Master’s thesis

Study of immune and haemostatic response induced by protein multilayers

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Department of Physics and Measurement Technology, Biology and Chemistry
Linköping University
AddBIO AB
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**Abstract**

FibMat2.0 is a fibrinogen multilayer developed by AddBIO. Other proteins such as immunoglobulin G (IgG) and human serum albumin (HSA) can also be used to build multilayers with the same technique. The aim of this study of FibMat2.0 was to investigate if the manufacturing of the protein multilayer would induce an immune or haemostatic response in the body. The multilayers of IgG and HSA were also studied. Methods such as null ellipsometry, imaging of coagulation and the cone-and-plate setup were used to study immune reactions, activation of the coagulation cascade, and stability of the multilayers.

Small amounts of plasma proteins were adsorbed to fibrinogen multilayers, but complement proteins adsorbed only to the IgG matrix and high molecular weight kininogen (HMWK) adsorbed only to the HSA monolayer. The imaging of coagulation method indicated that the titanium surface and the HSA monolayer activate surface induced coagulation rapidly, whereas fibrinogen and IgG multilayers demonstrated longer coagulation times. Platelets and a few white blood cells were bound to titanium surfaces and fibrinogen multilayers, but not to IgG multilayers or HSA monolayers.

A conclusion in this study is that the surface of an implant can be coated with FibMat2.0 without any risks, but more studies are needed to better understand the interactions between the surfaces prepared in the present study and the immune and the haemostatic systems of the human body.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>C1-C9</td>
<td>Complement factor 1-9</td>
</tr>
<tr>
<td>EDC/NHS</td>
<td>1-ethyl-3(dimethyl aminopropyl)carbodiimide/N-hydroxysuccinimide</td>
</tr>
<tr>
<td>Fib.C, B, X</td>
<td>Fibrinogen matrix (FibMat) with fibrinogen from manufacturer referred to as C, B, and X</td>
</tr>
<tr>
<td>FibMat</td>
<td>Fibrinogen matrix fabricated with a technology developed by AddBIO AB</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HMWK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>ProtMat</td>
<td>Protein matrix fabricated with a technology developed by AddBIO AB</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>VB++</td>
<td>Veronal buffer (VB⁻) supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström</td>
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Chapter 1

Introduction

This chapter begins with a short background of the presented project. Later on, the project aim is described, and also a short comment about the work method and limitations for the work. Information for the project comes mainly from earlier studies of the field, such as articles, and books. A few online databases have also been used and those are named and commented in the last part of the introduction chapter.

1.1 Background

Biomaterials that are inserted into the body may activate the immune system directly after the operation. The damaged tissues from the operation activate cells to repair the wounded body parts around the implant. Proteins in the blood will adsorb to the surface of the implant and the complement cascade may become activated. This acute inflammation is part of the normal healing process. However, if the cells continue to recognize the implant as a foreign object, even long after the operation, it can lead to a chronic inflammatory response and constant pain for the patient. A second operation could then be required, and consequently the risk for the patient is increased.

Cardiovascular biomaterials, e.g. stents, must not activate the coagulation system excessively when in contact with blood. However, in other biomaterial scenarios it is
perhaps a positive feature that the surface of a device induces coagulation. It is therefore
the specific application area that decides the necessary features of the device.

AddBIO has developed a protein multilayer technology called ProtMat2.0. The
fibrinogen based matrix, FibMat2.0, can be used as a drug delivery device, as have been
shown in previous work [5]. For example, a screw used to fix hip fractures coated with
FibMat2.0 is loaded with bisphosphonate. The molecules are in this way delivered
locally around the screw to the bone which will grow stronger. The risk for a
subsequent operation will thereby be reduced. This product is about to be tested in
clinical studies. Specific studies to document the biocompatibility of FibMat2.0 have
not previously been performed. The aim of this work was to study FibMat2.0 and
ProtMat2.0 of other proteins with respect to complement and coagulation activation.

1.2 Project aim

The aim of this project was to study the crosslinked protein multilayers manufactured
using the ProtMat2.0 technology, which has been developed by AddBIO. The
adsorption of complement and coagulation proteins to the matrix coated test surfaces
was studied to investigate if the surfaces possibly induce activation of the complement
system or the coagulation cascade. The adhesion of platelets and white blood cells onto
ProtMat2.0 was studied to observe differences at the cellular level of biocompatibility
between protein matrixes.

1.3 Limitations of work methods

Throughout this work plain titanium surfaces were used as a reference. In some
experiments the test surfaces were compared with surfaces subjected to spontaneously
preadsorbed proteins.

The studies of cell adhesion of platelets and white blood cells to different protein
multilayers were only tested qualitatively by microscopy imaging. If these experiments
are to give quantitative answers of the cell adhesion, more repeated tests have to be
performed, and more quantitative parameters should preferably be measured.
In the study of surface induced coagulation (chapter 2.6.2), the plasma was unfortunately spontaneously preactivated during the blood sampling procedure, and the measured clotting times are not fully reliable. The time for this project was unfortunately not enough for the performing of more assays.

1.4 Sources

The information and facts for this thesis have mainly been provided from reviewed and published books and articles, and from a few online databases. The databases are among others supported by national institutes and/or universities and offered to the public as freely available resources. As far as possible, the data from one database is compared to another to minimize the risk of incorrect facts. In a similar way, facts from older published books were compared with other reviewed books and articles.

DrugBank database is a bioinformatics and cheminformatics resource. The project was supported by the Departments of Computing Science & Biological Sciences, University of Alberta, and also by Genome Alberta and Genome Canada.

Genetic Home Reference is a service of the U.S National Library of Medicine, part of the National Institutes of Health, an agency of the Department of Health and Human Services.

Protein Data Bank is a databank with information about the 3D structures of large biological molecules, including proteins and nucleic acids. Research Collaboratory for Structural Bioinformatics (RCSB) are responsible for the management of the PDB.
Chapter 2

Theory

The first section in this chapter is used to describe the different test surfaces. Thereafter follows reviews of the complement cascade and the haemostasis, two important systems in the body. The instruments and methods that were used in the present study are described at the end of the chapter.

2.1 Protein matrix

ProtMat2.0 is a protein multilayer prepared with the proprietary technology of AddBIO. The multilayers were prepared on titanium surfaces using different proteins, i.e. fibrinogen, immunoglobulin G, and human serum albumin. The properties of the multilayers were then compared with monolayers of the respective proteins adsorbed onto titanium surfaces.

2.1.1 FibMat2.0

FibMat2.0 (fibrinogen matrix) is a protein multilayer developed by AddBIO and is described in another master’s thesis [4]. FibMat2.0 is developed from an earlier version, which in the present study is called FibMat1.0, and was described by Tengvall et al. (2003) [1]. The purpose of the development was to reduce the time for the manufacturing of the multilayers and to reduce the use of chemicals in the process [4]. Using the FibMat2.0, the fibrinogen will form a multilayer with a film thickness of
approximately 250 Å after a single incubation, and the thickness increases with subsequent incubations. Other proteins such as IgG and HSA can also be used with the same technique and are in the present study called for example IgG of ProtMat2.0 [5]. The fabrication of ProtMat2.0 is more accurately described in chapter 3.1.

The main purpose with FibMat2.0 is to act as a drug delivery device [6]. In the body, the drugs are released and the fibrinogen multilayer is dissolved. The drugs can, e.g. improve the healing of the damaged bone around a screw implanted in bone, or the drugs can be of antibiotic character and decrease the risk of infections in the wound.

2.1.2 Proteins used for the multilayers

Human serum albumin (HSA), fibrinogen, and immunoglobulin G (IgG) are proteins that were used for fabricating multilayers with the ProtMat2.0 technique.

*Human serum albumin* (figure 1) is a plasma protein produced in the liver. Its molecular weight is 66,5 kDa [7]. The protein is negatively charged at physiological pH [8]. It regulates the osmotic pressure in the blood and binds particles and toxic materials that naturally exist in the circulation system [9]. HSA is also a transport protein for fatty acids and insoluble molecules, which are transported to different parts of the body via the circulation system [10]. HSA also binds drug molecules such as ibuprofen. The protein is often used for the treatment of, e.g. hypovolemia, hypoalbuminemia, and nephrosis [9, 11].

![Figure 1. Human serum albumin (DrugBank database [7]).](image)
Fibrinogen is a glycoprotein that is synthesized in the liver [9]. The concentration is approximately 4 mg/ml and it is also an acute phase protein and part of the coagulation cascade and form fibrin. Fibrinogen has a molecular weight of about 340 kDa [9]. It is made up by three globular units connected by two rods, where each rod is three α-helices coiled around each other (figure 2A) [12]. The fibrinogen molecule consists of two Aα-chains, two Bβ-chains and two γ-chains (figure 2B) [9].

![A. B. C.](image)

**Figure 2.** Fibrinogen. **A:** A ribbon diagram. **B:** A schematic picture of the fibrinogen molecule. **C:** Formation of a fibrin clot. (J. M. Berg, et al., Biochemistry, 2002 [9])

At the end of the coagulation cascade, thrombin cleaves fibrinogen at the central globular region, and the fibrinopeptides A and B parts are released (figure 2B). The fibrinogen molecule without the fibrinopeptide A and B is called a fibrin monomer, with a subunit structure (αβγ)₂. The globular end of two other monomers can bind to the domain where the fibrinopeptide A and B were placed (figure 2C). Several monomers can then bind to each other and after binding with Factor XIIIa form a fibril that with other fibrils will form an insoluble gel. Fibrinogen from any mammalian source can be cleaved by thrombin from any other mammalian source. [9]

Immunoglobulins (Ig), also known as antibodies, are proteins that plasma cells start to produce when an antigen (antibody generator) binds to a surface receptor for antigens [13]. The molecular weight of an antibody is approximately 150 kDa [14]. The antibodies help phagocytes to ingest microorganisms and antigens, and to inactivate toxic substances produced by bacteria, attacking bacteria and viruses directly, and activating the complement system [15]. The antibodies are classified by structure and function; IgM, IgG, IgA, IgE, or IgD [16]. When an antigen is found in the body for the first time, IgM is produced, but the second time IgG is produced and also in greater
amounts [14]. IgG (figure 3) is the major class of antibodies in the bloodstream and is also present in tissues [15]. It is the most common antibody used in treatments of immune deficiencies and immune diseases [14].

