

# Attenuation of Acute Inflammatory Responses by Surface Nanotopography

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# **Attenuation of acute inflammatory responses by surface nanotopography**

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”Never be limited by other peoples limited imaginations”

-Mae Jemison



## **Abstract**

The interaction between biology and non-viable surfaces is crucial for many organisms and cells. For example, bacterial cells need to adhere to mineral surfaces in the soil, plants climb and adhere to walls and marine organisms produce adhesives to cling to underwater rocks etc. In the human body, tissue needs to firmly adhere to the mineral surface of bone, but also to foreign materials when for example a biomaterial is implanted. The knowledge of how biology interacts with surfaces is hence important and interesting in many aspects.

Within seconds after implantation of a biomaterial, proteins from the immune complement and coagulation systems adsorb to the surface with possible adverse consequences for the patient. To overcome this, chemical surface modifications are readily employed. However, recently the significance of surface nanotopography for the adsorption of proteins, and attachment of cells have been acknowledged.

To facilitate research on the interactions between biology and nanostructured substrates novel experimental surfaces with defined nanotopography and surface chemistry were developed. The surfaces are fabricated by binding gold nanoparticles to a gold surface, using a non-lithographic method and standard laboratory equipment. The surface chemistry was evaluated using XPS and ToF-SIMS. On these surfaces, the effect of surface nanotopography on the activation of the immune complement and activation of blood platelets was studied using QCM-D, SEM and fluorescence microscopy.

It was found that although nanostructured surfaces adsorbed greater amount of serum proteins, activation of the immune complement was attenuated by surface nanotopography. A suggested mechanism is that the curvature of the nanoparticles prevents interaction between complement proteins. It was also found that blood platelets were activated to a lower degree on nanostructured surfaces and were sensitive to changes in nanoparticle size and inter-particle distance. These nanostructured surfaces can hopefully facilitate research on protein/cell interactions on nanostructured surfaces.

## Populärvetenskaplig sammanfattning

Levande vävnad består till största delen av mjuka material som tex. proteiner, lipider och polysaccarider men en viktig del av allt liv är även att etablera kontakt med hårda icke levande ytor. Mikroorganismer fäster sig på mineralytor i sediment, klättrväxter kan klättra på stenar och murar, marina djur som tex blåmusslor håller sig fast på klippor i havet. Hos ryggradsdjuren är det interna skelettet en sådan yta, och där vikten av att mjukvävnad kan fästa är uppenbar. Medicinska implantat, sk. Biomaterial är ytterligare ett exempel på när vävnad möter en hård, icke levande yta.

När ett biomaterial introduceras i kroppen fäster proteiner genast till dess yta. Några av dessa proteiner tillhör immunkomplement och blodkoagulation som kan reagera negativt mot materialet i en så kallad ”främmande-kroppsreaktion”. På ett medicinskt implantat kan detta få förödande konsekvenser med förlorad funktion hos implantatet och lidande för patienten. Därför försöker man modifiera ytan på olika sätt för att minska reaktionen. Med hjälp av nanoteknik kan man fästa mycket små strukturer (några miljondelar av en millimeter) på ytan och de kan påverka både hur proteiner och celler fäster på materialet.

I min avhandling beskriver jag en metod för att fästa guld-nanopartiklar i storleksordningen 10-60nm på en slät yta. I hög förstoring med hjälp av elektronmikroskopi påminner ytorna om kullerstengator i miniatyr. Dessa ytor använde jag sedan till att studera hur immunkomplement och blodkoagulation aktiverades. Det visade sig att immunkomplementet nedreglerades på den nanostrukturerade ytan jämfört med den släta kontrollytan. En av förklaringarna till detta kan vara att kurvaturen hos nanopartiklarna rent geometriskt hindrar proteiner från immunkomplementet att samverka.

