coated with the complement fragment C3bi (Lew 1990).

### **Priming, an enhanced cell reactivity**

It has become apparent that neutrophil granulocytes can exhibit different levels of reactivity to stimulation. Neutrophil cytotoxic responses (respiratory burst and degranulation) can be obtained by a variety of stimuli. The cells may be "primed" by certain stimuli in a manner that amplifies the subsequent response to the same stimulus or to other stimuli. Most chemoattractants in low doses, such as the chemotactic peptide f-MLP (N-formyl-methionyl-leucyl-phenylalanine) etc. (Gallin 1992), induce priming. It is believed that priming of the neutrophils may already take place during the sticking adhesion to venular endothelium.

### **Transendothelial emigration**

Transendothelial emigration of neutrophil granulocytes across endothelial monolayers is obtained in response to a chemotactic stimulation, and requires the action of the CD11a/CD18, CD11b/CD18 receptors on the neutrophils and the PECAM-1 (CD31) receptors on the endothelial cells, which are concentrated at the junctions between them (Newman 1990). The activated neutrophils project pseudopods, formed by the focal polymerisation and depolymerisation of actin, and in this way gain mobility.



The cells then traverse between two adjacent endothelial cells (diapedese), pass the basement membrane of the vessel and enter the tissue.

### **Migration**

Neutrophils migrate from the circulation to accumulate at extravascular sites of inflammation in response to chemotactic mediators. Studies of the cellular actions of chemotactic factors have demonstrated that they bind to their specific cell-surface receptors and stimulate the biological responses by neutrophils such as actin polymerisation, integrin expression and priming (Harlan 1992). Migratory responses of neutrophils i.e. chemotaxis, are induced by chemoattractant doses that are at least 20-fold lower than those required to stimulate a secretory response. For example, the chemoattractant receptors share the property of being able to trigger directional movement in the presence of very low concentrations of ligand ( $10^{-11}$  to  $10^{-8}$  M), whereas at higher ligand concentrations there is an arrest of cell movement and activation of secretory processes ( $10^{-7}$  to  $10^{-6}$  M) (Lew 1990).

Chemotactic factors include f-MLP (bacterial peptides), C5a (a product of the complement cascade), PAF and LTB4 (platelet-activating factor and leukotriene B4; secreted products of stimulated phospholipid metabolism) and cytokines such as IL-8 (interleukin-8). Lipid chemoattractants (e.g. PAF and LTB4), while equally potent and active as chemotactic agents, are less effective secretagogues (Gallin 1992).

### **The intracellular calcium level**

Under native conditions, most agonists that elicit receptor responses in neutrophils and net actin assembly, also seems to induce transient rises in intracellular calcium concentration ( $Ca^{2+}$ )<sub>i</sub>. The ( $Ca^{2+}$ )<sub>i</sub> of a resting neutrophil is around 100nM, approximately 20,000-fold lower than the extracellular free  $Ca^{2+}$  in vivo. To obtain and maintain this low ( $Ca^{2+}$ )<sub>i</sub>, intracellular  $Ca^{2+}$  is pumped and sequestered into intracellular  $Ca^{2+}$  stores i.e. calciosomes, and the plasma membrane pump also transports  $Ca^{2+}$  to the extracellular space (Krause 1987; Lagast 1984a ; Lagast 1984b ).

The intracellular calcium level acts as an intermediate messenger in several processes included in different responses to neutrophil activation, evoked by particulate and soluble activators (Dore 1990; Haag Weber 1994 ). For example; during f-MLP activation , there is a rapid rise in ( $Ca^{2+}$ )<sub>i</sub> resulting from a transient  $Ca^{2+}$  release from internal stores, and a more sustained  $Ca^{2+}$  influx from the extracellular space (Andersson 1986).

A transient increase in intracellular  $Ca^{2+}$ -level, is a typical feature of chemotactic stimulation of neutrophils (Gallin 1992). It is known that intracellular calcium is an important signal for neutrophil degranulation (Haag Weber 1994 ; Lew 1990), actin polymerisation (Bengtsson 1986), integrin expression (Berger 1985) and phagocytosis

(Jaconi 1990). The respiratory burst is in some cases associated with an increased intracellular  $Ca^{2+}$  level (Ishihara 1990; Walker 1991). The pre-activation or priming that takes place upon weak stimulation of the neutrophils is in some cases (Walker 1991; You 1991), but not always (You 1993), associated with changes in the intracellular  $Ca^{2+}$  level.

## **Phagocytosis**

The specific process of phagocytosis may be divided into two steps (Gallin 1992):

- binding of the foreign particle to the surface of the neutrophil (which is a prerequisite for phagocytosis)
- engulfment of the bound particle

Engulfment of a bound particle occurs through the elaboration of broad, flat pseudopods which surround the particle on all sides. Remodelling of the cortical actin cytoskeleton causes advancing pseudopods to meet at a single point, and fusion of opposing membranes at this point pinches off the resulting phagosome into the cytoplasm of the neutrophil. The pseudopods are elaborated in response to signals in the cytoplasm arising from ligated receptors, and the movement of a phagocyte's plasma membrane along the surface of a ligand-coated particle is governed by the availability of receptors on the surface of the neutrophil, and is also guided by the distribution of ligands on the surface of the particle. The neutrophils are also primed for an enhanced cytotoxic response during phagocytosis of the foreign particles (Gallin 1992).

Phagocytic neutrophils express on their surfaces a large variety of phagocytic receptors such as receptors for IgG and complement etc. (Barclay 1994; Lew 1990).

### *Receptors for IgG.*

The antigen-combining sites of IgG molecules bind to surface antigens on foreign particles and expose the Fc domains on the particle surface, a process known as opsonisation. The opsonised particles are then recognised by three classes of receptors on human leukocytes, termed  $Fc\gamma RI$ ,  $Fc\gamma RII$  and  $Fc\gamma RIII$  (CD16). These receptors have been shown to be members of the immunoglobulin superfamily. The three  $Fc\gamma$  receptors mediate binding and phagocytosis of particles coated with immune complexes. During phagocytosis of IgG-coated particles, the ligation of  $Fc\gamma$  receptors appears sufficient to initiate production of toxic molecules which are secreted into the newly formed phagosomes. However, some leakage of proteolytic enzymes and oxygen free radical species into the extracellular space occurs during phagocytosis.



### *Receptors for complement.*

C3 is the most abundant complement protein in serum and it can be deposited on foreign particles by covalent binding, leading to the opsonisation of the particles. Upon proteolytic activation C3 is then rearranged to yield C3b, which is recognised by a receptor termed CR1 (CD35) on the cells. After C3b is deposited on the surface of foreign particles, its life time is short and the surface-bound C3b is subsequently degraded to C3bi, a form which is stable in serum for several hours and is recognised by a receptor termed CR3 (CD11b).

CR1 and CR3 mediate binding of foreign particles, and when appropriately stimulated, mediates phagocytosis. In contrast with the other opsonic receptors (CR1 and Fcγ receptors), the CR3 receptor requires divalent cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) for optimal ligand binding. Furthermore, CR3 is an integrin  $\alpha^{\text{M}}$  chain which is expressed as a heterodimer noncovalently associated with CD18 (integrin  $\beta_2$ ). Thereby, besides the interaction with the complement component C3bi, CD11b/CD18 also mediates the interaction with both extracellular matrix proteins and cell surface proteins such as CD54 expressed on activated endothelium.

### **Secretion**

During phagocytosis, the neutrophil granules move to the cell membrane and discharge their contents into the phagocytic vacuole and into the extracellular medium, a process known as degranulation (Sengeløv 1993). The degranulation also leads to the translocation of the NADPH oxidase to cell membranes, and the subsequent activation of this enzyme initiates a production of toxic oxygen metabolites i.e. the respiratory burst (Turner 1994). The activation of NADPH oxidase is associated with a sharp increase in  $\text{O}_2$  uptake and metabolism in the neutrophil, and a production of superoxide anions ( $\text{O}_2^-$ ). Through secondary reaction steps, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and single oxygen ( $^1\text{O}_2$ ) is generated. These molecules are oxidants, that are effective cytotoxic agents for foreign particles, by direct or indirect action with their DNA (Miller 1995). In addition, neutrophil degranulation discharges the enzyme myeloperoxidase, which catalyses the conversion of  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$  and  $\text{SCN}^-$  to the corresponding acids (HOCl, HOBr, etc.), which also acts as potent oxidants. The proteolytic enzymes in the discharged granules together with the myeloperoxidase enzyme act in a cooperative fashion with the  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$  and HOCl to produce a killing zone around the activated neutrophil, thus forming the last step in the inflammatory process (Gallin 1992; Ganong 1995).



### **Granulocyte markers of artificial material-induced activation.**

A very small percentage of the granulocytes in whole blood are in direct contact with the walls in a macroscopical flow device such as a dialyser or oxygenator (Kjellstrand 1991). Furthermore, it is shown that granulocyte adhesion will occur at a later time than platelet adhesion, thereby indicating that direct activation of the cells by a biomaterial surface is limited. In order to be harmful to the patient, the activation of granulocytes must be generalised in the vascular bed, where major complication for the patient occurs when activated granulocytes accumulate in vital organs such as the lungs, thereby causing subsequent tissue damage through the secretion of oxygen radicals and proteolytic enzymes. A minor complication for the patient is for example, the tendency to develop thrombophlebitis during use of polymer catheters commonly used in medical therapy (Dabir 1982 ; Gaukroger 1988 ; Larsson 1989 ; Reynolds 1995 ; Madan 1992 ; Lindblad 1987 ; Myles 1991 ; Payne-James 1991). However, there may be a number of possible pathways that generate plasma soluble activation products that end up in granulocyte adhesion and activation during blood-material contact.

Several studies have focused on complement activation of granulocytes (Cheung 1990 ; Haag Weber 1994) caused by the direct contact between a dialyser membrane surface and the complement factors present in plasma. Studies of patients under cardio-pulmonary bypass showed a possible interaction between platelets and granulocytes (Rinder 1992) where the circulating platelets were shown to express P-selectin with the possibility to bind and activate the granulocytes (Nagata 1993). Potential granulocyte activation is also provided by the release of platelet-derived microparticles during blood exposure to various biomaterial surfaces, shown in a previous study (Gemmel 1995).

Several markers of artificial material-induced granulocyte activation are used in the present thesis to register activation events such as ; expression and upregulation of the IgG-receptor (CD16) and complement receptors 1 and 3 (CD35 and CD11b) on granulocytes, evaluation concerning granulocyte intracellular  $Ca^{2+}$  concentration (by Calcium Green-1 fluorescence), evaluation concerning priming (increase of f-MLP induced respiratory burst) and respiratory burst (by chemiluminescence). In order to investigate the mechanisms behind the material-induced granulocyte activation, the possible role of complement was investigated by measurements of the complement protein C3 on material surfaces, and in serum from surface exposed blood. The occurrence of platelet-derived microparticles was examined by ultra centrifugation of serum from surface exposed blood, and evaluated in respect to their induced  $Ca^{2+}$  responses in granulocytes.