![Immunoglobulin G (IgG)](image)

**Figure 3.** Immunoglobulin G (Invitrogen AB [14]).

### 2.2 Difference between serum and plasma

Blood contains red blood cells, white blood cells (granulocytes, lymphocytes, and monocytes), platelets, and a wide variety of proteins [17].

*Plasma* is the yellowish solution of water (90%), electrolytes, plasma proteins, carbohydrates, lipids, and soluble salts that the blood cells are suspended in [18, 17]. The most abundant of proteins in plasma are albumin, immunoglobulins, fibrinogen and other coagulation factors [17]. Most of the globulins and coagulation factors are produced in the liver. The remaining globulins are the immunoglobulins synthesized by B lymphocytes. When the plasma coagulates the fibrinogen is converted to a fibrin clot and the remaining liquid is called serum [18].

*Serum* is the yellowish liquid that is expelled from the blood clott when it contracts [18]. Serum is the plasma without fibrinogen and the other proteins that are involved in the coagulation [17].


2.3 Protein adsorption

When a protein solution comes in contact with a solid surface, the surface could be covered by adsorbed proteins. Both the protein molecules, the solvent (e.g. water), the solid surface, and other components such as ions play a role in the adsorption process. The protein adsorption can only occur if Gibbs energy (formula 1) of the system, at constant temperature and pressure, decreases. [19]

\[
\Delta_{\text{ads}} G = \Delta_{\text{ads}} H - T \Delta_{\text{ads}} S < 0
\]

**Formula 1.** Gibbs energy, G, for a system (C A Haynes et al., Globular proteins at solid/liquid interfaces. Colloids and Surfaces, Biointerfaces, 2 (1993) 517-566, [19])

H is the enthalpy, T the temperature, S the entropy, and \( \Delta_{\text{ads}} \) the change in the thermodynamic functions of state resulting from the adsorption process. The adsorption process is a result of the net interactions within the system. [19]

Properties of the solid surface (e.g. hydrophobicity and charge distribution), of the protein, and of the solvent are important for the adsorption. Different kinds of interactions and forces act between atoms, molecules, proteins, the surface, and the solvent. All interactions can affect the adsorption process. During this process the structural conformation, and the characteristics of the protein change. [19]

Biomaterials in contact with blood, plasma, or serum, are instantly covered by proteins [8]. Therefore, living cells are not interacting directly with the surface of the biomaterial, but instead with the proteins adsorbed to the surface. Membrane bound receptors on the cell surface bind to the protein layer and these interactions may regulate determine the cell adhesion, shape, growth, differentiation, etc. [9]
2.4 The complement system

The complement system refers to more than thirty plasma and membrane bound proteins and is the non-cellular part of the immune system [9]. The involved proteins are called complement factors 1-9 (C1-C9). The system acts as a defence system in the body and it protects us against pathogenic agents such as bacteria, viruses, etc. Activation of the system lead to several biological effects, e.g. identification and opsonization of pathogens, and recruitment and activation of phagocytic cells that will try to destroy and digest the “intruder”. The activation also lead to damage of cell membranes, and clearance of immune complexes and apoptotic cells, etc. [20, 9]

The activation can proceed via three pathways (figure 4) [20]. The classical pathway is activated by immune complexes with IgG or IgM [20]. The lectin pathway is similar to the classical pathway but with a different initiation. The initiation of the alternative pathway is a spontaneous cleavage of C3 when bound to a pathogenic surface, i.e. the surfaces of plants, fungals, bacterials, etc. or artificial surfaces. [9, 20]

The complement system involves a few amplification steps, i.e. one activated complement factor will lead to the activation of many more complement factors. All three pathways will lead to the activation of large amounts of C3. The activation of C3 will result in the formation of C5 convertase that will cleave and activate C5. The activation of C5 will lead to the formation of the membrane attack complex (MAC). The complex can insert itself into a lipid membrane and create pores that will result in cell lysis and death. [17]
2.5 Haemostasis

Haemostasis originates from the Greek *hemos* (blood) and *stasis* (standing still). It is the process to stop the bleeding from damaged blood vessels. This can be achieved by vasoconstriction, increased tissue pressure and by formation of platelet plugs and formation of fibrin. Both platelet activation and fibrin are required for optimal clot formation. [17]

The interactions between an injured blood vessel (or an artificial surface), platelets, and complement proteins, play a role for the formation of a clot or a thrombus [9]. A blood clot is composed of platelets, fibrin, and in low flow vessels entrapped erythrocytes and leukocytes. Depending on where the thrombus formation occurs the
composition of the clot varies. If the clot is formed in the arteries the proportion of platelets will be higher, but if it is formed in the veins the amount of fibrin will be higher. [17]

### 2.5.1 Platelets

Different types of cells exists in the blood, red cells, white cells, and platelets. The red blood cells are important for the transport of oxygen, but seem not to be involved in haemostasis or thrombosis. The white blood cells are involved in inflammation, infection, wound healing, and the blood response to foreign materials. [9]

Platelets (figure 5) are discshaped cells without a nucleus with a diameter of 3-4 µm and an average volume of 10×10⁻⁹ mm³. They are produced in bone marrow and create plugs to stop the initial bleeding in injured blood vessels. By catalysing coagulation reactions, fibrin will be formed to stabilize the plug. [9]

![Figure 5. Platelets in fluorescence microscopy.](image)

The cytoskeleton in platelets and many eukaryotic cells consists of three types of protein filaments, intermediate filaments, microtubules, and actin filaments. The filamentous actin (F-actin), i.e. the polymer form of the globular protein actin, determine the shape of the cell and is necessary for the locomotion of the whole cell. They are flexible and in the cell most of them is concentrated just beneath the plasma membrane. F-actin has a diameter of 5-9 nm and the polymers are organized as linear bundles, a two-dimensional network, and a three-dimensional gel. [15]
Platelets are activated upon small stimulation and with F-actin polymerisation they become irregular in shape with pseudopods sticking out from their body, and bind to surfaces through multiple focal contacts. In vivo, platelets adhere to the underlying tissue elements, e.g. collagen, that are exposed when a vessel is damaged. The adhesion is mediated via specific membrane-bound receptors, such as platelet glycoprotein Ib (GPIb), using the plasma glycoprotein von Willebrand factor (vWF) as a cofactor. [9]

Platelets can only adhere to surfaces if proteins first have adsorbed to the surface. The conformational changes of the proteins activate membrane receptor GPIIb/IIIa. [9]

Platelets have cytoplasmic granules containing proteins (fibrinogen, albumin, fibronectin), Ca\(^{2+}\) ions, and adenosine disphosphate (ADP), etc. When the platelets are activated, the granules are released into the extracellular environment. If small amounts of thrombin is formed at the site of injury, this stimulates ADP release and formation of thromboxane A\(_2\) and fibrin. ADP recruits other platelets to aggregate. These are factors that help to recruit more platelets to the aggregate and stabilize the platelet thrombus. Fibrinogen is also important for platelet aggregation, since platelets bind to each other via fibrinogen molecules. The interactions are Ca\(^{2+}\)-dependent and the platelets will not bind to each other if fibrinogen, GP IIb/IIIa, or Ca\(^{2+}\) are eliminated. [9]

### 2.5.2 The coagulation cascade

The coagulation cascade (figure 6) involves at least twelve plasma proteins. The coagulation cascade can be activated either by negatively charged surfaces and follow the *intrinsic pathway*, or by damaged tissues and follow the *extrinsic pathway*. Inactive factors will become activated and lead to further activation of downstream factors. Both pathways lead to the final *common pathway* that ends with formation of thrombin that converts fibrinogen to fibrin. A blood clot will be formed and platelets be activated. [9]

The clotting is local, and not widespread, due to dilution of the activated factors by blood flow, and by the presence of inhibitors. Also, several reaction steps are not effective when not activated by surfaces of activated platelets or by damaged tissues. This is in order to prevent spontaneous coagulation in the blood vessels. [9]
2.5.2.1 Intrinsic pathway

The intrinsic pathway is initiated when contact factors, i.e. factor XII, XI, prekallikrein, and high-molecular-weight kallikrein (HMWK), adsorbs to a negatively charged surface [8], such as glass or titanium [17]. Factor XII is spontaneously surface activated to factor XIIa (the suffix “a” indicates an activated factor) [17]. HMWK is a cofactor that helps factor XI and prekallikrein to anchor onto the surface. Factor XIIa converts prekallikrein to kallikrein. Kallikrein accelerates the conversion of Factor XII to XIIa, creating a positive feedback loop. Factor XIIa, together with HMWK, cleaves factor XI to factor XIa. All these surface contact reactions are Ca\(^{2+}\) independent [8]. The first Ca\(^{2+}\) dependent step is when factor XIa, bound to HMWK, cleaves factor IX to IXa [17]. The enzyme thrombin activates factor VIII to VIIIa, which is a cofactor for the activation of factor X [9]. In the presence of Ca\(^{2+}\) (a large amount is released by activated platelets),
factors IXa and VIIIa forms a complex called “tenase” on phospholipid surfaces (on the surface of activated platelets). The reaction is slow in the absence of phospholipid surfaces, which help the reaction to take place on the platelet surfaces and not in the bulk fluid phase. The tenase complex converts factor X to Xa. [9, 17]

### 2.5.2.2 Extrinsic pathway

The extrinsic pathway is activated when blood vessels are injured. A membrane protein called tissue factor (tissue thromboplastin, or Factor III) interacts with Factor VII that turns to Factor VIIa [17]. The tissue factor (TF) is expressed by activated white cells and endothelial cells, but can also circulate in a soluble form. It is present in many tissues and becomes available to factor VII when blood vessels are injured and the underlying structures are exposed to flowing blood. Tissue factor, factor VIIa, and Ca$^{2+}$ forms a complex, analogous to a tenase, activating factor X to factor Xa [9].