Även celler från blodkoagulationen aktiverades i lägre grad på den nanostrukturerade ytan. Detta antas bero på att cellen ligger ovanpå partiklarna och därför har få kontaktpunkter med ytan och kan därför inte binda hårt till ytan.

Utvecklingen av de nanostrukturerade ytorna och de biologiska experimenten i denna avhandling kan förhoppningsvis leda till bättre förståelse om hur nanostrukturerade ytor samverkar med biologiska system.

## List of papers included in this thesis

This thesis is based on the following papers, referred to in the text by the roman numerals (I-IV):

- I**                    Mats Hulander, Jaan Hong, Marcus Andersson, Frida Gervén, Mattias Ohrlander, Pentti Tengvall and Hans Elwing  
*Blood interactions with noble metals: Coagulation and immune complement activation*  
Applied material & interfaces (1) **2009**, 1053-1062
- II**                    Mats Hulander, Anders Lundgren, Mattias Berglin, Mattias Ohrlander, Jukka Lausmaa and Hans Elwing  
*Immune complement activation is attenuated by surface nanotopography*  
International journal of nanomedicine (6) **2011**, 2653-2666
- III**                    Anders Lundgren, Mats Hulander, Joakim Brorsson, Malte Hermansson, Hans Elwing, Olle Andersson, Bo Liedberg and Mattias Berglin  
*Adsorption of gold nanoparticles and its application towards chemically functionalized gradient nanopatterns*  
Submitted
- IV**                    Mats Hulander, Lars Faxälv, Anders Lundgren, Mattias Berglin and Hans Elwing  
*The use of a gradient in surface nanotopography to study influence of nanoparticle size and inter-particle distance on platelet adhesion and activation*  
Submitted

## Abbreviations

C1-C9	Complement factors 1-9
DLVO	Derjaguin, Landau, Verwey and Overbeek
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESCA	See XPS
FITC	Fluorescein isothiocyanate
FLM	Fluorescent light microscopy
HMWK	High molecular weight kininogen
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MAC	Membrane attack complex
MBP	Mannan binding protein
PBS	Phosphate buffered saline
PMMA	Polymethyl metachrylate
QCM-D	Quartz crystal microbalance with dissipation monitoring
RSA	Random sequential adsorption
SAM	Self assembled monolayer
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TAT	Thrombin-anti-thrombin
TF	Tissue factor
TOF-SIMS	Time of flight secondary ion mass spectrometry
VWF	Von willebrand factor
XPS	X-ray photoelectron spectroscopy

# Table of Contents

1 Introduction.....	9
2 Blood.....	15
2.1 Blood cells.....	16
2.2 Proteins in blood.....	16
2.3 Plasma .....	18
2.4 Serum.....	18
3 Protein adsorption.....	19
3.1 Protein-surface interactions .....	20
3.2 Influence of surface chemistry .....	21
3.3 Vroman effect.....	22
3.5 Protein adsorption on nanoparticles and nanostructured surfaces.....	22
3.4 Quartz crystal microbalance with dissipation monitoring (QCM-D) ..	23
4 The immune complement system.....	27
4.1 Classical pathway .....	28
4.2 Alternative pathway.....	29
4.3 Lectin pathway .....	30
4.4 Regulation of the immune complement.....	30
4.5 Activation of the immune complement on biomaterial surfaces.....	32
4.6 Measurements of immune complement activation .....	32
4.7 Influence of surface nanotopography .....	33
5 Blood coagulation.....	35
5.1 Extrinsic pathway .....	35
5.2 Intrinsic pathway .....	36
5.3 Platelet binding activation on biomaterial surfaces.....	37
5.5 Influence of surface nanotopography .....	38