## 2. AIMS OF THE PRESENT THESIS

The aims of the present work were:

- To elucidate the relationship between kinetics, supramolecular structure, and equilibrium properties of surface adsorption of plasma adhesive proteins, with special emphasis on fibrinogen.
- To study the subsequent biological response to the surface adsorbed protein layer, such as cell adhesion and activation of platelets and granulocytes, using different established techniques.
- To develop a new method to study an immediate inflammatory response of a blood-biomaterial interaction, in respect to the liberation of granulocyte activating factors.
- To investigate if the properties of a biomaterial surface and the choice of anticoagulants, were important determinants of the outcome of humoral and cellular response to a blood-biomaterial interaction.



### 3. MATERIALS AND METHODS.

A general description is given below, whereas the experimental procedures are described in detail in the subsequent individual papers. All human and animal experiments were approved by the respective ethical committees of the University of Göteborg.

#### **3.1 Kinetics, supramolecular structure and equilibrium properties of fibrinogen adsorption at liquid-solid interfaces. (I).**

The present study was undertaken in order to elucidate the relationship between kinetics, supramolecular structure and equilibrium properties of fibrinogen adsorbed onto hydrophilic and hydrophobic quartz surfaces, studied by ellipsometry and transmission electron microscopy (TEM).

*Ellipsometry;* Quartz surfaces (silicon wafers) were either made hydrophobic with hexamethyl-disilazane (Jönsson 1985) or made hydrophilic by oxidation with dichromate sulphuric acid (Nygren 1988b). The static contact angle with water was measured from photographs of resting water drops placed at the surfaces (Nygren 1988b). Human fibrinogen was diluted in PBS (phosphate-buffered saline) and placed in droplets on the surfaces and incubated for various times of adsorption of up to 3 days. Dissociation of bound fibrinogen was studied by rinsing the plates in PBS for differing time periods up to 24 h. The amount of bound fibrinogen was determined by measurement of the optic mass of protein in each spot in a comparison ellipsometer (Stenberg 1982), equipped with a SiO<sub>2</sub>-reference surface and by calculation of the corresponding amount of protein as described previously (Stenberg 1983).

*Electron microscopy;* Negative staining of adsorbed fibrinogen, incubated 20h or 72 h, was performed on hydrophilic or hydrophobic quartz grids (Stenberg 1987) to allow comparison with ellipsometry data. The specimen were then dried and examined in a transmission electron microscope (TEM).

*Evaluation of data;* The reaction at the interface of two phases, the liquid-solid interface, is involved in a number of distinct processes. The reactants in the solution must first diffuse to the interface, be adsorbed there, and participate in a reaction mechanism on the solid surface, the products on the surface may then desorb and diffuse into the solution again. Often is the rate-determining step assumed to be the actual reaction on the surface, where the rate depends on the concentration of the reactants adsorbed on the surface (Berry 1980).



a) For an unstirred solution and a flat surface geometry, the maximum and diffusion-rate limited amount of bound fibrinogen could be described by the time-dependence equation (Trurnit 1954):

$$S=(2/\sqrt{\pi})c_0\sqrt{Dt} \quad (1)$$

where  $S$  is the surface concentration of protein ( $\text{mol}/\text{cm}^2$ ),  $c_0$  is the concentration of protein in solution at time zero ( $\text{mol}/\text{cm}^3$ ),  $D$  is the diffusion constant of the protein ( $\text{cm}^2/\text{s}$ ) for fibrinogen ( $D=2.02 \times 10^{-7} \text{ cm}^2/\text{s}$ ), and  $t$  is time (s).

b) Assuming the reaction-rate limited adsorption of protein to be an equilibrium reaction, the reaction rate is given by the equation (Berry 1980; Langmuir 1918):

$$dS/dt = k_1c(S_{\text{max}}-S) - k_{-1}S \quad (2)$$

where  $k_1$  is the forward reaction rate constant ( $\text{M}^{-1}\text{s}^{-1}$ ),  $k_{-1}$  is the dissociation rate constant ( $\text{s}^{-1}$ ),  $c$  (M) is the protein concentration in the bulk solution at time  $t$ ,  $S$  ( $\text{moles}/\text{cm}^2$ ) is the surface concentration of protein at time  $t$ , and  $S_{\text{max}}$  ( $\text{moles}/\text{cm}^2$ ) is the maximum amount of bound protein at the surface.

c) The rate of the reverse reaction, the dissociation of protein, is evaluated by measuring the surface concentration of protein at rinsing time  $t$ . For rinsing the Eq. (2) is used with  $c=0$ :

$$dS/dt = -k_{-1}S \quad (3)$$

separating the variables and integrating, gives the following equation:

$$\ln S/S_0 = -k_{-1}t \quad (4)$$

where  $S_0$  is the surface concentration of protein at time  $t=0$  ( $\text{moles}/\text{cm}^2$ ).

The dissociation rate constant  $k_{-1}$  is evaluated as the slope from plots of  $\ln(S/S_0)$  versus time  $t$ .

d) Assuming the reaction rate limited adsorption of protein to have reached a dynamic equilibrium where  $dS/dt = 0$ , Eq. (2) then could be written:

$$k_{-1}S = k_1c(S_{\text{max}}-S) \quad (5)$$

which could be rearranged to give:

$$S/c = K_a(S_{\text{max}}-S) \quad (6)$$

where  $K_a=k_1/k_{-1}$  is the equilibrium constant ( $\text{M}^{-1}$ ). A commonly used graphic method of analysing complex binding data, Scatchard plot (Creighton 1984; Scatchard 1949) could thereby be plotted. Thus, binding of a protein to one site on the surfaces could increase or decrease the affinities of the other sites; i.e. there could be either positive or negative cooperativity in the protein layer. The concentration-dependence of fibrinogen adsorption could thereby show deviations from a simple Langmuir isotherm described by Eq. (6), which should give a straight line in this plot. The Scatchard plots were obtained by plots of  $S/c$  versus  $S$  based on the data obtained by the ellipsometry.

The amount of fibrinogen adsorbed on the surface (abscissa, moles/cm<sup>2</sup>). The bound / free ratio of fibrinogen (ordinate, cm) was calculated as the ratio between the measured amount of bound fibrinogen, S (moles/cm<sup>2</sup>) and the remaining free concentration in the bulk solution of the droplets, c (moles/cm<sup>3</sup>) according to the equation;

$$c = c_0 - S(A/V) \quad (7)$$

calculated of concentration of protein in solution at time zero ( $c_0$ ), where  $V$  (cm<sup>3</sup>) is the volume of the droplet, and  $A$  (cm<sup>2</sup>) is the area of the spot.

e) The spatial distribution of the fibrinogen molecules during adsorption was shown by TEM by a negative staining of the protein layer. The average intermolecular distance (16 nm) was calculated from micrographs. Theoretical calculations of the maximum amount of bound protein at the surface in one monolayer of tight-packed fibrinogen molecules were performed. This was done by assuming different types of molecular orientation of the fibrinogen molecules, together with different average intermolecular distances. The intermolecular distance of 16 nm between parallel molecules was applicable to dehydrated fibrinogen. Fibrinogen molecules appear as hydrated protein molecules in the native state, and has the molecular dimensions with concomitant theoretical average intermolecular distance of 9 nm, as previously described (Bachman 1975).

### **3.2 Cooperativity in the adsorption of cellulose ethers and fibrinogen at liquid-solid interface, (II).**

The possibility of reducing fibrinogen adsorption to solid surfaces by competitive adsorption of water-soluble cellulose ethers (EHEC, HEC) was investigated by an ELISA assay. The ELISA assay was calibrated by ellipsometry, assuming the diffusion rate limitation of the initial adsorption of protein to the surface. The total depletion of fibrinogen from the bulk solution was also noticed as the maximum amount of fibrinogen that can bind to the surface when a droplet of bulk solution is depleted of all its protein.

*ELISA*; ELISA was performed as a spot-test on glass slides (SiO<sub>2</sub> content 72-74%), which were made hydrophobic with hexamethyl-disilazane (Jönsson 1985) or made hydrophilic by oxidation with dichromate sulphuric acid (Nygren 1988b). The static contact angle with water was measured from photographs of resting water drops placed at the surfaces (Nygren 1988b). Human fibrinogen was diluted in PBS and placed in droplets on the surfaces and incubated. The fibrinogen solution was in some experiments mixed with EHEC; ethyl(hydroxyethyl)cellulose (Bermocoll CST-103), or HEC; hydroxyethylcellulose (Natrosol 250 HBR), diluted in PBS. The fibrinogen and polymer solutions were mixed, placed in droplets and incubated. After rinsing, the primary antibody rabbit-antihuman fibrinogen was added, followed by peroxidase-conjugated sheep anti-rabbit immunoglobulin. The substrate reaction was performed by incubation with diaminobenzidine containing hydrogen peroxide. The optical density was measured by a spectrophotometer equipped for density measurements of glass slides.



*Ellipsometry*; The ELISA was calibrated by ellipsometry to allow interpretation of optical density values in terms of surface concentration of fibrinogen. The calibration procedure was similar to the procedure described previously (Nygren 1985), using the diffusion rate limitation of protein to the surface. A dilution series of human fibrinogen solution was placed in droplets on hydrophobic (Jönsson 1985) or hydrophilic (Nygren 1988b) quartz surfaces (silicon wafers), incubated, rinsed and blown dry with an air current.

*Evaluation of data*; The amount of bound fibrinogen on the surfaces was determined as described in section 3.1. The initial adsorption of fibrinogen at low surface concentrations was diffusion-rate limited, as shown in a previous study (Nygren 1988b). Experimental values of the amount of bound fibrinogen measured by ellipsometry were plotted against the calculated amount for fibrinogen according to Eq. (1) in section 3.1. The experimental values were seen to be close to the calculated amount below 0.90 pmol/cm<sup>2</sup>. The calibration curve of the ELISA was then obtained by plotting experimental light absorbance values measured by ELISA, versus the calculated surface concentration of bound fibrinogen according to Eq. (1) in section 3.1. It was seen from this plot that the absorbance values were adequately described as a function of surface concentrations, as evaluated from Eq.(1). For the absorbance values below 1.0 the response was almost linear with a correlation coefficient of  $r=0.95$  and a slope of the line of  $m=2.30$  abs. units per pmol/cm<sup>2</sup>.