### 2.5.2.3 Common pathway

Both the intrinsic and extrinsic pathways lead to the common pathway. The pathways lead to the activation of Factor X to Xa, and Factor V is a cofactor that is activated by thrombin. Factor Xa and Va forms, in the presence of calcium and platelet phospholipids, the “prothrombinase” complex that converts prothrombin (factor II) to thrombin. [9]

Thrombin catalyze the proteolysis of the soluble plasma fibrinogen. Fibrin monomers are released into the plasma and spontaneously polymerize to form a gel of fibrin polymers, called stable fibrin, that traps blood cells inside the thrombus [9, 17]. Thrombin is a strong catalyst for platelet activation, which causes them to release factors important for the haemostasis. Activated platelets also possess the optimal surface for the intrinsic pathway that leads to conversion of prothrombin to thrombin [17].

Factor XIII, trapped within the fibrin clot or provided by platelets, is activated by thrombin to become factor XIIIa and mediates crosslinking of fibrin polymers [9]. The action of the platelet actin cytoskeleton on the fibrin network make the clot shrink to a plug and serum is expelled from the clot [17].
2.5.3 Citrate and heparin

Citrate and heparin are two anticoagulants [21]. Citrate chelates Ca\(^{2+}\) ions in the plasma and thus prevents the coagulation process to continue [18]. When Ca\(^{2+}\) is added to the citrated plasma, it first neutralizes excessive citrate and secondly allows the coagulation to begin.

Heparin prevents the coagulation by inhibition of thrombin and not by chelation of Ca\(^{2+}\) and other ions [18]. Heparin is therefore better to use when working with blood cells, since ions are necessary for important cell functions [9]. The inhibition of thrombin is more difficult to reverse than the shortage of free Ca\(^{2+}\).

2.5.4 Fibrinolysis

Fibrinolysis is the degradation of stable fibrin after the thrombus formation (figure 7) [17]. The degradation of the clot is more generally called thrombolysis. The fibrinolytic system involves precursors, activators, cofactors and inhibitors [9].

The enzyme plasmin circulates in an inactive form, then called plasminogen [9]. Plasminogen adheres to blood clots, polymerases and is then incorporated into the network of fibrin. Plasminogen activators present in the blood or released from tissues, activates plasminogen to plasmin. The presence of fibrin accelerates the conversion. Two important plasminogen activators are the serine proteases tissue plasminogen activator (tPA) and urokinase (uPA). tPA comes from endothelial cells and uPA is present in the plasma. uPA must be attached to a cell surface receptor for the conversion of plasminogen to plasmin. [9, 16]

Plasmin digest the fibrin clot into soluble fibrin-fibrin products that are released out to the circulating blood. Fibrinolysis is inhibited by plasminogen activator inhibitors (PAIs), and by thrombin activated fibrinolysis inhibitor (TAFI) that promotes the stabilization of fibrin and fibrin clots. [9]
Figure 7. The fibrinolytic system. A plasminogen activator convert plasminogen to plasmin which cleaves the insoluble fibrin polymers into soluble degradation products (J. M. Berg, et al., Biochemistry, 2002 [9]).

2.6 Working methods and devices

The instruments and methods that have been utilized in this study are described in this chapter. Ellipsometry is a method that is simple to use and which do not destroy the sample. The fluorescence microscope was used to study blood cells that attached to the test surfaces. Methods such as the cone-and-plate setup are not detection instruments, but devices used when performing the experiments.

2.6.1 Ellipsometry

Ellipsometry is an optical technique originally developed for characterization of optical properties of materials [22]. Many properties and parameters, e.g. film thickness, optical constants, refractive index, surface roughness can be calculated from the data [24]. Film thickness in the range of 2-3000 Å can be determined. The technique is simple, non-destructive, and the measurements can be made in transparent mediums [23].

The light can be thought of as electromagnetic waves and the electrical field is used to describe the polarization state [22], e.g. how the light oscillates in space. If all the electromagnetic waves have the same amplitude and the same phase difference, the light is said to be polarized [24]. The polarization is in general elliptic [23]. The electrical
fields are both parallel ($p$-) and perpendicular ($s$-) to the plane of incidence (figure 8). When light interacts with the sample, surface and bulk, the polarization state changes. The change is described by two values of ellipsometric angles, $\Psi$ and $\Delta$, which are not very informative when separated, but if analyzed together different properties of the sample can be calculated. If the surface is too rough, the light beam will be scattered away from the detector and the light can not be measured. [24]

![Diagram](image)

**Figure 8.** Incident light to a surface and planes of incident, and the change of polarization (J. A. Wollam Co., Inc., USA [24]).

Changes in light properties before and after the reflection are measured by comparing the $p$- and $s$- components of light [23] and represent both a change in the amplitude ($\tan(\Psi)$) and a phase difference ($\Delta$) (formula 2)[24]. $R_p$ and $R_s$ are the Fresnel reflection coefficients for $p$- and $s$- polarized light, respectively:

$$\tan(\Psi) e^{j\Delta} = \frac{R_p}{R_s}$$

**Formula 2.** The change of polarized light by comparing light parallel ($p$-) and perpendicular ($s$-) to the plane of incident (J. A. Wollam Co., Inc., USA [24]).
2.6.2 Imaging of coagulation

Imaging of coagulation in vitro can give an indication to what extent a surface activates coagulation. The surfaces of interest are placed in plastic cuvettes filled with citrate plasma. Before the beginning of the experiment, Ca\(^{2+}\) is added to citrated plasma to allow the coagulation to start. Figure 9 demonstrates the course of the coagulation in a cuvette. To the left in the figure the plasma is not coagulated and is a transparent light yellow liquid. During the coagulation at the surface, the plasma becomes more opaque yellow gel (to the right). The polymerized fibrin network scatters more light than noncoagulated blood plasma. [25]

![Figure 9](image)

**Figure 9.** Photos of coagulation activated by a surface in a cuvette filled with plasma. The plasma changes color from a transparent light yellow liquid (left) to a non-transparent yellow gel (right).

The cuvettes are placed in front of a camera with the edges of the surfaces directed towards the camera. The camera stands at a distance from the cuvettes, enough to be able to photograph all the cuvettes, in this case four at the time, but also to minimize the angle in which the camera “see” the outer surfaces. The camera takes time-lapse images during the course of the coagulation. The coagulation activated by the surfaces will be visible as the plasma becomes less transparent.
2.6.3 Fluorescence microscopy

Fluorescence microscopy is a technique for observing samples that are prepared with specific fluorescent probes [26]. Fluorescence microscopy has made it possible to identify cells and cellular components with a high degree of specificity [27].

Certain molecules emit energy in the form of light when they are irradiated with the light of a specific shorter wavelength. Figure 10 shows the principle of fluorescence. When radiation of a specific wavelength reaches the sample (1), electrons in the sample becomes excited to a higher energy level (2). After a short time, the atom relaxes to a lower level and the electron falls back to the ground state, emitting excess energy as a photon. [27]

![Figure 10. Principle of fluorescence. A: Energy is absorbed by the atom. B: The electron gets excited to a higher energy level. C: The electron falls back to the ground state, emitting fluorescence light (Nobleprize.org [27]).](image)

2.6.4 Cone-and-plate setup

The cone-and-plate setup generates shear stress upon flow of a liquid over a surface, and cells affected by different flow conditions can be studied. The setup enables, among other things, testing of a film mechanical attachment strength upon applied shear stress. [25]

Figure 11 illustrates the cone-and-plate setup. The test surface is placed on an o-ring on the plate. A tube connects the plate with a vacuum pump and the vacuum holds the o-ring and the surface in place. A drop of blood or another liquid, e.g. buffer, is placed on the surface. The plate is lifted up in close proximity to the cone, so that the cone is in contact with the liquid drop. The cone is rotated with a desired velocity and thus creates a liquid flow over the surface with a specific shear rate. Low rotation speed of the cone
at approximately 100 rotations per minute (rpm) correspond to a shear rate at about 100 s$^{-1}$ and which is comparable to the shear rate in veins [25]. A high rotational speed of 1200 rpm (shear rate ~1200 s$^{-1}$) corresponds to blood flowing in the arteries. The cone has horizontal contact angle of 5°. The gap between the cone and the surface affects the shear rate on the surface. A larger distance between the cone and the surface decreases the shear rate. [25]

![Cone-and-plate setup](image)

**Figure 11.** Illustration of the cone-and-plate setup. The surface of interest is placed on top of an o-ring. The ring and the surface are held in place on the plate with vacuum. A drop of PBS or blood is placed on the surface and when the cone rotate, the liquid drop will simulate the blood flow in vessels.

The flowing liquid on the test surface will create a shear stress on the surface. Stress is defined as deforming force per unit area, where the force vector is parallel with the area. The viscosity of the liquid is a factor that affects shear stress on the surface. A liquid with lower viscosity, e.g. a buffer, creates a lower shear stress than a liquid with a higher viscosity, e.g. blood. [28]

The shear rate for the flowing liquid between two surfaces is a velocity gradient defined by the distance (d) between the stationary surface and the surface moving with a constant velocity (v) [25]. Formula 3 is valid at ideal conditions.
The shear rate is measured in $s^{-1}$, $\omega$ is the angular velocity of the rotating cone, and $f$ the frequency (revolutions per seconds), $r$ the distance from the centre of the cone, and $\alpha$ the horizontal contact angle of the cone. The ideal condition is, however, not possible to create, e.g. the moving surface can not be plane because it would crush the cells in a blood experiment, thus it is formed as a cone. [25]
Chapter 3

Materials and methods

The aim of this chapter is to describe the preparations and procedures of the experiments. Volumes, incubation times, etc. are described in connection with the specific experiment.

3.1 Preparation of surfaces

Silicon wafers with 2000 Å evaporated titanium on one side was cut into surfaces of the size 5×10 mm. The titanium surfaces were rinsed in destilled water (MilliQ), dried in flowing N$_2$-gas, and then cleaned 4 min in an UVO-cleaner (Jelight Company Inc., USA). The UVO-cleaner dissociates contaminant molecules by the absorption of short wavelength UV-radiation [29]. The surfaces were once again cleaned in MilliQ and dried in N$_2$-gas. The surfaces were measured with null-ellipsometry and the values of $\Delta$ and $\Psi$ acted as references for the measurements of the film thickness after the protein incubations.