6	Gold nanoparticles .....	40
6.1	Turkevich synthesis of gold nanoparticles .....	40
6.2	Other methods of gold nanoparticle synthesis.....	41
6.3	Stability of colloidal solutions.....	41
7	Nano-functionalization of surfaces .....	44
7.1	Self-assembly of gold nanoparticles.....	44
7.2	Controlling surface chemistry .....	46
7.3	Controlling inter-particle distance.....	49
7.4	Preparation of gradients in nanotopography.....	51
8	Summary of papers .....	54
	Paper I.....	54
	Paper II .....	56
	Paper III.....	57
	Paper IV.....	58
9	Conclusions & future outlook.....	60
10	Acknowledgements .....	61
11	References.....	62

# 1 Introduction

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*What is a biomaterial? A general consensus is that a biomaterial can be described as “A nonviable material used in a medical device, intended to interact with biological systems” (Williams 1987). The term biomaterial is sometimes confused with a material of biological origin. Although some derive from biological materials, most biomaterials are synthetic.*

The wish to heal, repair, improve or replace damaged tissue or body parts with the use of extracorporeal (non-self) materials is probably as old as mankind itself. The use of biomaterials can actually be traced thousands of years; The Mayans achieved bone integration of dental “implants” made of sea shells around 600 B.C., and sutures of various materials such as linen and thin gold wires were used by Egyptians and Greeks some hundred years B.C. [1] In the mid-20<sup>th</sup> century bone fixation and orthopedic implants were the first procedures to be regularly performed with success, much in favor to the development of stainless steel. During World War II it was found that air pilots that was injured from splinters from shattered polymethyl methacrylate (PMMA) from the airplane windshield, did not show any severe immunological response to the material [1, 2]. This observation led to the first use of the polymer as a biomaterial, and PMMA is still a widely used biomaterial in e.g. intraocular lenses and bone cement [3, 4].

Modern biomaterial science has undergone tremendous progress in the last decades, much owing to new and improved techniques that have allowed implementation of both chemical and structural functionalization of the surface. A wide range of highly specialized commercial biomaterials and medical devices are available. Depending on application, material properties such as e.g. surface chemistry, softness, load acceptance, and morphology must be optimized.

A coarse classification of biomaterials based on their material properties is shown below:

- Polymeric biomaterials
- Ceramic biomaterials
- Metallic biomaterials
- Structured biomaterials
- Functionalized biomaterials

Within the above classes additional types of biomaterials are of course found, as well as combinations of the different classes. Biomaterials can e.g. be designed to release a therapeutic drug [5], self-resorb or degrade after some time [6], or by carrying specific ligands on its surface, signal to selected cells in its environment to induce a desired host response [7]. In addition, biomaterials also include micro- or nano capsules that are designed to carry pharmaceutical agents or DNA to target cells via the circulatory system in for example cancer treatment [8-10].

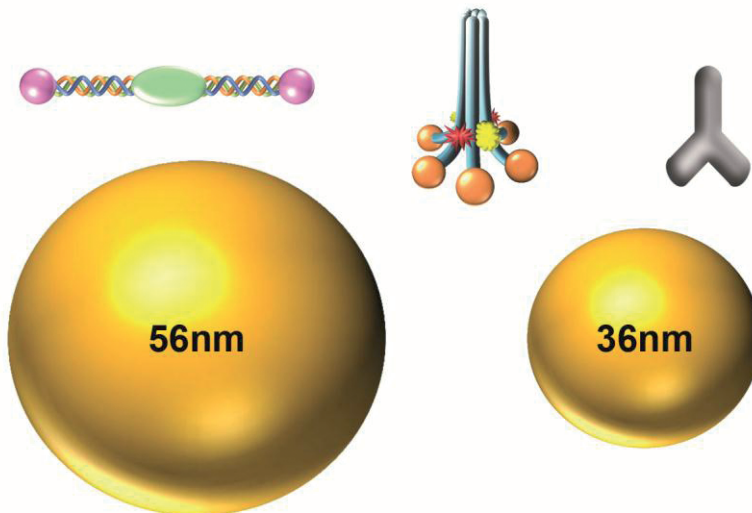
Unfortunately, introduction of a foreign material into the body is not free from complications. Any material that comes in contact with the physiological milieu of the human body will immediately trigger the innate immune system and subsequently a response from inflammatory cells [11-14]. In this thesis I have studied activation of the *Immune complement system* and activation of the *blood coagulation cascade*. These two cascade reactions occur in blood seconds to minutes after a biomaterial comes in contact with its host and are known to play a key role for the success of a biomaterial in terms of acceptance and integration [11, 15].