Calculations of total depletion of the maximal amount of fibrinogen present in the droplets of the solutions used for incubations, were performed according to the equation;

$$S_{\text{dep}}=c_0(V/A) \quad (8)$$

where  $S_{\text{dep}}$ (moles/cm<sup>2</sup>) is the maximum amount of fibrinogen that can bind to the surface when the droplet is depleted of all its protein,  $c_0$  (moles/cm<sup>3</sup>) is the concentration of protein in solution at time zero,  $V$  (cm<sup>3</sup>) is the volume of the droplet, and  $A$  (cm<sup>2</sup>) is the area of the spot.

Scatchard plots were obtained,  $S/c$  versus  $S$ , based on the data obtained by the calibrated ELISA and according to the equations (2,5,6,7) in section 3.1.

### **3.3 Pre-adsorption of a cellulose ether onto polymer surfaces, (III).**

The effect of pre-adsorbed cellulose ethers upon the adsorption of plasma proteins at polymer surfaces was measured by an enzyme-linked immunosorbent assay. Tubes of polyurethane and Teflon® were used and cellulose ethers EHEC A,B,C and HPC (Bermocoll CST-103; DVT 85007; SM 8713 and Klucel MF) were pre-adsorbed for 20h, the tubes were then emptied and carefully rinsed with saline.

The tubes were filled with human fibrinogen solution or human whole blood, anticoagulated with hirudin, a specific thrombin inhibitor. Retained Ca<sup>2+</sup> activity was



thereby made possible, keeping the initial coagulation cascade and calcium-dependent cell-protein interactions intact during the experiments. The final concentrations of hirudin (50 units / ml) allowed the blood to remain liquid in the tubes after 2h (Elam 1992). The blood or fibrinogen solution were incubated in the tubes and after 5 min the tubes were emptied and rinsed.

In one group of tubings, the relative amounts of adhesive proteins were investigated using ELISA (Elam 1992) with specific antisera directed against human fibrinogen, fibronectin and monoclonal antibody against human vitronectin. In the second group of tubings, the amount of platelet released  $\beta$ -thromboglobulin was measured and the test was performed as described by the manufacturer.

Animal *ex vivo* studies were performed using male, New Zealand white rabbits. PUR tubes were connected as a shunt between arteria carotis and vena jugularis for 2h. The first half of the tubes were pre-filled with PBS and the second half pre-treated with cellulose ethers, Bermocoll CST-103 or SM 8713. After being rinsed with saline, fixed in glutaraldehyde and gold sputter coated, the tubings were examined in a scanning electron microscope and the number of adhering platelets were counted.

#### **3.4 Protein-platelet and platelet-leukocyte interaction at materials in contact with human blood, (IV).**

The adhesion and activation of human platelets and leukocytes together with analysis of adsorbed plasma proteins at blood-material interfaces, was studied by using fluorescence microscopy and photometry. Three groups of experimental models were investigated by using specific anti-CD and anti-plasma protein antibodies, and the calcium probe Fura-2.

In the first group, hydrophilic or hydrophobic (methylised) glass slides were prepared and human blood was placed in droplets on the glass surface from 15s to 2h incubation time and the slides were then rinsed. The adsorption of plasma proteins was detected by incubation with FITC (fluorescein isothiocyanate) labelled antibodies directed against albumin, IgG, fibrinogen, fibronectin, v. Willebrand factor (VIII : vWF), prothrombin / thrombin and complement factor C3c. The adhesion of platelets was shown by FITC-labelled antibodies directed against CD61 (GPIIIa), specific for this cell type. Adhesion of leukocytes was measured by staining their DNA with 0.1% acridine orange, which is a specific stain for DNA-containing cells i.e. leukocytes in blood. The glass slides were examined in a fluorescence microscope and the fluorescence intensity was recorded and photographed.

In the second group of experiments, human granulocytes were isolated by a one-step Percoll<sup>®</sup> technique (Braide 1986), suspended, recalcified and loaded with the calcium indicator Fura-2/AM<sup>®</sup> together with pluronic acid, added to solubilise Fura-2 in

physiological media (Poenie 1986). The supernatant of hirudin-anticoagulated blood (Elam 1992) (from blood previously exposed to hydrophilic or hydrophobic glass surfaces) was added to the loaded granulocytes and the fluorescence was recorded by a spectrofluorometer. A rapid peak was seen, indicating calcium influx into the cytoplasm. An attempt was also made to remove the activating substance from the supernatant by filtering through a 0.1-0.45  $\mu\text{m}$  Millipore<sup>®</sup> filter and then adding it to the cell preparation. As a biological control of the system, the chemotactic peptide for leukocytes, f-MLP was used as stimulus, with a final concentration of  $10^{-7}$  M in the cuvette. After the measurements, an identical reference recording was made with unloaded cells to compensate for medium autofluorescence.

Thirdly, blood samples were taken from patients undergoing cardiopulmonary bypass operations for measurement of the expression of cell surface receptors on leukocytes. Consecutive blood samples were drawn from 5 patients at defined stages: 1. before surgery; 2. after 1h of surgery; 3. after cooling and 15 minutes of oxygenator circulation; 4. after 45 min of extra corporal circulation; 5. after blood warming before disconnection; and 6. 30 min after disconnection. Blood samples were anticoagulated with hirudin, cooled, incubated with antibodies for 30 min and fixed with formaldehyde. Fluorochrome-conjugated monoclonal mouse antibodies directed against human CD11b (CR3), CD16 (IgG-receptor), CD35 (CR1), CD61 (platelet GPIIIa) and CD62 (platelet P-selectin) were used in order to evaluate the occurrence of platelet-granulocyte aggregates and the activation state of the platelet and granulocyte in any such aggregates (Rinder 1992). In the following FACSCAN (fluorescence activated cell sorting) evaluations, the granulocyte cells were discriminated by size and granularity and evaluated by the mean fluorescence in relation to controls with non-related antibodies. In each patient the obtained values were normalised to the values obtained before the operation. Results were expressed as the mean of all patients for each antibody and stage of surgery.

### **3.5 Exposure of blood to biomaterial surfaces liberates substances that activate PMNGs, (V).**

The present study shows an experimental model, where the intracellular calcium level of test granulocytes in suspension reflects the presence of granulocyte activating factors, produced by the previous interaction between human blood (anti-coagulated in different ways) and hydrophilic or hydrophobic (methylised) glass surfaces.

Human blood was obtained by vein puncture of a healthy donor, anti-coagulated with different concentrations of heparin (10, 1 and 0.1 units/ml whole blood) or hirudin (Revasc<sup>®</sup>; 500, 250 and 50 units/ml whole blood), a specific inactivator of thrombin. In some experiments, native serum was obtained instead. The whole blood was transferred



under saline to the bottom of a glass test tube with either hydrophobic (Jönsson 1985) or hydrophilic (Nygren 1988a) inner surfaces. After 10 minutes of incubation, the test tube was spun and the platelet-poor plasma layer or serum layer was removed and transferred to a suspension of fluorescent test granulocytes.

Human granulocytes, used as test granulocytes in the study, were obtained from the same donor and isolated by a one-step Percoll® technique (Braide 1986), suspended, recalcified and loaded with the long-wavelength calcium indicator Calcium Green-1/AM® together with pluronic acid (Poenie 1986). The use of a long-wavelength fluorochrome such as Calcium Green-1® minimised the background autofluorescence of the suspending medium.

The rise of intracellular calcium level of the test granulocytes after addition of the supernatant from surface-exposed blood (constituted 1/4 of the total fluid volume of the cuvette), was used to quantitate the degree of granulocyte activation. The signal was detected by the fluorescence intensity and measured in a spectrofluorometer. The calcium level was also related to the  $Ca^{2+}$  signal obtained after a standardised activation of the same granulocyte suspension by  $10^{-7}$  M f-MLP used as a positive biological control, where HBSS was used as negative control. A dose-response relationship for the  $Ca^{2+}$  rise upon f-MLP (from  $10^{-11}$  to  $10^{-7}$  M) stimulation was established in separate experiments and used to evaluate the effects of the test surfaces.

In a series of control experiments, the direct effect of the anti-coagulant heparin on the test granulocytes was investigated. In those experiments, the fluorescence increase upon f-MLP ( $10^{-7}$  M) stimulation or stimulation with hydrophilic serum were compared between samples of the same granulocyte suspension that had been preincubated for 10 min with or without heparin (10 unit/ml).

In order to further characterise the granulocyte activation, detected by the intracellular  $Ca^{2+}$  responses, measurements of integrin CD11b (CR3) and respiratory burst in response to f-MLP stimulation were performed on activated granulocytes. The CD11b expression on the granulocytes was investigated by addition of a stimulus (serum from blood exposed to the test surfaces) to granulocytes in suspension, which was immediately placed in droplets on hydrophilic or hydrophobic glass slides. Preparation control was performed by excluding the stimulus. Double specimens were prepared from each stimulation, allowing staining for CD11b integrins and cell nuclei in each pair of specimens, to normalise the obtained values. CD11b integrin was detected by incubation with primary antibody; specific mouse monoclonal antibodies directed against human CD11b, followed by the secondary antibody; FITC-conjugated rabbit anti-mouse Ig-antibodies. The granulocyte nuclei were detected by staining their DNA with 0.1% acridine orange, a specific stain for DNA-containing cells. The glass slides were examined in a fluorescence microscope and the fluorescence intensity was recorded, photographed and analysed with a computerised image analysis. The % surface coverage

of fluorescence with an intensity above background was used as quantitative measurements.

In order to examine the possible role of complement in the granulocyte activation, measurements of complement activation on the test surfaces and in serum from surface exposed blood were performed. Hydrophilic or hydrophobic (methylised) glass slides were prepared and human blood was placed in droplets on the glass surface for 10 min incubation time and the slides were then rinsed. The adsorption of complement on the test surfaces, was detected by incubation with the primary antibody (specific rabbit anti-human C3 complement) and the secondary antibody (FITC-conjugated swine anti-rabbit IgG). The glass slides were examined in a fluorescence microscope and the fluorescence intensity was recorded, photographed and analysed with a computerised image analysis. The % surface coverage of fluorescence with an intensity above background was used as quantitative measurements.

The content of human complement C3 in the serum from blood exposed 10 min to hydrophilic or hydrophobic glass tubes was also analysed by using a commercially available radial immunodiffusion kit (RID-kit C3-NL). The test was performed as described by the manufacturer and the concentration of C3 was obtained in mg/ml.