3.1.1 Protein matrix onto titanium surfaces

The proteins used for the matrixes were IgG and HSA from Sigma-Aldrich, and fibrinogen from three different manufacturers. The fibrinogens were obtained as freeze
dried plasminogen depleted human fibrinogen from Calbiochem (USA), Haemocomplettan® from CSL Behrings (USA), and fibrinogen from a third manufacturer which in the present study will be referred to as X. The product from Calbiochem (which in this study is called Fib.C) had the lowest purity of fibrinogen compared to fibrinogen from Behrings (Fib.B) and fibrinogen from X (Fib.X) which has the highest concentration of pure fibrinogen. The exact proportion of fibrinogen in each product is not known. In the following text the protein concentrations are referred to the total weight of the products.

The proteins were dissolved in 10 mM acetate buffer at a concentration of 2 mg/ml. The pH and weight percent (wt%) of salt in the buffers were specific for each protein [4, 5]. Acetate buffer with pH 5.5 for fibrinogen and IgG films and pH 4.3 for HSA films were verified and adjusted before use.

For protein monolayers, titanium surfaces were incubated in the protein solution for 30 min at room temperature. For protein multilayers, titanium surfaces were incubated in the protein solution in a heating block (Grant QBD digital block heater, Camlab Limited, UK) at the temperature required for each protein [5]. The work to determine the buffer pH, the salt concentration, and the incubation time for the buffers for IgG and HSA is described in another master’s thesis [5]. Surfaces with size 5×10 mm were incubated in 0.5 ml protein solution in 0.5 ml microfuge tubes for 10 min. Surfaces with size 5×20 mm were incubated for 15 min in 1.5 ml fibrinogen solution, or for 10 min 1.5 ml IgG solution. The surfaces were then rinsed in MilliQ and dried with N₂-gas. The film thickness was measured by ellipsometry in air before and after the incubation.

3.2 Plasma, serum and antibody incubations

The differently prepared surfaces were incubated in plasma or serum before incubation in an antibody solution. The adsorption of plasma proteins and the subsequent binding of antibodies were measured with ellipsometry. Normal human serum and normal human heparinized plasma from two healthy donors were frozen and stored at -80°C until use. Serum and plasma were used for the study of deposition of complement and coagulation proteins, respectively, onto the different surface modifications.
The titanium surfaces with the protein films were placed in 0.5 ml microfuge tubes in 0.5 ml serum or plasma and incubated in a heating block for 10 or 60 min in 37 °C. After incubation the surfaces were rinsed in MilliQ and dried with N₂-gas. The adsorption of serum or plasma proteins onto the protein matrixes was measured with null-ellipsometry.

After serum or plasma incubation, the surfaces were incubated in an antibody solution. The deposition of serum and plasma proteins was analysed through the binding of polyclonal antibodies. The antibodies rabbit anti-human C3c and rabbit anti-human C3d (DAKO Sweden AB) have affinity to the complement protein C3b and degradation fragments of C3b, respectively [1]. The antibody goat anti-human high molecular kininogen (anti-HMWK) (The Binding Site, UK) have affinity to the coagulation factor binding protein HMWK.

Veronal buffer saline (VBS⁻), containing 0.15 mM NaCl, was supplemented with 0.15 mM CaCl₂ (Merck) and then called VB⁺++. The antibodies were diluted 1:50 in VB⁺++. The surfaces were incubated in the antibody solution for 30 min at room temperature. After incubation the surfaces were rinsed in MilliQ and dried with N₂-gas. The binding of antibodies was measured with ellipsometry.

3.3 Protein film thickness measured with null ellipsometry

The thickness of the protein film was measured in air by null ellipsometry (AutoEL III null ellipsometer, Rudolph Research, USA) before and after incubation. Assumed refractive index for proteins was \( n_f = 1.465 \) [30]. Five points on the surface were measured and the protein film thickness for each point was calculated according to the McCracking evaluation algorithm [31]. The mean value and standard deviation of the points were then calculated according to formula 4.

\[
S = \sqrt{\frac{n^2 \sum x^2 - (\sum x)^2}{n(n-1)}
\]

**Formula 4.** Standard deviation.
The standard deviation, s, is a measured variation over n data points \((x_1,...,x_n)\) of an average value. \(\Sigma\) is the sum of all \(x\) and \(x^2\).

### 3.4 Studies of coagulation and blood cells

The following chapter describes the procedure and analysis of the imaging of coagulation method, and of cell adhesion tests of the surfaces in whole blood.

#### 3.4.1 Imaging of coagulation

Titanium surfaces with multilayers of fibrinogen (Fib.C, B, and X), IgG and monolayer of HSA, were prepared as described in chapter 3.1. Titanium surfaces were used as positive controls. Blood was drawn from one donor into citrate containing tubes. The tubes were centrifuged with 2500×g for 15 min in order to separate plasma and blood cells. The plasma supernatant was transferred to another tube. CaCl\(_2\), 36 µl/ml plasma, was added to the plasma right before the start of the experiment. The Ca\(^{2+}\) first neutralizes the citrate in the plasma, the excessive amount of Ca\(^{2+}\) can then be used by the coagulation factors to allow the coagulation to start.

![Figure 12. Imaging of coagulation. Four cuvettes are placed in front of a camera. The cuvettes are filled with plasma (1). The test surfaces lean against the left wall of the cuvettes (2) and are held in place by cuvette tips (3).](image-url)
Figure 12 shows the surfaces placed in transparent cuvettes, leaning against one side of the cuvettes. 0.5 ml of the plasma was then added to the each cuvette.

The camera (Canon Eos 400D digital) was set to photograph the coagulation process every 15th second, until the plasma in all the cuvettes had coagulated. With MatLab® (version 7.2, The Mathworks Inc., Natick, USA), the time of coagulation for each surface was calculated [25].

### 3.4.2 Adhesion of platelets and white blood cells

Titanium surfaces (5x10 mm) with multilayers of fibrinogen (Fib.C, B, and X) and IgG, and monolayer of HSA were prepared (chapter 3.1). Blood was drawn from a donor and filled into tubes, containing the anticoagulantia heparin, within an hour before use. The surfaces were placed in 0.5 ml microfuge tubes. The tubes were filled with blood and placed on a rotator to generate a slow flow of blood over the surfaces. The surfaces were under these conditions incubated for 40 min at room temperature and then gently dipped in PBS, to rinse the surfaces from blood.

The surfaces were directly placed in a petri dish filled with 3.7 % paraformaldehyde (PFA) (Sigma-Aldrich) in PBS, without drying, and incubated in PFA for 15 min at room temperature. The PFA creates covalent crosslinkings over the cells on the surface in order to fixate the cells to the surface and to help them hold their form when Triton® X (0.1 % in PBS) was added in the next step. After incubation in PFA, the surfaces were rinsed in PBS and transferred to a petri dish without drying the surfaces.

PBS buffer containing 0.1 % Triton®X (Sigma-Aldrich) was added to the surfaces, just enough to cover them. Triton®X is a detergent, a nonionic surfactant that solubilizes the cell membrane [12]. The surfaces were incubated for 2 min at room temperature, rinsed in PBS and transferred to a new petri dish.

The F-actin in the cells was labeled with the fluorescent probe Alexa Fluor® 546 Phalloidin (Invitrogen AB), diluted 1:100 in PBS. Small droplets were placed over the surfaces which were then gently shaken to ease the staining of the cells. The surfaces were incubated for 20 min at room temperature in darkness, then first rinsed in PBS and then directly rinsed with destilled water. The surfaces were dried with flowing N₂-gas.
On a slide for microscopy, small droplets of ProLong® Gold (Invitrogen AB) were placed. The surfaces were placed upside down on top of each drop. ProLong® Gold is an antifade reagent that suppress photobleaching and preserves the signals from the fluorescantly labelled target molecules [14]. It also contains the blue-fluorescent nuclear counterstaining DAPI, which labels the nucleus in white blood cells. DAPI is visible at wavelength for UV-light (10-400 nm), and Alexa Fluor® 546 at wavelength 546 nm.

Microscopy of the cells was done with the microscope Zeiss AxioObserver Z1 and the software Zize AxioVision 4.6 (Carl Zeiss MicroImaging GmbH, Germany) [26].

### 3.5 Stability tests of protein multilayers

Different stability tests of the protein multilayers were performed. ProtMat2.0 of fibrinogen, HSA and IgG were incubated in different buffers. FibMat2.0 was also exposed to enzymes and to mechanical shear forces to study the mechanical stability.

#### 3.5.1 Incubation in VB++ and HEPES buffer.

The stability of fibrinogen, IgG and HSA multilayers onto titanium surfaces was studied in VB++ buffer in both room temperature (22°C) and 37°C for 10, 30 or 60 min. The HSA multilayer was also incubated in HEPES buffered saline (HBS) in a heating block in 37°C for 10, 30 or 60 min. Both the VB++ and HBS buffer had pH 7.4. The multilayers were incubated in 0.5 ml microfuge tubes with 0.5 ml of one of the buffers. The surfaces were rinsed with MilliQ and dried with N₂-gas. The change in the multilayer thickness was measured with null ellipsometry.

#### 3.5.2 Incubation in plasmin and thrombin

Multilayers of fibrinogen C, B, and X were prepared on titanium surfaces (size 5×10 mm). The surfaces were placed in 0.5 ml microfuge tubes and the tubes were filled with 0.5 ml citrate plasma. Tissue plasminogen activator (tPA) and thromboplastin were then
added to plasma. Thromboplastin initiates the conversion of prothrombin to thrombin, which catalyzes the formation of stable fibrin. Thromboplastin is a composite of both phospholipids and tissue factor and both are required in the activation of the extrinsic pathway. tPA turns plasminogen to plasmin that digest the fibrin clot.

Ca$^{2+}$ was added to the plasma, to allow the coagulation to start. The surfaces were incubated in the plasma at room temperature until both the coagulation and fibrinolysis process were activated and ended. The surfaces were then rinsed with destilled water and dried with flowing N$_2$-gas. The multilayer thickness was measured with null ellipsometry before and after the incubation.

To investigate if both thrombin and plasmin, or only one of them, had any affect on the fibrinogen multilayers, other surfaces were incubated in only thrombin. Droplets of thrombin in PBS were placed on top of the surfaces with fibrinogen multilayers. The incubation lasted for 20 min and the surfaces were then rinsed with MilliQ and dried with N$_2$-gas. The multilayer thickness was measured before and after incubation with null ellipsometry.