From a material science point of view, the main challenge is naturally to enhance and improve mechanical properties of a biomaterial such as material strength, corrosion resistance, wear resistance etc. On the other hand, a well-designed and mechanically perfected biomaterial is of little use if the biological response from the host to the material is not appropriate. Thus, biomaterials science must span a broad range of disciplines including material science, analytical sciences, molecular biology and medicine.



During the last decade surface modifications in the nano-size regime has received substantial amount of attention in biomaterial research. A number of recent findings indicate that surface bound nano-sized features in the size below 100nm influence a variety of cell types on polymeric, ceramic and metallic surfaces [16-23]. The underlying reasons for this are still much unknown, but may lay in the fact that surface features in this size range structurally mimics the cells natural environment in the extra cellular matrix (ECM), or that proteins that creates the link between the surface and the cells, alter their three-dimensional structure upon adsorption on nanostructures [24-26]. Although the active use of nanotechnology in the field of biomaterials is still in its infancy, it is a fast growing field and it is expected that the use of nano-materials will change the concept of biomaterials in the near future [10, 27, 28].

In this thesis, gold nanoparticles in the same size regime as three important proteins in the immune complement and blood cascade reactions were used to study how surface nanotopography affects these events. A size comparison of the particles and the proteins are given in **fig 1.1**.



**Figure 1.1** Size comparisons of three proteins, each with essential influence on early response to biomaterials, with gold nanoparticles of two different sizes used in this thesis. From left: fibrinogen, complement factor 1 (C1) and immunoglobulin G (IgG).

The study of interactions between nanostructures and biological events is far from straightforward. One reason for the difficulties is associated with the fabrication of nanostructured materials which often require clean room facilities and expensive high vacuum techniques.

In addition, due to the complex nature of biological processes around biomaterials (of which many are still unknown) formulating hypotheses to predict a response is difficult. As an example, Titanium (or rather its oxide), often regarded as a keystone in biomaterial development, was shown in the early 1980's to have excellent properties for bone tissue integration [29], but underlying mechanisms of its successful biocompatibility are still not fully understood.

By introducing nanotopography on a surface, the level of complexity will further increase. The two main goals of this thesis were therefore;

- I. To develop well defined nanostructured platforms where interfacial biological events can be studied with a parametrical approach
- II. To study the impact of surface nanotopography on acute inflammatory responses

One of the prerequisites for this was that the nanostructured surfaces should be affordable and convenient to fabricate in a standard laboratory environment.

Early in the planning of the research in this thesis, the idea of fabricating model surfaces to facilitate the study of specific contributions from e.g. surface chemistry or nanotopography was launched; in **paper I** in this thesis, I performed a study of blood compatible properties of a commercial nanostructured biomaterial coating using a model for studying whole blood interactions with surfaces [30]. By deposition of the coating on medical devices the incidence of nosocomial (hospital acquired) infections can be significantly reduced [31-34].

The coating is comprised of randomly distributed nanometer sized deposits of gold, silver and palladium. Hence, the chemistry of three metals, the chemistry of one metal in particular, surface nanotopography, or a combination of all of the above could be responsible for any result seen in the study. To overcome this, smooth model surfaces was prepared by sputtering the individual metals on

standard microscope glass slips and compared with the nanostructured coating. From the results in **paper I** we concluded a contributing effect from surface nanotopography in attenuating immune complement and coagulation reactions.