Four types of granulocyte respiratory burst response were analysed separately in each experiment: spontaneous activity, response to f-MLP, response to serum stimuli and response to f-MLP after exposure to serum stimuli. The measurements were performed using luminol-amplified chemiluminescence. Spontaneous activity was measured after mixing the granulocyte suspension with HBSS-buffer and Luminol. Response to f-MLP and serum stimuli was measured after replacing HBSS with f-MLP or serum stimuli respectively. Response to f-MLP after exposure to serum stimuli, was measured in order to detect a priming effect of the serum from blood exposed to hydrophilic or hydrophobic surfaces. In this case, serum was added (1/4) to the cell suspension and the mixture was incubated at 37°C for 10 min, before addition of f-MLP and Luminol. All samples were analysed immediately in a luminometer, controlled by a desk-top computer. The final results were presented in terms of priming effect and direct response to serum stimuli. The priming effect was calculated as the increase in the responses to f-MLP induced by a prior exposure to serum stimuli. The magnitude of the direct response was calculated as the difference between the response to serum and the spontaneous activity. Both types of measurements (priming and direct response) were normalised in relation to the f-MLP response.



### **3.6 Biological characterisation of biomaterials: Initial inflammatory response as manifested by granulocyte activation induced by a blood-material interaction, (VI).**

The following study focused on the possibility to characterise and compare biomaterials concerning the immediate inflammatory response of a blood-biomaterial interaction, by using the recently developed analysis method in the fifth paper in the present thesis, together with analysis of biophysical measurements of the biomaterial surfaces.

According to the previous experimental procedure, human whole blood was transferred under saline to glass test tubes with either hydrophilic or surface modified inner surfaces, modified with two different pre-adsorbed cellulose ethers (EHEC Bermocoll CST-103 and EHEC SM 8713). The whole blood was also syringed into tubes of different polymer materials commonly used in medical therapy, such as polyvinylchloride (PVC), polyurethane (PUR), silicon rubber (SIL), polytetrafluoroethylene (PTFE, Teflon®) and polypropylene (PP). Tubes for preparative ultra centrifugation, such as Ultra-Clear® (UC), polyallomer (PA) and cellulose nitrate (CN) were also examined.

After 10 min blood incubation, the test tubes were spun and the sera were removed and transferred to suspensions of human granulocytes, loaded with the calcium sensitive fluorochrome Calcium Green-1/AM®. The rise of granulocyte intracellular  $Ca^{2+}$  after addition of the serum was used to quantify the degree of granulocyte activation in relation to the  $Ca^{2+}$  signal, obtained after a standardised activation of the same granulocyte suspension by  $10^{-7}$  M f-MLP. HBSS was used as the negative control.

Since preliminary data showed that sera from Ultra-Clear® ultracentrifugation tube, induced a rise of the granulocyte intracellular  $Ca^{2+}$ -level, additional samples of those sera were treated by ultra centrifugation at 100,000 g for 120 min and re-evaluated.

In order to investigate the mechanisms behind the granulocyte activation, measurements of complement C3 in sera from hydrophilic surface and Ultraclear® tube exposures were performed. The content of human C3 was analysed by using a commercially available radial immunodiffusion kit (RID-kit C3-NL). The test was performed as described by the manufacturer and the concentration of C3 was obtained in mg/ml.

The material surfaces in the present study were also analysed biophysically with respect to their surface qualities by studying the static contact angle (Zisman 1964), i.e. the angle formed between a sessile drop of water (with a volume of 10  $\mu$ l) and a flat material surface. The static contact angle was used to characterise and compare the wettabilities of the solid material surfaces of glass, polymer tubes and tubes for preparative ultra centrifugation, and the critical surface tension was then estimated.

## 4. RESULTS AND DISCUSSION

### 4.1 Surface adsorption from single plasma protein systems.

#### 4.1.1 Fibrinogen, (I)

##### *Kinetics*

Adsorption of fibrinogen onto hydrophobic and hydrophilic quartz surfaces was studied by ellipsometry and TEM. The experimentally found amount of bound fibrinogen onto hydrophobic or hydrophilic quartz surfaces versus time of adsorption, was shown in the first paper in the present study. A logarithmic plot was also given, together with the theoretically calculated amount of adsorbed fibrinogen, assuming a diffusion-rate limited adsorption. The experimentally found amount of bound fibrinogen was close to the theoretically calculated amount at low bulk concentrations, showing that the initial binding was rapid and transiently became diffusion-rate limited. The increase in surface concentration was proportional to the logarithm of time, with plateau levels or saturation levels of adsorption dependent on the bulk concentration of fibrinogen and on the surface energy of the quartz surfaces. The plateau levels of adsorption was lower at the hydrophilic quartz surfaces than at the hydrophobic surfaces, the results being in accordance with similar measurements made by others (Chuang 1978 ; Jönsson 1982).

Assuming a reaction-rate limited adsorption of fibrinogen, the initial adsorption to a hydrophobic surface could be described by an apparent forward rate constant  $k_1$ , which decreases with time. The rate decrease was proportional to the logarithm of time, so that the rate coefficient was  $k_{app} \propto k_1 t^{-h}$ , where  $h$  is a fractional number. The apparent forward rate constant of adsorption to a hydrophilic surface was both time-dependent and concentration-dependent.

Chemical reactions with time-dependent forward reaction rate constants have collectively been named fractal kinetics (Blumen ; Kopelman 1988 ). Fractal kinetics as a phenomenon is related to situations where the reactants are spatially constrained by phase boundaries, but the molecular mechanism is still unclear. The findings in the present study that the apparent forward rate constant was both time- and concentration dependent, indicates that the adsorption of new molecules was dependent on the presence of previously adsorbed molecules. This process can also be described as cooperative process, and positive and negative cooperativity can be analysed by the use of a Scatchard plot (Scatchard 1949).

The dissociation of bound fibrinogen from both hydrophilic and hydrophobic surfaces shows an initial rapid dissociation. Assuming first-order kinetics of the initial desorption, it was possible to calculate a dissociation rate constant  $k_{-1}$ . The initial desorption rate of fibrinogen bound to hydrophilic surfaces was slightly higher than the desorption from the hydrophobic surfaces. A more stable plateau level was reached after



about 15 min rinsing time, down to 80-90 % of the initial surface concentration. The stable level was lower at the hydrophilic than at the hydrophobic surface. Even some other studies (Chan 1981 ; Jönsson 1982) like the present one, report an initial dissociation of protein from hydrophilic quartz surfaces followed by an establishment of a stable level.

Assuming the reaction-rate limited adsorption of fibrinogen to have reached an ideal dynamic equilibrium after 24h and 72h long incubation time, a graphic method of analysing binding data was used (Scatchard plot). For the hydrophobic surfaces, the curves were concave (concave up) and the concavity increases with time. The concavity was extremely sensitive to a small change in surface-concentration, indicating negative cooperativity of the adsorbed layer. At the hydrophilic surfaces the curves were convex (concave down), indicating a positive cooperativity between fibrinogen molecules at low surface concentrations. The positive cooperativity may be seen in the structure of the adsorbed layer analysed by TEM, as the formation of polymers of fibrinogen. The part of the Scatchard plot showing positive cooperativity, corresponds to the surface concentrations where a phase transition to an orderly polycrystalline orientation of fibrinogen molecules were adsorbed side-on.

In a recent study (Alaeddine 1996), the adsorption of fibrinogen onto hydrophilic and hydrophobic silicon surfaces was investigated by using off-null ellipsometry, thereby showing that the highest surface concentration of fibrinogen appeared on the hydrophobic surface. The kinetics for both surfaces show an initial concentration-dependent lag-phase, followed by an accelerating binding rate. The reaction rate then decreased abruptly at a surface concentration far below monolayer coverage and the rate became proportional to the logarithm of time. The saturation levels of adsorption were in accordance with the number of the initial binding sites on the surface, i.e. the levels were lower at hydrophilic surfaces. The kinetics in the study was described by a logistic law of limited growth function. The lateral surface diffusion of adsorbed fibrinogen was not detectable on either of the surfaces, but fibrinogen was still adsorbed with accelerated kinetics, thus indicating that the accelerated kinetics must probably be due to cooperative interactions between the adsorbed molecules and the molecules in the bulk solution.

#### *Molecular and supramolecular structure of fibrinogen*

Adsorption from fibrinogen solution at low bulk concentrations, resulted in a heterogeneous distribution of fibrinogen molecules found at the hydrophilic quartz surface, below monolayer coverage. The dried and negatively stained fibrinogen molecule appears as a 5 nm x 45 nm rod. The supramolecular structure was characterised by the formation of end-to-end dimers and trimers laying down at the hydrophilic surface, whereas on a hydrophobic surface the adsorbed fibrinogen was distributed as single molecules or smaller aggregates, as reported in a previous study (Nygren 1988b).

Thus, the adsorption of fibrinogen to hydrophilic and hydrophobic quartz surfaces reveals differences in the adsorbed interaction layer.

An increase of the bulk concentration of fibrinogen adsorbed onto a hydrophilic surface, showed protein-protein interactions that resulted in an orderly layer of end-to-end polymerised fibrinogen molecules along with polycrystalline-like structure where different domains were visible with parallel orientation of the molecules adsorbed side-on. The average intermolecular distance between the parallel molecules was 16 nm.

The molecular dimension of the dried fibrinogen molecule seen in our experiments is smaller than that of the hydrated fibrinogen molecule with a diameter of 9 nm (Bachman 1975). The intermolecular distance of 16 nm seen in the orderly layer of fibrinogen molecules adsorbed side-on at the hydrophilic quartz surface, suggests the existence of a 7 nm layer of water between two parallel molecules. This indicates the formation of a two-phase system at the solid surface, where more or less orderly structured water is included. Therefore all parameters affecting the solubility of the protein (Leavis 1974), also affects the properties of the adsorbed protein layer at solid surfaces and the partition of proteins between the surface phase and the bulk.

#### **4.1.2. Surface modifications by cellulose ethers, part 1, (II, III).**

The possibility of reducing fibrinogen adsorption to hydrophilic and hydrophobic glass surfaces by competitive adsorption of cellulose ethers (EHEC, HEC) was investigated using an ELISA assay calibrated by ellipsometry, in the second paper in the present study. The measurements of fibrinogen adsorption were made at low surface concentration in the study. The surface adsorption of fibrinogen increases with increasing bulk concentration up to a surface concentration of  $0.1 \text{ pmol/cm}^2$ , where a stable level is seen at a hydrophilic surface. At a hydrophobic glass surface the amount of bound fibrinogen increases with the bulk concentration up to a stable plateau level at a surface concentration of about  $0.12 \text{ pmol/cm}^2$ , and at the lowest measurable surface concentration, the bulk solution was completely depleted of fibrinogen.

These results were in accordance with results from a previous study (Nygren 1988b). The surface concentration at the plateau level corresponds to a protein layer below a monolayer of fibrinogen molecules adsorbed side-on. Thus, the interactions that are studied are taking place in spaced layers of adsorbed fibrinogen molecules, containing significant amounts of solvent as previous discussed in the section 4.1.1 above.