### 3.5.3 Cone-and-plate

Multilayers of Fib.C, Fib.B, and Fib.X (prepared as described in chapter 3.1), were tested for mechanical stress caused by flowing fluid in contact with the matrix.

A surface was placed on the plate (chapter 2.6.4). 40 µl PBS was then dropped on the surface. The cone was lowered until it was in contact with the liquid drop and as close as possible to the surface. The rotational speed of the cone was set to 1200 rpm, which corresponds to a shear rate of approximately 1200 s$^{-1}$, for 3 min. The surfaces were rinsed with destilled water and dried with flowing N$_2$-gas. The thicknesses of the multilayers were measured with null ellipsometry before and after the experiment.
Chapter 4

Results

This chapter presents the results from the performed studies. Bar diagrams illustrate protein film thicknesses before and after the specific incubation steps. Photographs illustrate platelets and white blood cells at surfaces after incubation in blood. At the end of the chapter some crucial results are summarized in a table.

4.1 Protein film thickness

Different kinds of protein films were prepared and the characteristics were compared. Figure 13 shows the protein film thickness of the multilayers and monolayers of the fibrinogens, IgG, and HSA. The HSA multilayer had the highest multilayer thickness, about 450 Å, Fib.B the lowest film thickness of the three fibrinogen multilayers, about 170 Å. All three fibrinogen multilayers showed a lower film thickness than IgG multilayers. All monolayers were about 30 Å thick.
Figure 13: Protein film thickness for multilayer of fibrinogen, monolayer of fibrinogen C (Fib.C), multilayer of IgG and HSA (n = 3). Protein conc. 2 mg/ml.

4.2 Adsorption of native human serum and plasma

Spontaneous adsorption of serum to protein multilayers and monolayers on titanium were studied (figure 14). The surfaces were incubated in normal serum for 10 or 60 min at 37°C. The fibrinogen multilayers (no. 1-3) adsorbed small amounts of serum (16 Å), as did the fibrinogen monolayer (no. 4, Fib.C, 15 Å). Adsorption of serum to IgG multilayer was 34 Å, i.e. larger than the serum protein binding to the fibrinogens, which was less than 15 Å. The largest amount of serum adsorbed to IgG monolayer, 44 Å after 10 min and 140 Å after 60 min. The HSA monolayer adsorbed low amounts of serum, up to 10 Å after 60 min of incubation. Incubations in serum resulted in a reduced thickness of the HSA multilayers. The decreased film thickness is shown in the figure as negative values. After 60 min of incubation, the film thickness decreased 267 Å, with a standard deviation of 35 Å. Clean titanium surfaces were used as positive controls; about 50 and 60 Å of serum protein adsorbed to titanium at 10 and 60 minutes respectively.
Serum adsorption

Figure 14. Serum adsorption to protein films (n = 3). Up to 15 Å of serum adsorbed to fibrinogen (no. 1-4) and HSA monolayer (no. 8). HSA multilayer (no. 7) decreased in film thickness with about 260 Å. Serum adsorbed to IgG multilayer (no. 5) and monolayer (no. 6) and to titanium surfaces (no. 9), used as positive controls.

The adsorption of heparinized plasma proteins to different prepared surfaces is shown in figure 15. Low amounts of plasma adsorbed to fibrinogen multilayers (no. 1, 2, 3) and monolayer (no. 4). 6 Å plasma adsorbed onto IgG multilayer after 60 min (no. 5) and 26 Å onto the monolayer (no. 6). 19 Å plasma proteins adsorbed to HSA monolayer (no. 7). HSA multilayer dissolved during the incubation and is thus not shown in the figure. Titanium surfaces (no. 8) were used as positive controls with 47 Å adsorbed plasma.

Plasma adsorption

Figure 15. Plasma adsorption to differently prepared surfaces (n = 3). Low amounts of plasma adsorbed to fibrinogen multilayers (no. 1-3) and monolayer (no. 4). Plasma adsorbed to IgG multilayer (no. 5) and monolayer (no. 6). Plasma also adsorbed to HSA monolayer (no. 7) and to titanium surfaces (no. 8) which were used as positive controls. HSA multilayer dissolved during the incubation and is thus not shown in the diagram.
4.3 Binding of antibodies

The adsorption of complement proteins and coagulation factor HMWK after incubation in serum or plasma, respectively, were analysed by the binding of specific antibodies to adsorbed plasma or serum films. Degradation fragments of C3b was probed with anti-C3c and anti-C3d, respectively.

4.3.1 Binding of anti-C3c and anti-C3d

The binding of anti-C3c to the protein films can be seen in figure 16. At the most 11 Å of anti-C3c adsorbed to fibrinogen multilayers (no. 1-3), fibrinogen monolayer (no. 4), and HSA monolayer (no. 8), demonstrating that the complement protein C3 had not bound to the surfaces during serum incubations. Antibodies did not adsorb to IgG multilayer (no. 5) that were incubated in serum for 10 min, but did so when incubated for 60 min. IgG monolayer (no. 6) showed most anti-C3c binding to the surface. HSA multilayer (no. 7) dissolved during the incubation, so antibodies could not be used. Titanium surfaces were used as positive controls and anti-C3c bound to some degree onto the surfaces.

![Anti-C3c binding](image)

Figure 16. Anti-C3c binding to multi- and monolayers of protein films (n = 3). Up to 11 Å of anti-C3c adsorbed to multi (no. 1-3)- and monolayer (no. 4) of fibrinogen. Anti-C3c adsorbed to IgG (n 5, and 6). Anti-C3c adsorbed to monolayer of HSA (no. 8) after 60 min in serum. The HSA multilayer (no. 7) dissolved during the incubation. Titanium surfaces (no. 9) were used as controls (n =3).
The adsorption of C3-fragments onto different surfaces was detected with the binding of anti-C3d (figure 17). No anti-C3d bound to to fibrinogen multilayers (no. 1-3). The multilayer thickness decreased with about 17±4 Å. Approximately 15±21 Å anti-C3d bound to IgG multilayer after 60 min of serum incubation.

Figure 17. Anti-C3d binding to the three fibrinogen multilayers (no. 1-3) and to IgG multilayer after 10 and 60 min of incubation in serum (n = 3).

4.3.2 Binding of anti-HMWK

Anti-HWMK binding to different surfaces after plasma incubation for 10 or 60 min is shown in figure 18. Both fibrinogen and IgG multi- and monolayer adsorbed at most 5 Å. No anti-HMWK bound to fibrinogen multilayers (no. 1-3) and monolayer (no. 4), IgG multilayer (no. 5) and monolayer (no. 6). 57 Å anti-HMWK bound to HSA monolayer (no. 7) after incubation in plasma for 10 min. 22 Å anti-HMWK adsorbed to titanium surfaces, that were used as positive controls.
Figure 18. Binding of anti-HMWK to protein multilayers (n = 3). No binding of anti-HMWK to the fibrinogen multilayers (no. 1-3) and monolayer (no. 4). No binding of the antibody to IgG multilayer (no. 5) and monolayer (no. 7). Anti-HMWK bound to titanium surfaces, which were used as positive controls.

4.4 Coagulation times induced by different proteins

The imaging of plasma coagulation was done for different protein films. Titanium surfaces were used as positive controls. Figures 19 and 20 show the coagulation times, i.e. the time for the recalcified citrate plasma to be activated at the surface and for coagulation of the plasma in the proximity of the surface. Figure 19 shows two tests (series 1 and 2) performed with fresh plasma from two different healthy donors and figure 20 shows two tests (series 1 and 2) with frozen plasma from two different healthy donors.

The coagulation times measured in frozen and thawed plasma were longer than in fresh plasma. Titanium and the HSA monolayer induced faster coagulation than fibrinogen and IgG multilayers, both with frozen and fresh plasma, best demonstrated in the diagram for frozen plasma. The plasma coagulated spontaneously in tests performed with fresh plasma and one time with frozen plasma (series 2). The coagulation times with fresh plasma were between 11 to 16 min in series 1 and between 21 to 26 min in series 2.
Figure 19. Coagulation times for different protein films with fresh plasma, which were spontaneously activated. The coagulation times for series 1 were between 11-16 min and for series 2 between 21-26 min.

Figure 20, where frozen plasma was used, give a better indication that the coagulation times for fibrinogen and IgG were longer in comparison with Ti and HSA monolayer.

Series 1 demonstrates a bigger difference between the surfaces’ coagulation times. The clotting time in series 1 for fibrinogen C, B, and X was 55, 70, and 60 min, respectively. A monolayer of HSA induced a clotting time almost at the same time as the titanium surface, after about 25 min. The longest time for the plasma to start the coagulation was in the proximity of the IgG multilayer, with a clotting time at 90 min.

The plasma used for series 2 appeared to already be activated when the experiment began and thus the coagulation times became shorter than for series 1. The coagulation times in series 2 also had smaller differences between the surfaces than series 1.
Figure 20. Coagulation times for different protein films, from the imaging of coagulation setup with frozen plasma. Series 2 demonstrates bigger differences between the coagulation times than series 1. In series 2, fibrinogen multilayer C, B, and X induced coagulation after 55, 70, and 60 min, respectively. Titanium and HSA monolayer activated coagulation after about 20 min.

4.5 Adhesion of platelets and white blood cells

The adhesion of cells to surfaces was studied with fluorescence microscopy and photos were taken at different magnifications. Both platelets and white blood cells adhered onto titanium surfaces (figure 21), that were used as positive controls. Red blood cells have no special receptors for adhesion to surfaces and therefore only platelets and white blood cells were studied at surfaces. Photos 21.A and 21.B were taken at the wavelength specific for the different probes. The actin filaments of the cells were stained with Alexa 546 Phalloidin, that fluoresce when illuminated with light at wavelength $\lambda=546$ nm and is visible as orange in the photos (figure 21.A). To distinguish the white blood cells from platelets, the nucleus of the white blood cells were stained with DAPI, that fluoresce when illuminated with UV-light and is visible as blue spots (figure 21.B). Platelets do not have a nucleus and are therefore not affected by DAPI staining.
Figure 21. A: Platelets, with the visible as orange spots, and B: White blood cells, with the cell nucleus visible as blue spots, adhered to a titanium surface. A and B are photos of the same area and surface, but at different wavelengths to distinguish between the different cell types.