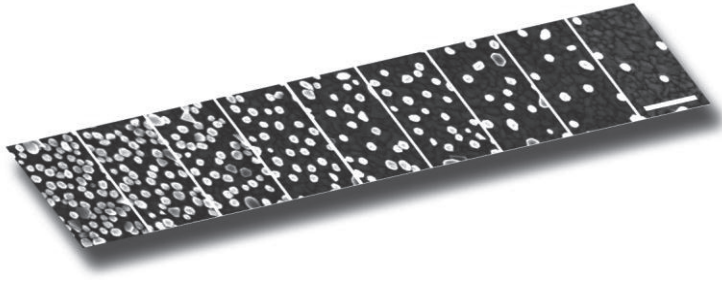
To further investigate the influence of surface nanotopography, the need for a nanostructured model surface was evident, and in **paper II** a nanostructured surface was developed by immobilizing gold nanoparticles on a smooth substrate of gold (described in chapter 7). Due to the relatively convenient method of particle fabrication, along with the possibility of producing “large” (20-100nm) nanoparticles, gold was the metal of choice. Gold is considered an inert metal that unwillingly partake in chemical reactions. One of the exceptions is with sulfur, and thiolated (sulfur containing) molecules can therefore be used to functionalize the gold particles and the surface. By binding e.g. hydrophobic molecules to the surface, influence of surface nanotopography versus surface hydrophobicity can easily be studied.

In **paper II** surfaces with and without particles, and with different hydrophobicity, were compared by means of their ability to activate the immune complement system. To make sure that any result in the study was a result of changes in surface nanotopography and not in surface chemistry, much effort was put in developing surfaces that differed only in topography, but not in chemistry.

Recently it has been shown that size and spacing of nano-sized surface features can influence both protein adsorption and cellular response [20, 35-38] However, to study how variations of these two parameters influences these phenomena by varying particle sizes or spacing on a substrate, a very large amount of experimental surfaces would ultimately be required. If a third parameter was to be included, (e.g. surface hydrophobicity), the sample set would quickly grow to huge proportions. Also, with increased number of individual preparations, the larger the risk of errors between the preparations becomes.

To conveniently study variations in size, spacing and chemistry of nano-sized surface features, a protocol was developed in **Paper III** where gold nanoparticles was attached to a gold surface in a gradient fashion (described in chapter 7). This was used in **paper III** to study bacterial adhesion to a gradual change in amount of hydrophobic patterns by functionalizing the particles with

octanethiol. As this was carried out with particles  $\sim 10\text{nm}$  in size, the contribution from surface nanotopography was considered negligible.



**Figure 1.2** Collage made from multiple SEM images taken 0.5mm apart along a gradient in nanotopography, realized by binding gold nanoparticles to the surface. The total length of the gradient stretches over approximately 9mm. Scale bar is 200nm.

In **paper IV** a gradient with two different sizes of particles (36 and 56nm) was prepared (see **fig 1.2** above). These gradient surfaces were used to study the influence of size, spacing and hydrophobicity of the particles on adhesion and activation of platelets.

By introduction of the above nanostructured model surfaces, hopefully the results in this thesis can contribute to the understanding of processes involved on the nano-bio interface on biomaterials and medical devices.

## 2 Blood

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*Blood or blood products are often the first encounter a biomaterial makes with its host. Knowledge of the processes involved during this event is therefore of great value to understand in order to control adverse reactions to a biomaterial.*

The first medical perspective on blood was probably that of Hippocrates (460 B.C). In his concept of Humors, blood was one of the four bodily fluids blood, phlegm, yellow bile and black bile. According to this teaching, each of the fluids represented four temperaments that corresponded to four different human traits and personalities. Imbalance between these fluids was thought to bring illness and disease that healed only when the balance was restored [39].