The effects of cellulose ethers on the adsorption of fibrinogen shows that the adsorption increases at the hydrophilic surface in the presence of the EHEC and HEC in the solution. The presence of HEC in a mixture of fibrinogen in the solution, caused complete depletion of fibrinogen from the solution. The effect was less prominent in the presence of the more hydrophobic EHEC. The adsorption of fibrinogen to a hydrophobic surface was reduced in the presence of cellulose polymers. The reduction was most



obvious for the hydrophobic EHEC, which completely inhibits the adsorption of fibrinogen. The effect was less prominent in the presence of the hydrophilic HEC, which showed a reduction of the surface concentration from the stable level and a decrease of the adsorption of fibrinogen at low concentrations.

Scatchard plots for analysing binding data of fibrinogen adsorption onto hydrophilic and hydrophobic glass surfaces were also shown. The Scatchard plot of fibrinogen adsorption to a hydrophilic surface, shows a convex curve indicating a strong positive cooperativity of adsorption at surface concentrations below  $0.05 \text{ pmol/cm}^2$ . Similar results were shown in the previous first study using a different technique, where the fibrinogen-fibrinogen interaction at the hydrophilic surfaces resulted in positive cooperativity at low surface concentrations as seen in the Scatchard plot of the concentration dependence of fibrinogen adsorption.

For the fibrinogen adsorption to a hydrophilic surface and in the presence of EHEC, a break point was shown in the adsorption curve, and a concave curve was seen at higher surface concentrations. This breakpoint disappeared in the presence of HEC, showing that the adsorption at low concentrations had resulted in complete depletion of the fibrinogen from the droplet. A tendency to a concave curve at higher surface concentrations was observed.

A Scatchard plot of fibrinogen adsorption to a hydrophobic surface shows a concave curve indicating negative cooperativity in the adsorption. In the presence of HEC, a breakpoint was seen, with decreased binding at both low and high surface concentrations.

The results above reveal an interesting effect on the adsorption of fibrinogen by the presence of cellulose ethers. The results indicate that the polymer compatibility of mixed polymer solutions and the polarity of the solid surface on which the protein adsorption takes place, must be considered as important factors in the protein adsorption.

The decrease of adsorption of fibrinogen to a hydrophobic surface could be explained as a direct competition between fibrinogen and the cellulose ether (EHEC and HEC), where the more hydrophobic EHEC has a higher apparent affinity of binding to the hydrophobic surface. However, the increase of fibrinogen adsorption onto the hydrophilic surface seen in the presence of the same polymer, indicates that the binding strength between fibrinogen and the surface is not the only factor that determines the concentration dependence of fibrinogen adsorption to a solid surface. Phenomena such as protein-protein interactions, which could be seen as cooperativity of adsorption between different polymers, must also be taken in account. Similar effects of positive cooperativity between plasma proteins on hydrophilic surfaces has previously been reported (Chuang 1978).

Addition of HEC abolished the positive cooperativity of fibrinogen adsorption to hydrophilic surfaces seen in the Scatchard plots, thereby suggesting a positive

cooperative interaction between fibrinogen and the hydrophilic HEC itself. The hydrophobic EHEC most likely interacts with fibrinogen and itself, or with the surface, since the positive cooperativity of fibrinogen adsorbed to hydrophilic surfaces, was still partially present after addition of this polymer. At a hydrophobic surface, the hydrophobic EHEC interacts with the surface and the effect could be explained as a direct competition between fibrinogen and EHEC as mentioned above, which completely abolished the surface-adsorption of fibrinogen.

In conclusion, the presence of different water-soluble cellulose ethers in a protein containing solution, may increase protein adsorption to hydrophilic surfaces or abolish surface adsorption of the protein onto hydrophobic surfaces. Phenomena such as protein-protein interaction and ordering of protein molecules during surface adsorption, have been suggested on the indirect basis of kinetics of adsorption and have also been shown directly by TEM. The supramolecular structure of the adsorbed and ordered fibrinogen layer as seen by TEM, also showed that the space between the ordered fibrinogen molecules could accommodate amounts of solvent, such as water, present in the interaction layer, as discussed in the section 4.1.1 above. This even suggests the existence of a solvent-protein interaction in the presence of water-soluble cellulose ethers within the adsorbed layer. The effect on the adsorption of fibrinogen was thus dependent on the polarity of the cellulose polymer, and on the surface energy of the solid surface.

The possibility of reducing fibrinogen adsorption by pre-adsorption of hydrophobic cellulose ethers (EHEC, HPC) onto polymer surfaces commonly used in medical therapy, was investigated using an ELISA assay, in the third paper in the present study. There was a statistically significant decrease of adsorbed fibrinogen on PUR surfaces pre-adsorbed with EHEC (Bermocoll CST-103, DVT 85007 or SM 8713) compared with the native PUR surface. On PTFE surfaces, pre-adsorption with EHEC (Bermocoll CST-103 or SM 8713) resulted in a statistically significant decrease of the adsorbed fibrinogen. Pre-adsorption with cellulose ether HPC (Klucel MF) showed no decrease of adsorbed fibrinogen either on PUR or PTFE surfaces. Adsorbed fibrinogen onto PTFE surfaces first pre-adsorbed with EHEC (Bermocoll CST-103) and then stored dry for various times up to 30 days, showed significant decreases compared with native PTFE surfaces, regardless of dry storing time. Thus, surface modifications with EHEC had a significant effect on the fibrinogen adsorption onto PUR and PTFE surfaces.



## **4.2 Surface adsorption within complex protein systems**

### **4.2.1 Plasma proteins, (IV)**

Almost any medical device introduced into the human body will interact initially with blood. Plasma proteins, being present in high concentrations, will be the first components present at the blood-material interface, where the adsorption is a rapid process. This initially formed protein layer is not always stable, but may be exchanged upon prolonged exposure to blood (Vroman 1969 ; Vroman 1980) and these exchange reactions will differ between surfaces (Nygren 1988a).

This may be expressed in a theoretical model of protein adsorption (Alaeddine 1996; Nygren 1994 ) in which the surface water acts as one phase and the bulk solution as a separate phase. According to Brønsted partition (Brønsted 1931), proteins will be distributed in the two phases according to their molecular size and surface properties as  $C_1 / C_2 = e^{-lA / k T}$  where  $C_1/C_2$  are the concentration of the molecules in two phases (bulk and surface),  $A$  is the molecular area and  $l$  relates to molecular properties e.g. hydrophathy,  $k$  is the Boltzmann's constant and  $T$  is the absolute temperature. This formalism has been used in order to explain the partition of proteins in macromolecular two-phase systems (Albertsson 1986).

The adsorption of the adhesive proteins from native blood onto hydrophilic and hydrophobic glass surfaces, was investigated in the fourth paper in the present study using FITC-labelled antibodies directed against the proteins. The surface concentration of plasma proteins was analysed by measuring the fluorescence intensity, showing that the surface concentration of fibrinogen was twice as higher than that of albumin and IgG. The fluorescence from the FITC-labelled anti-fibrinogen antibodies was also found evenly distributed onto the material surfaces, with a higher intensity at the material surfaces near adhering platelets. For anti-von Willebrand factor (a-VIII:vWF) and anti-fibronectin antibodies, the fluorescence was found localised at the cell surface of adhering platelets. The fluorescence from FITC-labelled anti-C3c antibodies was found localised at platelet cell surfaces and on microvesicles adhering to the material surfaces. Fibrinogen was thus the most abundant plasma protein occurring onto the material surfaces, whereas von Willebrand factor (VIII: vWF) and fibronectin were found at the cell surfaces.

Thus, the pattern of surface-adsorbed protein molecules suggests that platelets can adhere to surface-immobilised fibrinogen, since the other adhesive proteins were found localised at the cell surface and not on the material surface. Fibrinogen is thus suggested to be the substrate for platelet adhesion to material surfaces, in accordance with the results of other studies (Nagai 1993). Platelet adhesion to surface-immobilised fibrinogen has been shown to induce tyrosine phosphorylation, cause reorganisation of the cytoskeleton resulting in cell spreading (Haimovich 1993), but does probably not induce

secretion, because no detectable secretion was seen in the surrounding medium. However, the study still suggests that platelet adhesion to surface-immobilised fibrinogen may stimulate the release of small potentiating amounts of ADP from platelet-dense granule into the local platelet milieu. The suggestion was that it may appear to be a "co-stimulatory" role for ADP to ligand-occupy GP IIb-IIIa complex (CD61) on platelets.

Possible ligands and candidates for platelet activation of secretion are von Willebrand factor (VIII:vWF) that has been shown to affect platelet aggregation (O'Brien 1987), or thrombin which has previously been shown to be activated on hydrophilic but not on hydrophobic glass surfaces (Nygren 1988a). Thrombin is a potent activator of platelet secretion (Haimovich 1993 ; Tapparelli 1993) and may increase the binding of fibrinogen to the platelets (Huzoor 1993). The immunofluorescence method used in the fourth paper failed to demonstrate prothrombin/thrombin on the cell surfaces, that may be due to lack of sensitivity of the immunoassays. The results from the fourth paper thereby suggests, that von Willebrand factor (VIII: vWF) may be a strong candidate for platelet activation and secretion, at the material surfaces described in the paper.

#### **4.2.2 Surface modifications by cellulose ethers regarding complex protein system, part 2, (III).**

Fibrinogen, fibronectin, vitronectin and von Willebrand factor acting as adhesive proteins for platelets are often to be enriched at the surface of solid biomaterial surfaces in contact with blood (Elam 1992). Several investigations have been made in order to find ways of reducing the adsorption of the adhesion-promoting proteins onto material surfaces. The effect of pre-adsorption of hydrophobic cellulose ethers (EHEC) onto polymer material surfaces was investigated in the third paper in the study, with the expectation that this would prevent the enrichment of adhesive proteins on the surface, according to the second paper in the study. Cellulose ethers readily soluble at room temperature, but insoluble at 37°, was chosen with the assumption that the cellulose ether would not be exchanged with plasma proteins in the blood, which would prevent platelet activation, thrombus formation and a further inflammatory response. Studies on surface-induced thrombus formation at polymer materials show little or no activity of the serine-protease coagulation factors suggesting that the most important pathway of thrombus formation at these surfaces is via cell adhesion and activation (Elam 1992 ; Nygren 1988a).

Whole blood interacting with polymer tubes consisting of PUR and PTFE, showed significant reductions of the adsorption of the proteins fibrinogen and vitronectin, for the EHEC-modified surfaces (Bermocoll CST-103 or SM 8713) compared with native PUR and PTFE surfaces. A significant reduction in the amount of adsorbed fibronectin from whole blood was observed for PUR and PTFE surfaces pre-coated with EHEC Bermocoll CST-103.