Two titanium surfaces were used as controls at each time the experiments were performed. Figure 22.A and 22.B demonstrate the difference between two titanium surfaces tested in the same assay and treated in the same way. More platelets and white blood cells were visible on the surface illustrated by photo A than on the surface in photo B. The differences between these two surfaces demonstrate the variance that could occur between similar surfaces in the one and same experiment.

Figure 22. Two titanium surfaces, A and B, treated in the same way during the same experiment, but showing a difference between the adhesion of platelets (orange) and white blood cells (blue). The differences can be considered as the variation between similar surfaces in an experiment.
After incubation in whole blood neither platelets nor white blood cells could be detected on IgG multilayer and HSA monolayer (figure 23) and the photos therefore appears black.

Figure 23. No cell adhesion to the surfaces A: IgG multilayer, or to B: HSA monolayer. The photos therefore appear black.

Platelets adhered to Fib.C, B, and X, shown in figure 24.A, 24.B, and 24.C, respectively. The cell adhesion to the three fibrinogen multilayers differed from each other, but similar to the titanium surfaces (figure 22), the cell adhesion to one fibrinogen multilayer could vary between repeated incubations.

Figure 24. Platelets and white blood cells adhered to fibrinogen multilayer. The platelets are visible as orange and the white blood cells are visible as blue spots. A: Fib.C with only a few cells adhered to the surface. B: Fib.B with more cells on the surface than both Fib.C and Fib.X. C: Fib.X with a few cells adhered to the surface.
Figure 25.A and 25.B show photos of fibrinogen B and X and the cells adhered to the multilayers. The platelets that adhered to Fib.B (25.A) spread over the surface and pseudopods spread out from the cell bodies. The platelets on the Fib.X (25.B) were more clustered together with just a few pseudopods stretched out over the surface.

![Figure 25. Platelets adhered to multilayers of fibrinogen. A: Fib.B, the platelets are spread over the surface and pseudopods are reaching out from the cells. B: Fib.X, the platelets are grouped together and only a few short pseudopods are visible from the cell bodies.](image)

To study if the fibrinogen multilayers were dissolved or not during any of the steps from the incubation in blood to the microscopy, the fibrinogen surfaces were incubated with fluorescent fibrinogen antibodies. Figure 26 shows a titanium surface. The area of the titanium surface was the same as for photos A-C. Areas where nothing has bound to the surface appear black. The platelets appear orange and the fibrinogen antibodies appear blue. Photo 26.A shows both platelets and fibrinogen antibodies. The platelets (figure 26.B) are not spread all over the surface, but are partly grouped together. The fibrinogen antibodies (figure 26.C) bound to the same area as the platelets, indicating that the cells have bound plasma fibrinogen and could be activated.
Figure 26. A titanium surface, at the same area in all three pictures A-C. In the black areas, nothing bound to the surface. A: Platelets and antibodies against fibrinogen. B: A photo of platelets only. C: Fibrinogen antibodies, indicating that the platelets have bound plasma fibrinogen.

Figure 27 are photos of titanium prepared with multilayer of Fib.B. Photo 27.A shows platelets (orange) adhered to the fibrinogen multilayer, and it also shows fibrinogen antibodies (blue). 27.B illustrates the platelets (orange) bound to the fibrinogen multilayer (black). The fibrinogen antibodies (blue) shown in 27.C were mainly bound to the fibrinogen multilayer in the areas where no platelets were bound. The photos 27.B and 27.C are almost mirror images of each other, i.e. where B is orange (platelets) C is black, and where C is blue (fibrinogen antibodies) B is black.

Figure 27. Fib.B, the same area at all three photos. A: Platelets and fibrinogen antibodies. B: Platelets. C: Fibrinogen antibodies cover the areas where the platelets in B have not adhered to the surface. Photos B and C are almost mirror images of each other: where one of them is coloured, the other looks black.
4.6 Stability tests

The multilayers were tested for the stability in different buffers and the fibrinogen multilayers were also tested for the stability of shear forces.

4.6.1 Effect of VB$^{++}$ and HEPES buffer on multilayer protein films

Multilayer of HSA decreased in thickness in serum incubation at 37°C and when incubated in antibodies diluted in VB$^{++}$ buffer at room temperature. The stability of the multilayer was then tested in VB$^{++}$ buffer and HEPES buffered saline (HBS) at pH 7.4, for different incubation times at 37°C (figure 28). The graph shows that the multilayer thickness of HSA decreased rapidly during the incubation. The film dissolved in both buffers at 37°C. The multilayers decreased to about 55 Å.

![Graph showing the effect of VB$^{++}$ and HBS on HSA film thickness](image)

**Figure 28.** Multilayer of HSA incubated in VB$^{++}$ or HBS buffer for 0-60 min at 37°C. The thickness of the multilayer start to decrease shortly after the incubation is initiated, n = 3.

The stability of fibrinogen and IgG multilayers in the VB$^{++}$ at room temperature (22°C) or at 37°C is shown in figure 29. The thickness of the fibrinogen and IgG multilayer did not decrease more than 5 Å, with a maximum standard deviation of 12 Å, during the incubation time. No difference was observed after incubation at room temperature or at 37°C.
4.6.2 Effect of plasmin and thrombin on fibrinogen multilayers

The effect of plasmin on fibrinogen multilayers was tested. The clotting of the plasma took about 5 min, and the plasma formed a gel inside the microfuge tubes. The fibrinolysis was activated and finished after about 20 min, and the plasma was again liquified. The multilayer thicknesses were measured with null ellipsometry before and after the coagulation and fibrinolysis of the plasma. The fibrinogen multilayers decreased in thicknesses, which indicates that the FibMat2.0 were digested by plasmin during the fibrinolysis (figure 30). All the multilayers decreased to a film thickness of approximately 20 Å, even though the film thickness varied between 225 and 300 Å before incubation.
Fibrinogen multilayers were incubated in thrombin for 20 min. The effect of thrombin on the multilayer thickness was very small, which can be seen in figure 31.

**Figure 30.** Fibrinogen multilayers before and after fibrinolysis (n = 3). Most of the multilayers have dissolved but not all of it. Thickness before incubation was $277 \pm 38 \, \text{Å}$ and after $38 \pm 2 \, \text{Å}$.

**Figure 31.** Fibrinogen multilayers (n = 3) before and after incubation in thrombin. The effect of thrombin to the multilayers was insignificant. Thickness before incubation was $260 \pm 31 \, \text{Å}$ and after $253 \pm 34 \, \text{Å}$.

### 4.6.3 Effect of applied shear stress on fibrinogen multilayers

Multilayers of fibrinogen C, B, and X were tested for their stability by applying shear stress to the multilayers. The rotation velocity of the plate was set to 1200 rpm and rotated for three minutes. The bars in figure 32 show that the thickness of the fibrinogen multilayers decreased with no more than 50 Å.
Figure 32. The stability for applied shear stress on fibrinogen multilayers \((n = 3)\) tested in the cone-and-plate setup. The multilayers decreased at most 50 Å. Thickness before tests 256±12 Å, after 233±25 Å.

4.7 Results summarized in a table

Table 1 is a simplified summary of some of the results presented above. Plus signs represent positive results, e.g. binding of proteins or cell adhesion to the surfaces. Minus signs represent experiments where proteins and cells did not bind to the surfaces. Empty boxes means that the experiments were not performed. Small amounts \((0±5 \text{ Å})\) is written in the table as ±.

| Summary of results |
|-------------------|-----------------|-----------------|-----------------|--------------|------------------|
| ProtMat2.0         | Serum           | Plasma          | Anti-C3c        | Anti-C3d      | Anti-HMWK       | Cells            |
| Titanium           | +               | +               | +               | +            | +                | +                |
| Fib. mono          | +               | ±               | -               | ±            | ±                | ±                |
| Fib. multi         | +               | ±               | -               | ±            | ±                | +                |
| IgG mono           | +               | +               | +               | ±            | ±                | -                |
| IgG multi          | +               | ±               | +               | +            | ±                | -                |
| HSA mono           | +               | +               | ±               | +            | +                | -                |
| Adsorption (+)     | No or low adsorption (-) |
| Adsorption 0±5 Å (±) |                 |                 |                 |              |                  |                  |

Table 1: A summary of some of the results in this project. Detected binding of plasma, serum, antibodies and cells are marked with a plus (+), no detection is marked with a minus sign (-) and amounts of 0±5 Å is marked with (±).
Chapter 5

Discussion

5.1 Protein film thicknesses differs between used proteins

In this study, different protein films have been studied. Fibrinogen, immunoglobulin (IgG), and human serum albumin (HSA), were used for preparation of protein multilayers. The proteins became different in multilayer film thicknesses (figure 13), and factors that primarily affect the thickness are protein concentration, buffer composition and pH, incubation time and temperature. Variations of these factors have been shown in previous work [4, 5].

Fibrinogen from three manufacturers (Calbiochem, CSL Behring, and X) differ in concentrations of proteins and other additives, but the exact compositions of the products are not known. Differences between fibrinogen multilayers could be observed, and the differences in product composition could be a reason for that.

5.2 Adsorption of serum and anti-C3c to titanium

For adsorption of complement proteins, titanium was used as positive reference. The surfaces were incubated in native human serum for 10 or 60 min at 37°C. The amount of adsorbed serum increased during the incubation from about 47±3 Å after 10 min to about 70±4 Å after 60 min (figure 14). To detect the binding of complement protein
C3b, the surfaces were incubated in anti-C3c. Antibody binding increased from 12±2 Å after 10 min in serum, to about 39±2 Å after 60 min (figure 16).

Arvidsson et al. also demonstrated binding of anti-C3c onto titanium [32]. The surfaces were then incubated in plasma, instead of in serum. Plasma was adsorbed after 1 min and increased with time to about 60 Å after 60 min, which is comparable to the result in this study. The anti-C3c binding was higher than in the present study; about 60 Å after 60 min, but in the same range.