Without the concepts of cell biology (cells had not yet been discovered), the view of blood as a mysterious life-sustaining liquid persisted over hundreds of years, remaining a central part of health and disease with e.g. blood-letting as a popular treatment for various conditions. The discovery of cells was not made until the late 1700 century, when Dutch fabric handler and microscopist Anton Van Leeuwenhoek for the first time described the presence and morphology of red blood cells [40]. This was the first step towards modern cell biology and a view on blood and diseases in a mechanistic way.

More than 2000 years after Hippocrates's attempt to understand the physiology of blood, we now know that blood is composed of a complex aqueous mixture of many different cell types, proteins, lipids, carbohydrates and electrolytes. Below a short overview of the constituents of blood is given, highlighting components used to study blood compatibility in this thesis.

## 2.1 Blood cells

Red blood cells (erythrocytes) are by far the most abundant cell type in human blood. Their primary task is to transport oxygen to the cells, and carry back CO<sub>2</sub> from the cells to the lungs. Erythrocytes account for around 45% of the total blood volume and nearly a fourth of all cells in the body. They lack cell nucleus and many organelles in favor of high abundance of the oxygen carrying protein hemoglobin.

White blood cells (leukocytes) are divided in granulocytes and agranulocytes (lymphocytes and monocytes) and constitute less than 1% of the blood in healthy adults. The primary function of leukocytes is to protect tissue from infection and to respond to early inflammatory responses. Granulocytes carry toxic proteins and enzymes that act directly upon invading bacteria, but can also approach foreign materials such as biomaterial surfaces via chemotaxis to peptides from the immune complement. Lymphocytes are responsible for cell mediated immune response by e.g. secretion of antibodies, but also ingest and destroy bacteria and foreign objects by phagocytosis.

Platelets (thrombocytes) are small disc-shaped non-nuclear cell fragments derived from megakaryocytes. They are the smallest cells in the body except from the sperm cell. The main task for platelets is to prevent blood loss by forming a plug together with the plasma protein network fibrin. They respond to a breaking blood vessel by rapidly flattening their cell body to stop hemorrhage [41], but can also be activated by contact with biomaterial surfaces [12].

## 2.2 Proteins in blood

Recent development of protein separation and sequencing techniques has resulted in the rapid discovery of a great number of “new” proteins present in blood plasma. As of today, more than 15,000 proteins and their isoforms have been found in the human blood [42]. The biological function of many of these proteins is still unknown.

Proteins that are present in great abundance in plasma are typically those involved in metabolic, regulatory, or immune-related processes where fast transport of substances or rapid response to a change in physiology is required. In table 1, twenty of the most abundant proteins found in normal human plasma are presented. Among these, three highly important proteins involved in reactions against biomaterials are found; fibrinogen, immunoglobulin G (IgG) and complement factor 3 (C3).

**Table 1.** Normal concentration of the 20 most occurring plasma proteins. Proteins involved in reactions towards biomaterials and studied in this thesis are in bold. Values in parenthesis are mg/ml. Data from [43].

<b>Protein</b>	<b>Normal abundance (<math>\mu\text{M}</math>)</b>
Albumin	500-800 (35-52)
<b>IgG</b>	40-100 (7-16)
Apolipoprotein A-I	36-72 (1-2)
Transferrin	25-45 (2-36)
Apolipoprotein A-II	22-60 (0,2-0,6)
$\alpha_1$ -Proteinase inhibitor	18-40 (0,9-2)
$\alpha_1$ -Acid glycoprotein	12-30 (0,5-1,2)
Transthyretin	15-30 (0,2-0,4)
Haptoglobin	3-20 (0,3-2)
Hemopexin	15 (0,9)
IgA	4-24 (0,7-4)
Apolipoprotein C-III	6-20 (0,06-0,2)
$\alpha_2$ -Macroglobulin	7-17 (1,3-3)
$\alpha_2$ -HS-glycoprotein	12 (0,6)
Gc globulin	8-14 (0,4-0,7)
Apolipoprotein C-I	6-12 (0,04-0,08)
<b>Fibrinogen</b>	6-12 (2-4)
$\alpha_1$ -Antichymotrypsin	7 (0,5)
<b>Complement C3</b>	5-10 (0,9-1,8)
$\beta_2$ -Glycoprotein I	4-8 (0,2-0,3)

When studying blood interactions on biomaterial blood is usually divided into its major components.