The data showed that it was possible to significantly reduce the adsorption of fibrinogen, fibronectin and vitronectin at polymer surfaces by surface-modification with cellulose ethers. A concomitant platelet adhesion and activation were also reduced, indicating that the amount of adhesive proteins adsorbed at the surface is important for cellular reactions at the blood-material interface.

### 4.3 Interactions within complex cell systems (whole blood).

#### **4.3.1 Whole blood interactions, (IV, V, VI)**

##### *Kinetics*

The kinetics of platelet and leukocyte adhesion from native blood onto hydrophilic and hydrophobic glass surfaces was studied in the fourth paper in the present thesis. Adhering platelets were found after 15 s of blood-material contact on both surfaces. The number of adhering platelets rapidly decreased at the hydrophilic surface, but remained high for more than 8 min at the hydrophobic surface. The kinetics of leukocyte adhesion from native blood show only a few scattered cells on both surfaces from 2 through 32 min of blood-material contact. After 64 min the number of cells increases significantly at the hydrophobic surface, and after 2 h of blood-material contact a significant number of leukocytes were seen on both surfaces. Thus, the study shows that platelets seems to be the first reactive cell type initially adhered to foreign surfaces, and thereby possibly having an effect on the ensuing cell-surface reactions, e.g. leukocyte-surface reactions during blood-material contact.

##### *Cell surface antigens*

A plausible effect of an extensive blood-material surface contact, was studied in the fourth paper in the present thesis, where blood samples were drawn from five patients at defined stages of cardiopulmonary bypass (CPB) and thoracic surgery (coronary bypass). The blood samples were drawn for measurements of the expression of cell surface receptors on granulocytes. The evaluations of the FACSCAN measurements of the blood samples, showed an upregulation of the IgG receptor (CD 16, Fc $\gamma$ RIII) and the complement receptor 1 (CD 35) and 3 (CD 11b), starting at the onset of extracorporeal circulation and remaining after disconnection. The time histories of the three receptors were almost identical, suggesting that the underlying mechanisms for upregulation were closely connected.

The presence of the platelet-associated antigens CD 61 and CD 62P (P-selectin) on granulocytes was ascribed to adhering platelets (Rinder 1992). The number of adhering platelets increased during surgery. Oxygenator circulation induced a decrease of non-activated platelets (CD 61) and a concomitant increase of activated platelets expressing P-selectin (CD 62P) adhering to the granulocytes. There was no apparent correlation in time between the expression of the platelet antigens and the granulocyte surface receptors, but the granulocytes were indeed shown to be activated rapidly after the onset of oxygenator circulation.



*Material-induced cell activation; hydrophilic and hydrophobic surfaces, and the outcome of anticoagulants*

To be harmful to a patient, an activation of granulocytes must be generalised in the vascular bed. This requires an efficient mechanism of activation, and a direct activation of granulocytes on a biomaterial surface (Betz 1988) should be an inefficient mechanism, because a very small percentage of the granulocytes is in direct contact with the walls in a macroscopic flow device such as an oxygenator or dialyser (Kjellstrand 1991). Thus, an experimental system designed to measure blood biocompatibility in terms of granulocyte activation, should emphasise the release of plasma-soluble granulocyte activators capable of acting in the entire vascular bed. Such an experimental model system was shown mainly in the fifth paper in the present thesis, where intracellular calcium level of test granulocytes in suspension, reflected the presence of granulocyte activating factors. These granulocyte activating factors were produced by the previous interaction between human blood (anti-coagulated in different ways) and hydrophilic or hydrophobic glass surfaces.

The changes in intracellular  $\text{Ca}^{2+}$  level of test granulocytes in suspension were expressed as fluorescence increase over time, normalised in relation to the peak Calcium Green-1 fluorescence change obtained after stimulation with f-MLP. Serum from human blood exposed to hydrophilic surfaces, produced a 40 % mean fluorescence peak increase (f-MLP=100 %), significantly larger than the corresponding 10 % from a hydrophobic surface.

The addition of the specific thrombin blocker, hirudin (500, 250 or 50 U/ml whole blood), before exposure of the whole blood to hydrophilic or hydrophobic surfaces did not affect the level of granulocyte activators in plasma after the exposure. Unlike the results with hirudin, the addition of heparin at concentrations of 10, 1 or 0.1 U/ml whole blood significantly affected the liberation of granulocyte activators (intracellular  $\text{Ca}^{2+}$  increase in test granulocytes) during the interaction between blood and hydrophilic surfaces. The data clearly showed a dose-response relationship with a negative correlation between heparin concentration and mean relative peak fluorescence. The granulocyte calcium levels obtained with 10 U heparin per millilitre of blood, were significantly smaller than the values obtained from untreated blood. Heparin had no significant effects on the calcium response after the exposure of blood to hydrophobic surfaces.

To clarify the mechanism behind the blocking effect of heparin on granulocyte activation, heparin (10 U/ml) was added directly to the Calcium Green-1 loaded granulocytes before f-MLP and serum from hydrophilic surface exposures were tested. For both stimuli, the similarity between heparin-pretreated and untreated granulocytes was confirmed by  $p$  levels above 0.95. Thus, the heparin effect was not exerted through a direct blocking of the test granulocytes.

Intracellular  $\text{Ca}^{2+}$  levels after exposure of granulocytes to different concentrations of f-MLP were also investigated. The dose-response relationship between f-MLP concentration and Calcium Green-1 fluorescence was presented as a family of average curves, and the curve family was used as a tool for semiquantitative evaluation of the responses to surface exposures.

In order to examine the possible role of complement in granulocyte activation, measurements of complement activation were performed on the test surfaces and in the serum from surface exposed blood. The adsorption of human complement factor C3 from human whole blood onto the treated glass surfaces showed a much higher surface concentration at the hydrophobic surfaces, than at the hydrophilic surfaces. Furthermore, the analyses of the concentrations of human complement factor C3 in serum samples from blood exposed to hydrophilic and hydrophobic surfaces, were compared with standard male and female serum samples. The C3 concentrations were lower than normal after exposure to hydrophobic surfaces, indicating a consumption by complement activation in this case. Serum from hydrophilic surface exposures showed C3 concentrations at a normal level for male and female serum.

The integrin CD 11b (CR 3) was generally expressed by a large fraction of the granulocytes after stimulation with serum from material exposures. Measurements were expressed as the ratios between CD 11b surface fractions and the acridine orange stained cell nucleus surface fractions in the paired specimens obtained after each experiment. Serum samples from hydrophobic surface exposures produced slightly higher surface ratios, than serum samples from hydrophilic surface exposures, although the difference was not statistically significant.

The chemiluminescence measurements of respiratory burst were presented in terms of the priming effect and direct response of granulocytes on stimulation by serum samples from hydrophilic and hydrophobic surface exposures of blood. Serum from hydrophobic surface exposure generally induced a stronger direct response and priming effect when added to granulocyte suspensions, than did serum from hydrophilic surface exposure.

The results indicate that humoral factors formed during the surface exposure of blood were able to activate the test granulocytes. A transient increase in intracellular  $\text{Ca}^{2+}$  is known to accompany the chemotactic stimulation of granulocytes, suggesting that the granulocyte activator or activators produced by the surface exposure of blood had chemotactic properties. The established dose-response relationship between f-MLP concentrations and Calcium Green-1 fluorescence, allows a quantification of the calcium transients induced by serum samples from the different surfaces. Thus, the calcium transients induced by surface exposures in the present model were equal to those of f-MLP concentrations, which ranged from  $10^{-11}$  to  $10^{-9}$  M, concentrations known to initiate a chemotactic response. Chemotactic factors include f-MLP (bacterial peptides),



C5a (a product of the complement cascade), PAF (platelet-activating factor) and LTB4 (leukotriene B4) (both secreted products of stimulated phospholipid metabolism), and a number of cytokines such as IL-8 (interleukin-8). There are differences between peptide and lipid chemotactic factors with regard to their ability to stimulate secretory responses *in vitro*. Lipid chemotactic factors (e.g. PAF or LTB4), while equally potent and active as chemotactic agents, are less effective secretagogues (Gallin 1992).

The control experiments showed evidence of complement activation during the exposure of blood to hydrophobic glass surfaces. Consequently, the fact that priming, integrin expression and respiratory burst took place in parallel with the calcium transient in the granulocytes, suggests that the complement factor C5a may have been liberated at the hydrophobic surface.

The serum samples from the hydrophilic surfaces led to a calcium transient three times the magnitude of that from hydrophobic surfaces, although, the effects on integrin expression, priming, and respiratory burst were weaker than those obtained after hydrophobic surface exposure. Because there was no evidence of significant complement activation at the hydrophilic surface, C5a may be excluded as a mediator in this case. A lipid chemotaxins, PAF or LTB4, could be suggested as the mediator in this case, because those substances are known to produce a chemotactic response without the accompanying respiratory burst produced by other chemotaxins. The source of PAF or LTB4 may be platelets or granulocytes in the exposed blood (Ganong 1995). A previous study (Lorant 1993) discussed that PAF and P-selectin may be components with distinct roles in a common adhesion and activation cascade for granulocytes; where P-selectin tethers and captures the granulocytes, whereas PAF mediates activation of the granulocytes.

Alternative mediator candidates are interleukins produced from mononuclear cells at the biomaterial surface. The only cytokine known to bind to a chemotactic receptor that produces a calcium transient is IL-8. This cytokine is produced by monocytes (Agace 1993), but the short incubation time used in this method contradicts this possibility, because cytokine production generally is a slow process that requires DNA transcription (Kasahara 1994 ; Tufano 1994).

Results of the calcium measurements performed after the addition of heparin to the probe granulocytes were in accordance with earlier results, showing that the intracellular calcium increase after f-MLP stimulation was not affected by heparin (Webb 1993). Thus, heparin exerted its blocking effects during the surface exposure of the blood. The heparin effect is, however, not conclusive evidence of any specific underlying mechanisms, because in addition to its anticoagulatory effect, principally mediated through a potentiation of anti-thrombin (Jordan 1987 ; Kestin 1993), heparin has a great number of different effects on various blood cells and compounds released from blood cells.

The adhesion/activation of platelets is an early event during the surface exposure of blood, studied in the fourth paper in the present thesis, and has a potential for a subsequent release of granulocyte activators (Gemmel 1995). Besides blocking platelet activation by thrombin (Kestin 1993), different heparin preparations completely prevent human platelet aggregation, serotonin release, and thromboxane B2 production (Renesto 1991). The fact that hirudin, present at adequate anticoagulatory doses (Elam 1992), failed to affect the liberation of granulocyte activators, clearly excludes the involvement of thrombin in this process.