Wälivaara et al. detected serum on titanium surfaces after 10 and 30 min [33]. About 40 Å serum was detected on titanium after both 10 and 30 min. In the following antibody incubation, about 30 Å anti-C3 bound to surfaces after 10 min and the amount increased to about 60 Å after 30 min of incubation.

Both Arvidsson et al. and Wälivaara et al. suggest that the adsorption of C3c to titanium, which was also observed in this study, after incubation in plasma, is an indication on activation of the complement system. Wälivaara et al. suggested that the activation possibly proceeded via the alternative pathway due to the increasing amounts of anti-C3c, which was also observed in this study.

### 5.3 Adsorption of plasma and anti-HMWK to titanium

The adsorption of the coagulation protein HMWK onto titanium surfaces was studied. The multilayers were incubated in plasma for 10 or 60 min in 37°C. 26±3 Å plasma proteins adsorbed to the surfaces after 10 min and 47±22 Å after 60 min (figure 15). After the plasma incubation the multilayers were incubated in anti-HMWK. After 10 min 22±2 Å anti-HMWK bound to the surfaces, and after 60 min the amount decreased to about 7±19 Å (figure 18).

Wälivaara et al. detected about 16 Å plasma on titanium after 1 min and 10 min of incubation, and about 60 Å anti-HMWK in the following antibody incubation [33]. They suggested that titanium had high affinity for the procoagulant factor HMWK after short time exposure to heparin plasma. Arvidsson et al. also detected plasma adsorbed to titanium, about 40 Å after 1 min and up to 60 Å after 60 min [32]. In the anti-HMWK incubation, about 50 Å could be detected after 1 min and the amount increased during
the next 10-15 min, but then decreased and had disappeared after about 2 hours. The adsorption of HMWK to titanium after 1 min was not studied in the present study, but the antibody could be detected after 10 min of plasma incubation, and according to Arvidsson et al., the adsorption of HMWK after 1 and 10 min could be an indication on rapid activation of coagulation.

5.4 Binding of anti-C3c to FibMat2.0

The adsorption of complement proteins to FibMat2.0 was studied. After a 60 minutes incubation in serum, about 15±7 Å adsorbed to the surfaces (figure 14). The multilayers were then incubated in anti-C3c (figure 16) and anti-C3d (figure 17). No anti-C3c was bound to the surface after 10 min, but after 60 min it had increased up to 11±5 Å. The multilayer thickness decreased with about 17±4 Å in the anti-C3d incubation. When fibrinogen monolayers were incubated in serum, 46±6 Å was adsorbed after 60 min, and up to 15±7 Å anti-C3c was adsorbed in the following incubation.

Both Tengvall et al. and Ericsson et al. studied fibrinogen multilayers preparation with the EDC/NHS technique [1], here referred to as FibMat1.0. The multilayers were incubated in plasma for studying both adsorption of complement and coagulation proteins. Tengvall et al. detected about 46±8 Å plasma proteins onto FibMat1.0 after 60 min, to be compared to the 15 Å adsorbed to FibMat2.0 in this study. In the following incubation, no antibodies were detected on the surfaces after 5 min, and less than 10 Å was detected of either C3c or C3d even after 60 min. The fibrinogen monolayers adsorbed 5±5 Å plasma after 60 min, and then adsorbed 5±4 Å anti-C3c, which are smaller amounts than adsorbed to the multilayer in this study (46Å serum). In the study of Ericsson et al., FibMat1.0 multilayers were used as negative controls for plasma adsorption [3]. About 8 Å plasma proteins adsorbed to the multilayer, and no anti-C3c was detected on the surface.

Both Tengvall et al., and Ericsson et al., suggest that low binding of anti-C3c to FibMat1.0 indicate a low activation of the complement system was induced by the surface. Tengvall et al. also proposed that the increased amount of anti-C3c from 5 to 60 min suggested that the surface became C3b opsonized with time [1]. In this study,
adsorption of anti-C3c to FibMat2.0 also increased with incubation time. Following the suggestion of Tengvall et al., FibMat2.0 also became C3b opsonized with time, but to a lower extent, and has a low activation of the complement system.

5.5 Binding of HMWK to FibMat2.0

The adsorption of HMWK to FibMat2.0 was studied. After the plasma incubation, about 5±7 Å plasma was detected on the FibMat2.0 multilayer, and about 4±2 Å on the monolayer (figure 15). After incubation in anti-HMWK about 5±9 Å was detected on the multilayer and -4±7 Å on the monolayer (figure 18).

In the study of Tengvall’s et al., 46±8 Å plasma was adsorbed to the FibMat1.0 multilayer and after incubation in anti-HMWK the film thickness decreased with -12±7 Å [1]. Ericsson et al. also detected a low binding, about 5 Å, of anti-HMWK to FibMat1.0 after incubation in plasma [3]. Their result is similar to the results in the present study. Both Tengvall et al., and Ericsson et al., suggest that the low binding of anti-HMWK indicate a low activation of the coagulation cascade [1, 3]. According to this, the FibMat2.0 multilayer and monolayer induce a low activation of coagulation.

5.6 Binding of anti-C3c to IgG multilayer

IgG multilayers, prepared according to the ProtMat2.0, were incubated in serum for 10 and 60 min. The adsorbed amount of serum increased during the incubation to about 34±16 Å after 60 min (figure 14). In the following incubations, about 25±8 Å anti-C3c and 15±12 Å anti-C3d were adsorbed to the surface. IgG monolayer were used as a positive control, and 140±53 Å serum (figure 14) and 56±47 Å anti-C3c (figure 16) adsorbed to the monolayer.

Tengvall et al. also studied IgG multilayers with EDC/NHS coupling chemistry, here called ProtMat1.0 [1]. They observed a rapid adsorption of plasma, to about 42±3 Å, to the multilayer, and increasing amounts anti-C3c (38±5 Å) and anti-C3d (12±3 Å) were detected. To IgG monolayers, about 116±4 Å plasma, 102±4 Å anti-C3c, and 41±4 Å anti-C3d was adsorbed.
In another study, Sjöwall et al. used IgG preadsorbed to hydrophobic silicon surfaces as positive references for activation of the complement cascade [34]. Serum bound to the surfaces and anti-C3c increased during the 60 min of incubation.

Tengvall et al., have in other previous studies also demonstrated a rapid adsorption of serum to precoated surfaces with IgG [35, 36]. In both studies, the binding of anti-C3c increased rapidly during the first 5 min in the serum incubation and was then almost constant. Both Tengvall et al. and Ericsson et al. suggested that IgG coated surfaces activate the complement cascade. Tengvall et al. also suggested, due to their results from several tested antibodies to different complement factors, that IgG surfaces activated the classical pathway and with time also the alternative pathway [35, 36]. According to this, the binding of anti-C3c and anti-C3d onto IgG multilayers prepared according to ProtMat2.0, would then suggest surface induced activation of the complement system, even though the results in this study can not determine via which pathway the activation was initiated.

5.7 Binding of anti-HMWK to IgG multilayers

The adsorption of the coagulation factor HMWK onto IgG ProtMat2.0-preparation was studied. The multilayer adsorbed 6±2 Å plasma after 60 min of incubation. In the following anti-HMWK incubation, no binding of antibodies was detected (figure 15). To the IgG monolayer, 2±8 Å of anti-HMWK was bound to the surface after incubation in plasma.

When IgG (ProtMat1.0-prepared) was studied, the surfaces were first incubated in plasma (42±3 Å) and then in anti-HMWK [1]. The multilayer decreased with -9±3 Å in the anti-HMWK incubation, but 4±3 Å anti-HMWK adsorbed to the monolayer. Due to the low binding of anti-HMWK to IgG surfaces of both FibMat1.0, both multilayer and monolayer, Tengvall et al. suggested the activation of coagulation induced by the surfaces was low. The adsorbed amounts of anti-HMWK onto IgG of both ProtMat1.0 and 2.0 were similiarly low. If low binding of anti-HMWK indicate a low activation of coagulation, it could be suggested that IgG of ProtMat2.0 induce a low activation.
5.8 Binding of anti-C3c onto HSA monolayer

In this study, the adsorption of complement proteins to HSA monolayer was studied. Up to 10±3 Å serum was adsorbed after 60 min, but during the anti-C3c incubation, the film thickness decreased with about -5±2 Å. Unfortunately, HSA multilayer was not stable enough to be tested.

Tengvall et al., studied the adsorption of complement proteins to HSA multilayer of ProtMat1.0 [1]. Increasing amounts of plasma (31±4 Å), anti-C3c (37±7 Å), and anti-C3c (15±5 Å) could be detected after the respective incubation. The monolayer adsorbed higher amounts of both plasma (51±5 Å) and anti-C3c (56±9 Å) after 60 min plasma incubation, than in the present study. Tengvall et al. suggested that the increasing amounts of antibodies indicated opsonization with C3b with time.

Both the multilayer and monolayer in the study of Tengvall et al. adsorbed more plasma and antibodies than the monolayer in the present study, suggesting a higher opsonization of C3b than the monolayer in this study. The surfaces in the two studies are differently prepared. Proteins denature and change structure when adsorbed onto a solid surface [19]. The EDC/NHS prepared surfaces were possibly identified by the plasma proteins as more foreign than the HSA monolayer in the present study, and thus leading to a higher activation of the complement.

5.9 Binding of anti-HMWK onto HSA

The adsorption of coagulation factor HMWK onto HSA monolayer was studied in the present study. About 19±5 Å plasma was adsorbed to the surface, and about 57±3 Å anti-HMWK was (figure 15) adsorbed to the monolayer.

In the study of Tengvall et al., 2±2 Å anti-HMWK adsorbed onto the HSA monolayer and 5±5 Å onto the multilayer, after incubation in plasma [1]. They suggested that both the HSA monolayer and multilayer, built with EDC/NHC technique, had low activation of coagulation due to the low Binding of antibodies. Ericsson et al. used HSA multilayers (with EDC/NHS) as negative reference for surface initiated coagulation [3].
The amounts of adsorbed anti-HWMK in the present study were higher than the amounts in the study by Tengvall et al., possibly due to the different fabrication methods of the surfaces. It is possible that the HSA monolayer does not cover the whole titanium surface so that some areas of the titanium are in contact with the plasma and affect the binding of anti-HMWK.