There are, however, numerous pathways that lead to platelet adhesion/activation, and earlier data from the fourth paper in this thesis, have demonstrated the presence of activated platelets during blood exposure to the surfaces used in the present study. Assuming that the surface activation of platelets is important for the subsequent formation of soluble granulocyte activators, the heparin must have been interfering with processes further down the chain of events. Recently it has been shown that platelet-derived microparticles are liberated during the exposure of blood to biomaterial surfaces (Gemmel 1995) and that they have a potential for granulocyte activation. Platelet activation also leads to the expression and secretion of P-selectin (CD 62P) (Chong 1994) and earlier data from the fourth paper in this thesis, have demonstrated the presence of activated platelets expressing P-selectin during blood exposure to artificial surfaces, where CD 62P has the ability to bind and activate the granulocytes (Nagata 1993). In this case, heparin may interfere with the process of granulocyte activation by binding to and blocking P-selectin, because heparin oligosaccharides, including non-anticoagulant tetrasaccharides, are effective L- and P-selectin inhibitors *in vitro* and have anti-inflammatory activity *in vivo* (Nelson 1993).

Although further studies are needed to explain the heparin effect in the present experiments, this study provides information on the importance of anticoagulants in systems designed to evaluate the blood biocompatibility of biomaterial surfaces. According to the present results, hirudin seems to be the best choice of anticoagulant to evaluate the blood biocompatibility of biomaterial surfaces, because it did not interfere with the measurements. This was in agreement with an experimental study (Nygren 1988a) of human whole blood, anticoagulated with hirudin or citrate. In this study the results emphasised that the adsorption of plasma proteins at the blood-solid interface and the subsequent adhesion/activation of platelets, were dependent on the anticoagulant used.

To conclude, the present study clearly showed that the properties of a biomaterial surface and the choice of anticoagulants were important determinants of the outcome of a blood-biomaterial interaction. Hydrophobic surfaces seem to induce complement activation in contact with blood, leading to the liberation of C5a, which activates granulocytes. Hydrophilic surfaces show no evidence of complement activation, but



nevertheless induce the production of soluble granulocyte-activating substances in contact with blood, thereby suggesting the presence of a lipid chemotactic factor.

#### *Material-induced cell activation; polymer material surfaces*

In the sixth paper in the present thesis, it was intended to characterise and compare biomaterials, such as polymer materials, concerning an immediate inflammatory response of a blood-biomaterial interaction by using the recently developed method in the fifth paper. In this method, the intracellular calcium level of test granulocytes in suspension, reflects the presence of granulocyte activating factors, produced by the previous interaction between human blood and biomaterial.

The results show that the changes in intracellular  $Ca^{2+}$  were expressed as fluorescence increase over time, normalised in relation to the peak Calcium Green-1 fluorescence change obtained after stimulation with f-MLP  $10^{-7}$  M. Serum from blood exposed to polymer tubes, commonly used in medical therapy, such as; PUR, PVC, SIL and PP did not significantly affect the level of granulocyte activation compared with the negative control HBSS. However, a statistically significant early peak was shown induced by serum from blood exposed to polymer tubes of PTFE.

Intracellular  $Ca^{2+}$  levels following exposure of granulocytes to serum from preparative ultra centrifugation tubes consisting of PA and CN induced no significant effect compared with HBSS. Serum addition from Ultra-Clear<sup>®</sup> ultra centrifugation tubes showed a significant calcium peak. Statistical comparison between serum addition from UC tubes and additional samples of those sera, treated by ultra centrifugation at 100, 000 g for 120 min (UC-cent) did not reveal any significant difference regarding the calcium response.

The content of human complement C3 was measured in the sera from blood exposed to UC, ultra centrifugation tubes and glass tubes with a hydrophilic inner surface and were compared with standard male and female sera. The measurements (using commercially available radial immunodiffusion kit) showed no significant complement activation in sera from the hydrophilic surface or in sera from UC tubes.

In the sixth paper in the present study, the investigation of the polymer tubes commonly used in medical therapy, show that sera from PTFE tubes, in contrast to that from the other tubes, induced a significant granulocyte activation. Some previous studies of polymer material surfaces (Gaukroger 1988 ; Larsson 1989 ; Linder 1984; Madan 1992 ; Myles 1991 ; Payne-James 1991 ; Reynolds 1995 ; Woodhouse 1980 ), used in catheters, have focused on clinical evaluations such as the incidence of thrombophlebitis and the degree of thromboresistance. Several of those studies have shown that clinical use of PTFE cannulae is limited by a high incidence of thrombophlebitis.

The initial events at the material surfaces in contact with human whole blood and common polymer materials used in this study have been investigated in a previous experimental study (Elam 1992). On most of the surfaces, the amounts of adsorbed protein seemed stable after initial adsorption, but showed significant differences between the polymer materials. There was a correlation between the adsorption of adhesive proteins onto the material surfaces and the release of the specific protein  $\beta$ -thromboglobulin from platelets. The highest amount of adhesive proteins was detected on PTFE, together with more antigenically detectable  $\beta$ -thromboglobulin in blood incubated in PTFE tubing's. PUR demonstrated the lowest values of adhesive proteins, and also show the lowest value of  $\beta$ -thromboglobulin. Thereby, the previous results suggest that the polymer surfaces in the experimental study, induced adhesion of adhesive proteins followed by platelet activation, and only to a lesser extent was initiating plasma coagulation at the surfaces.

The adhesion/activation of platelets have been shown to be an early event during surface exposure of blood and the platelets also have a potential for a subsequent release of granulocyte activators. Platelet-derived micro particles with the ability for granulocyte activation have been shown to be liberated during the exposure of blood to biomaterial surfaces (Gemmel 1995). It is possible to remove platelet-derived microparticles by ultracentrifugation because they have a known size and density. The failure in the present study to show any effects of ultracentrifugation in the sera from Ultra-Clear<sup>®</sup> tubes, suggests that the granulocyte activators present in these sera were humoral rather than particulate. Filtration of sera through a Millipore filter have previously been shown to have decreased the ability for granulocyte activation. This effect may have been produced by the contact between serum and filter-material surfaces, rather than by a removal of particulate activators from the sera.

Biophysical measurements of the material surfaces in hydrophilic-hydrophobic glass tubes, polymer tubes and tubes for preparative ultra centrifugation were performed in this study using static contact angle measurements. The Ultra-Clear<sup>®</sup> tube showed the lowest value of static contact angle with concomitant highest values of critical surface tension of the polymer materials, thereby having the most hydrophilic surface of the chosen polymer materials. The measurements of human complement, C3, in blood exposed to UC tubes or hydrophilic glass tubes produced normal concentrations and thus no evidence of complement activation. It was previously shown, in the fifth paper, that sera from hydrophilic surface exposure of blood induced a low level of granulocyte respiratory burst in relation to the calcium transients, thereby suggesting the presence of a humoral lipid chemotactic factor as the mediator that may have been liberated from the hydrophilic surface.

In conclusion, the data in the sixth paper showed that biomaterials in clinical and laboratory use differed in respect to the induction of an early inflammatory response



during exposure to blood. The inflammatory response to various polymer materials seemed to correlate with the wettability, since the most hydrophilic polymer material produced a similar inflammatory response as that of hydrophilic glass. The results in the study were also partly in line with clinical data on the biocompatibility of the same materials.

#### **4.3.2 Surface modifications by cellulose ethers regarding complex cell system (whole blood), part 3, (III, VI).**

The third paper in the present thesis demonstrates the number of platelets adhering to polymer tubes consisting of native PUR or PUR pretreated and surface-modified with EHEC Bermocoll CST-103 or EHEC SM 8713, after exposure to whole rabbit blood in an *ex vivo* shunt for 2h. There was a reduction in the number of adhered platelets on the PUR surfaces with pre-adsorbed cellulose ethers. This reduction was statistically significant on the surfaces pre-adsorbed with EHEC SM 8713.

Studies on human whole blood anticoagulated with hirudin, and exposed to different polymer tubes pre-adsorbed with cellulose ethers, were also performed in the third paper. The polymer surfaces consisted of native PUR or native PTFE surfaces, with corresponding surface modifications with EHEC Bermocoll CST-103. The release of the platelet specific protein  $\beta$ -thromboglobulin into the blood plasma, was measured by ELISA. There was a significant reduction of released  $\beta$ -thromboglobulin from the polymer surfaces with pre-adsorbed EHEC Bermocoll CST-103, thereby indicating less activation of the platelets at these surfaces. Thus, there was a good correlation between the reduced surface concentrations of adhesive proteins onto the polymer surfaces pretreated with cellulose ethers discussed in section 4.2.2, and the reduced activation of platelets, measured as the release of  $\beta$ -thromboglobulin from the cells. A reduction in the number of adhered platelets was also observed at the polymer surfaces with pre-adsorbed cellulose ethers, thereby decreasing the attachment of blood cells, minimising this pathway of thrombus initiation at polymer surfaces.

In the sixth paper of the present thesis, in which the immediate inflammatory response of a blood-biomaterial interaction was studied, the effect of surface-modification by cellulose ethers was also investigated. Serum from human blood exposed to hydrophilic surfaces, reveal a significant increase regarding the intracellular calcium response, compared with the negative control HBSS. The fluorescence-peak increase was larger than the response from corresponding hydrophilic surface with pre-adsorbed cellulose ethers, EHEC Bermocoll CST-103 and EHEC SM 8713, which seemed to block the production of granulocyte activators.

Statistical comparison between the hydrophilic glass surfaces and the surface-modified glass surfaces reveal significant difference between the surfaces, with the

response inhibited by pre-adsorption with cellulose ethers. Thus, the study clearly showed that surface modifications by cellulose ethers onto hydrophilic glass, reduced the liberation of granulocyte activators during the interaction between blood and material surfaces, thereby minimising the immediate inflammatory response of a blood-biomaterial interaction .



## 5. GENERAL DISCUSSION

Fibrinogen, fibronectin, vitronectin and von Willebrand factor (VIII:vWF) acting as adhesive proteins for platelets, are often enriched at the surface of solid biomaterial surfaces in contact with blood (Elam 1992). Patterns of surface-adsorbed plasma proteins (demonstrated with fluorescence-labelled antibodies) from native whole blood interacting with hydrophilic or hydrophobic surfaces in the present study, showed that fibrinogen was the most abundant plasma protein during the initial blood-material contact. In contrast, von Willebrand factor and fibronectin were mainly located on the cell surface of scattered adhering platelets, suggesting that platelets can adhere to the surface-immobilised fibrinogen, since the other adhesive proteins were not found on the material surface.

To elucidate the relationship between kinetics, supramolecular structure and equilibrium properties, the adsorption of plasma adhesive proteins onto material surfaces was studied in the present thesis, with special emphasis on fibrinogen. The adsorption of fibrinogen from solution to quartz surfaces, show that the initial adsorption at low bulk concentrations was rapid and diffusion-rate limited. The increase in surface concentration at the hydrophobic surface was found to be proportional to the logarithm of time, and the adsorption to a hydrophilic surface was both time-dependent and concentration-dependent, indicating cooperative processes between the fibrinogen molecules. These processes were demonstrated by Scatchard plots, indicating negative cooperativity between the fibrinogen molecules at hydrophobic surfaces and positive cooperativity at hydrophilic surfaces. The plateau levels of adsorption were dependent on the bulk concentration of fibrinogen and on the surface energy of the quartz surfaces, and was lower at hydrophilic than at hydrophobic surface quartz surfaces. The dissociation of bound fibrinogen was also studied, and the data showed an initial dissociation of protein followed by an establishment of a stable level. These findings are in agreement with those reported previously by others (Brash 1987 ; Brynda 1986).

Adsorbed fibrinogen showed different supramolecular structures at hydrophilic and hydrophobic quartz surfaces. On a hydrophobic surface the adsorbed fibrinogen was distributed as single molecules or smaller aggregates as reported in a previous study (Nygren 1988b). Adsorption from low bulk concentration of fibrinogen to a hydrophilic surface, resulted in a heterogeneous distribution of fibrinogen molecules, and the supramolecular structure was characterised by the formation of end-to-end dimers and trimers lying down at the surface. At higher surface concentrations, adsorbed molecules showed polycrystalline structure with repeated nearest neighbour distances of 16 nm in the present study. The distribution of adsorbed fibrinogen molecules indicates that surface-adsorbed fibrinogen may form a two-phase system containing significant amounts of water. Therefore, all parameters affecting the solubility of fibrinogen, also

affect the properties of the adsorbed protein layer at solid surfaces and the partition of proteins between the surface phase and the bulk (Nygren et al 1994).

When blood or plasma is exposed to an artificial solid surface, the initially formed protein layer shows varying stability upon prolonged exposure to blood. Protein exchange reactions on solid surfaces seem to occur in sequence, with kinetics dependent on the properties of the surface. Earlier studies have shown that fibrinogen initially adsorbed on hydrophilic glass surface is replaced by high molecular weight kininogen (HMWK), a phenomenon known as the "Vroman effect" (Vroman 1980 ; Schmaier 1983; Wojcieszowski 1986 ; Vroman 1986). The HMWK carries prekallikrein and the coagulation factor XI to the surface (Mandle 1976 ; Thomson 1977), where they interact with initially adhered and activated factor XII, initiating the coagulation cascade. Other protease cascades are initiated on the surface in a similar way. On hydrophobic surfaces, however, the initial layer of fibrinogen is stable (Brash 1987 ; Horbett 1982 ; Vroman 1969 ; Vroman 1971), and no exchange reactions occur. Fibrinogen is an adhesive protein for cell-interactions (Ginsberg 1988) and has a tri-peptide, Arg-Gly-Asp, present in the amino-sequence of the protein, acting as a cell-attachment site. This leads to the adhesion and subsequent activation of platelets via specific membrane-located receptors (Ginsberg 1988 , Zwaal 19869). Thus, both hydrophilic and hydrophobic surfaces finally can cause thrombus formation, although via different parts of the initiating pathway of the coagulation process. The hydrophilic surfaces mainly induce plasma coagulation and the hydrophobic surfaces can initiate the cellular pathway of blood coagulation by platelet activation.

The kinetics of platelet and leukocyte adhesion from native blood onto hydrophilic and hydrophobic surfaces was also studied. Adhering platelets were found after 15 s of blood-material contact on both surfaces, and a significant numbers of leukocytes were seen after 2h of blood-material contact. Thus, the study showed that platelets seem to be the primary reactive cell type adhered to artificial material surfaces, thereby indicating that platelet adhesion is an early event during the initial blood-material contact.

The granulocyte is the second type of cell that will interact with an artificial surface. It is known that this event can cause a generalised inflammatory response during an extensive blood-material contact and has been associated with cardiac, pulmonary and renal dysfunction. To be harmful to a patient, an activation of the granulocytes must be generalised in the vascular bed. A direct activation of granulocytes on a biomaterial surface is not a likely mechanism for such generalised activation due to the small percentage of the granulocytes in direct contact with the walls in a macroscopic flow device such as an oxygenator or dialyser. Thus, an experimental system designed to measure blood biocompatibility in terms of granulocyte activation, should emphasise the release of plasma-soluble granulocyte activators capable of acting in the entire vascular bed. A new method, introduced in the present thesis, was therefore developed to study



the immediate inflammatory response of a blood-biomaterial interaction with respect to the liberation of granulocyte activating factors.

In this method, the intracellular calcium level of test granulocytes in suspension reflects the presence of granulocyte activating factors, produced by the previous interaction between human blood (anticoagulated in different ways) and biomaterial. The results, mainly from the fifth paper in the present study, indicate that humoral factors formed during the surface exposure of blood were able to activate test granulocytes in suspension. A transient increase in intracellular calcium is known to accompany the chemotactic stimulation of granulocytes (Gallin 1992), suggesting that the granulocyte activator produced by the surface exposure of blood had chemotactic properties.

Exposure of blood to hydrophobic surfaces showed evidence of complement activation in serum, with accompanying integrin expression, priming and respiratory burst of the test granulocytes. These processes took place in parallel with the calcium transient of the granulocytes, suggesting that the complement factor C5a may have been liberated at the hydrophobic surface. The serum samples from hydrophilic surfaces produced an intracellular calcium increase three times the magnitude of the early peak from blood exposed to hydrophobic surfaces, although the effects on integrin expression, priming and respiratory burst were weaker. Measurements of human complement in blood exposed to hydrophilic surfaces produced normal concentrations and thus no evidence of complement activation. Because there was no evidence of significant complement activation, C5a may be excluded as the mediator in this case. The low level of granulocyte respiratory burst in relation to the calcium transients instead suggests the presence of a lipid chemotaxin (PAF or LTB<sub>4</sub>), as the mediator of granulocyte activation from hydrophilic surface exposure. The source of PAF or LTB<sub>4</sub> may be platelets or granulocytes in the exposed blood.

The induced calcium response in this assay was not affected by the presence of hirudin (a specific thrombin blocker), but heparin exerted a blocking effect during the surface exposure of blood. Heparin has besides its anticoagulatory effect, a great number of different effects on various blood cells and compounds released from blood. The adhesion/activation of platelets is an early event during the surface exposure of blood, and has a potential for a subsequent release of granulocyte activators. Besides blocking platelet activation by thrombin, different heparin preparations completely prevent human platelet aggregation, serotonin release, and thromboxane B<sub>2</sub> production. The fact that hirudin, present at adequate anticoagulatory doses, failed to affect the liberation of granulocyte activators, clearly excludes the involvement of thrombin in this process.

There are, however, numerous pathways that lead to platelet adhesion/activation. Assuming that surface activation of platelets is important for the subsequent formation of soluble granulocyte activators, heparin must have been interfering with processes further down the chain of events. Platelet activation leads to the expression and secretion of P-

selectin (CD 62P) (Chong 1994), which has the ability to bind and possibly activate the granulocytes (Nagata 1993). In this case, heparin may interfere with the process of granulocyte activation by binding to and blocking P-selectin, because heparin oligosaccharides, are effective L- and P-selectin inhibitors *in vitro* and have anti-inflammatory activity *in vivo*.

Although further studies are needed to explain the heparin effect in the present experiments, this study provides information on the importance of anticoagulants in systems designed to evaluate the blood biocompatibility of biomaterial surfaces. According to the present results, hirudin seems to be the best choice of anticoagulant to evaluate the blood biocompatibility of biomaterial surfaces, because it did not interfere with the measurements.

In the present thesis an attempt was made to characterise and compare biomaterials, such as polymer materials commonly used in medical devices, with respect to an immediate inflammatory response of blood-biomaterial interaction using the method discussed above. The sera taken from blood exposed to Teflon® (PTFE) tubes induced a significant granulocyte activation, measured as intracellular  $Ca^{2+}$  response. Blood contact with other polymers e.g. polyvinyl chloride, polyurethane, polypropylene or silicon rubber did not induce this response.

Platelet-derived microparticles with the ability for granulocyte activation have been shown to be liberated during the exposure of blood to biomaterial surfaces (Gemmel 1995). It is possible to remove these microparticles by ultracentrifugation, since they have known size and density. The failure in the present study to show any effects of ultracentrifugation in the sera taken from blood exposed to hydrophilic ultracentrifugation tubes, suggests that the granulocyte activators present in these sera were humoral rather than particulate.

A further aim of the present thesis was to investigate the effect of surface modifications onto biomaterial surfaces by competitive adsorption or pre-adsorption of water-soluble cellulose ethers (e.g. EHEC). The results obtained showed that it was possible to significantly reduce the adsorption of adhesive proteins on material surfaces by surface-modification with certain cellulose ethers. There was also a good correlation between the reduced surface concentrations of adhesive proteins on biomaterial surfaces pre-treated with cellulose ethers and the reduced activation of platelets, measured as the release of  $\beta$ -thromboglobulin from the cells. A reduction in the number of adhered platelets was also observed at material surfaces with pre-adsorbed cellulose ethers, thereby decreasing the attachment of blood cells, minimising this pathway of thrombus initiation at material surfaces. A possible granulocyte activation was also evaluated regarding intracellular calcium response from sera taken from blood exposed to cellulose ether-modified surfaces. The study showed that surface modification by cellulose ethers on material surfaces, reduced the liberation of granulocyte activators during the blood-



material contact, thereby minimising the immediate inflammatory response of a blood-biomaterial interaction.

It was shown that the presence of different water-soluble cellulose ethers in a protein containing solution may affect the protein adsorption to material surfaces, suggesting the probable existence of a solvent-protein interaction within the adsorbed protein layer. The effect on the adsorption of fibrinogen and other adhesive proteins was thus dependent on the polarity of the cellulose polymer, and on the surface energy of the solid surfaces, which may trigger the subsequent adhesion and activation of blood cells, primarily platelets and granulocytes.

To conclude, the experimental model surfaces (hydrophilic or hydrophobic quartz surfaces) used in the present thesis reveal significant differences in kinetics, supramolecular structure and equilibrium properties of protein adsorption at solid surfaces. Phenomena such as protein-protein interaction and ordering of protein molecules during surface adsorption have been suggested on the indirect basis of kinetics of adsorption and have also been shown directly by electron microscopy. Hydrophilic or hydrophobic surface treatment seem also to induce a significant effect on the subsequent biological response to the surface adsorbed protein layer, during an initial blood-biomaterial interaction. The present thesis shows that the properties of a biomaterial surface and the use of anticoagulants are important determinants of the outcome of a blood-biomaterial interaction.

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