5.10 Coagulation times induced by ProtMat2.0

In the present study, surface induced coagulation was studied. Unfortunately, the plasma was spontaneously activated before the tests and resulted in unsure coagulation times. Clotting in fresh plasma (figure 19) was induced by clean titanium surfaces and by HSA monolayer after approximately 11 min. Surfaces with FibMat2.0 and IgG of ProtMat2.0 had clotting times at about 15 min. With frozen plasma (figure 20), it was more obvious that the clotting was more rapidly induced by titanium and HSA (about 25 min) than by fibrinogen (55-70 min) and IgG (about 90 min).

The binding of anti-HMWK to the different prepared surfaces (figure 15) could be compared with the coagulation times. Both titanium and HSA monolayer bound more anti-HMWK than fibrinogen and IgG multilayers, in this study, and the coagulation times were also shorter for these surfaces. The binding of anti-HMWK and the short coagulation time for titanium follow the suggestion of both Arvidsson et al. and Wålivaara et al. that titanium is an activator of the coagulation cascade [32, 33].

In Ericsson’s study of glycerol monooleate (GMO) on top of HSA [3]. HSA of ProtMat1.0 was used as negative reference for surface initiated coagulation. The silicon activated the coagulation after 10 min, GMO after 85 min, and HSA after 60 min. The coagulation times for HSA monolayer in this study were shorter than for HSA in the study by Ericsson. One factor that could possibly affect the clotting times in this study was that the plasma was activated during the sampling procedure. Another reason could be the different surface preparations. Possibly, the HSA monolayer does not cover the whole titanium surface so that areas of the titanium are in contact with the plasma during the coagulation tests and therefor affect the clotting times.
5.11 Blood cell adhesion to FibMat2.0

In this study, the adhesion of blood cells to different prepared surfaces were studied. Blood cells bound to the reference titanium surfaces (figure 22) and to the fibrinogen multilayers (figure 24). Surface located platelets on the surfaces showed variation in behavior, they were either spread evenly over the surface or clustered together (figure 25). Fibrinogen antibodies bound mainly to the areas where no platelets had bound.

Platelets can only adhere to artificial surfaces if proteins first have adsorbed to the surface [8]. When proteins adsorb to a surface they undergo conformational changes, which potentiates the fibrinogen binding of platelet surface receptor GPIIb/IIIa. The ligand binding may support further binding and spreading of the platelets over the surface through multiple focal contacts [8]. Hansson et al. observed platelet adhesion to fibrinogen coated gold surfaces, using surface plasmon resonance (SPR) [37]. Göransson et al. studied the inflammatory response induced by titanium, with FibMat1.0 as a positive control [2]. After incubation with mononuclear cells, the number of adhered cells, the viability, etc. were measured. They suggested that fibrinogen surfaces had a stronger inflammatory response than titanium. Tang et al., also observed that fibrinogen enhanced the adhesion and activation of inflammatory cells [38].

In this study, it was evident that blood cells adhered to the titanium surfaces and to fibrinogen multilayers. The variations of the cell adhesion on titanium or fibrinogen surfaces treated in the same way could be due to one of the incubation steps.

5.12 Blood cell adhesion to IgG and HSA

In the present study the blood cell adhesion to IgG multilayer of ProtMat2.0 and to HSA monolayer (figure 23) was also studied. No cells could be found on the two surfaces.

Wetterö et al. observed that neutrophils adhered to IgG coated hydrophobic glass surfaces [39]. A correlation was found between precoated IgG, adsorption of C3, and adhesion of neutrophils. Wetterö et al. mention a hypothesis that C3 adsorb to IgG coated surfaces, with an increasing complement reaction and/or amplification of
neutrophil adhesion. In this study, no cells adhered onto the IgG multilayer, even if complement factor C3 was preadsorbed to the surface (figure 14). A reason for the differences could be the different fabrication methods of the surfaces. Proteins denature and change conformation upon adsorption onto a solid surface [19], but the proteins coupled with the EDC/NHS technique are more similar its soluble native form and could possibly give different immune reactions and different adhesion of blood cells to the surface than ProtMat2.0.

Tang et al. observed that albumin precoated surfaces recruited only a few neutrophils or macrophages, and suggested that albumin induce a low inflammatory response [38]. They also suggested that one or more elements present in plasma, but absent in serum, were responsible for proinflammatory effects of implanted materials. One of the elements could be fibrinogen, which also could be a primary component of phagocyte recruitment. In the present study, HSA monolayers were incubated in whole blood, which means that fibrinogen molecules were present, but still no cells adhered to the surfaces. The results support the suggestion that albumin induce a low inflammatory response even if soluble fibrinogen is present.

5.13 Different stability for different protein multilayers

This chapter discusses the stability tests of the multilayers of ProtMat2.0. HSA multilayer appeared to be instable, and IgG and fibrinogen multilayers were more stable than HSA. Fibrinogen was also stability tested for plasmin, thrombin, and against shear stress.

5.13.1 ProtMat2.0 stability in buffers

The stability of ProtMat2.0 procedure was investigated in this study. The HSA multilayer dissolved rapidly during incubations in serum (figure 14), VB++, and HBS buffer (figure 28). Both fibrinogen and IgG multilayers changed little or nothing during the incubations.
Stability tests of EDC/NHS coupled protein multilayers, in this study called ProtMat1.0, had been performed in previous studies [1, 40]. In the work by Tengvall et al., a 600 Å thick multilayer showed no decrease in thickness after 10 weeks incubation in PBS-buffer at 4°C [1]. The fibrinogen multilayer was then incubated in a trypsin solution overnight, and the film thickness decreased with 17 %. No change was observed when trypsin was exchanged for tPA, to form plasmin in heparin plasma. In another study by Gratzer et al. showed that EDC treated collagen surfaces appeared to be stable against degradation enzymes [40]. When the stability of type II collagen-chondroitin sulfate (CS) with variable concentrations of EDC/NHS was tested, the surfaces showed improved mechanical stability with increasing EDC concentration [41].

ProtMat2.0 multilayers were tested in an earlier study for the stability in buffers [3]. ProtMat2.0 incubated in deionized water with added NaCl at 37°C for one hour, one day and one week. Fibrinogen multilayer did not decrease in thickness, but IgG multilayers decreased with 3 % after one week. HSA multilayer decreased rapidly and decreased from about 425 Å to 125 Å after one week. After incubation in PBS with added NaCl, HSA multilayer decreased significantly, fibrinogen decreased with 14 %, but no change was observed for IgG. These incubation tests support the indications, in this study, that fibrinogen and IgG multilayer of ProtMat2.0 are relatively stable in buffer incubations, but the HSA multilayer is not.

### 5.13.2 Plasmin digest FibMat2.0

FibMat2.0 incubated in citrate plasma with added thrombin and tPA (figure 30). Thrombin catalyzes the formation of stable fibrin. tPA convert plasminogen to plasmin, that normally digest stable fibrin to fibrin degradation products, which occurs after the clot formation. The multilayer thickness decreased from about 300 Å to about 40±7 Å after the incubation. After incubation in thrombin only, no change in the multilayer thickness was detected with null ellipsometry (figure 31).

Tengvall et al. studied the stability of fibrinogen multilayer (FibMat1.0) [1]. When the multilayer incubated in heparin plasma and tPA to form plasmin, no change in the
film thickness was observed. Compared to FibMat1.0, the version 2.0 is less stable in plasmin. The effect of thrombin on FibMat1.0 was not studied.

Normally, thrombin cleaves fibrinogen at the end of the coagulation cascade and the fibrinopeptides A and B parts become released (figure 2B). The globular end of two fibrin monomers can bind to the domain where the fibrinopeptide A and B were placed, and the monomers can spontaneously polymerize to form stable fibrin (figure 2C) [9]. If changes in the fibrinogen multilayer had been detected, that could be an indication of cleavage of the fibrinogen molecules by thrombin. The results from thrombin and the plasmin incubation suggest that thrombin did not affect the fibrinogen multilayer during the clot formation, but plasmin digested the multilayer during the fibrinolysis.

5.13.3 Mechanical stability against applied shear stress

In this study, FibMat2.0 was tested against an applied shear stress with the cone-and-plate setup, and with PBS as test liquid. The multilayer thicknesses decreased then with less than 50 Å (figure 32).

Ericsson et al. also tested surfaces for stability against shear stress with the cone-and-plate [3]. HSA of ProtMat1.0, were used as reference surfaces. The rotational speed of the cone was 1000 or 1500 rpm, which corresponds to approximately shear rates of 1000 and 1500 s⁻¹, respectively. The multilayer of HSA showed no difference in thickness before and after shearing with PBS. Comparing this with the present study, FibMat2.0 appears to be less stable than HSA of ProtMat1.0, possibly due to different fabrication processes.
Chapter 6

Conclusions

Adsorption of complement factors and coagulation factors onto multilayers and monolayers prepared according to the FibMat2.0 technique were studied. Low amounts of the complement protein C3b adsorbed to fibrinogen multilayers (FibMat2.0) and to HSA monolayer, and larger amounts to IgG multilayer (ProtMat2.0-prepared). The coagulation factor HMWK adsorbed to HSA, but only low amounts were detected on the fibrinogen and IgG multilayer.

The suggestion that titanium rapidly activate the intrinsic pathway of coagulation was supported when in situ imaging of coagulation was used. HSA monolayer surfaces also appeared to rapidly induce coagulation. The coagulation times of fibrinogen multilayers were longer than for titanium and HSA, but shorter than for IgG. This suggests that IgG multilayer (ProtMat2.0-prepared) has a lower activation of the intrinsic pathway of coagulation compared to FibMat2.0 and HSA monolayer.

Preadsorbed fibrinogen on a surface is suggested to activate and recruit blood cells. Platelets and a few white blood cells bound to titanium surfaces and fibrinogen multilayers (FibMat2.0), but not onto IgG multilayer (ProtMat2.0) or HSA monolayers.

A conclusion in this study is that the surface of an implant can be coated with FibMat2.0 without any risks, but more studies are needed to better understand the interactions between the surfaces prepared in the present study and the immune and the haemostatic systems of the human body.
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