## 2.3 Plasma

Apart from blood cells, whole blood contains a plethora of different blood proteins. To form blood plasma, a coagulation inhibitor e.g. sodium citrate, EDTA or heparin is added to prevent the blood from clotting. All blood cells are then separated from the fluid by centrifugation at ~5000 g. The remaining supernatant consists of an aqueous solution of proteins and ions and is denoted plasma.

## 2.4 Serum

Different from plasma, serum is prepared by letting the blood clot. Normally this is achieved by leaving the blood undisturbed at room temperature for around 30 minutes. During this time the abundant plasma protein fibrinogen is polymerized to fibrin to which primarily platelets adhere, forming the blood clot. The clear liquid surrounding the clot is serum, and the sample is usually further centrifuged at ~5000 g to remove the clot. The clear serum contains blood proteins (except fibrinogen), water and electrolytes.



# 3 Protein adsorption

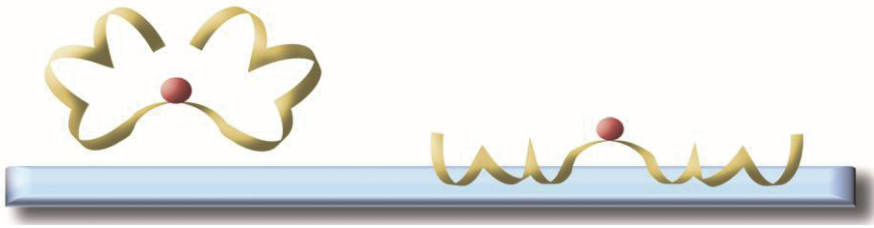
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*When a biomaterial is introduced in a physiological milieu, proteins from the blood will immediately adsorb on the surface. This early event plays a key role in determining the fate and success of a biomaterial.*

Adsorption of proteins to surfaces is a common but complex phenomenon that have implications in a variety of fields. Apart from its importance in biomaterial research and design, knowledge of protein adsorption is important in areas such as medical diagnostics, sensing, and food industry [44].

Seconds after a biomaterial comes in contact with blood or interstitial fluids, proteins will adsorb to its surface [45-49]. Proteins are small and mobile, and arrive at the surface far ahead of any cells. In fact, cells may not encounter the material at all, but instead senses the thin film of proteins covering the surface of the material [50-52].

Adsorption of a protein to a surface is always associated with changes in the proteins three dimensional structure and upon adsorption, specific domains or epitopes that are otherwise obscure in the center of the protein can therefore be exposed (see **fig 3.1**). These epitopes can function as docking sites for cells that recognizes the site and bind through specific receptors present on the cell surface [47, 48, 53, 54]. The initial adsorption of proteins on the surface of a biomaterial thus creates an important link between material and biology, and host response to a biomaterial therefore depends not only on the material itself, but also on *which* and *how* proteins are adsorbed on the surface [50, 55].



**Figure 3.1** Illustration of how surface induced conformational changes to a protein can expose epitopes normally hidden inside the native protein.

### 3.1 Protein-surface interactions

Protein adsorption is a non-specific process strongly influenced by physicochemical properties of the surface. Interactions between proteins and the surface involve Van der Waals forces, hydrophobic and electrostatic interactions and hydrogen bonding [47, 56, 57]. The complexity in interpreting protein adsorption further increases when adding the influence of e.g. protein concentration, type of protein, and time to the above. The driving force for protein adsorption is to lower the energy in the system between the protein and its surrounding (decreasing Gibb's free energy). This can be achieved by e.g. expelling water associated with the protein, structural changes to the protein that leads to a decrease in energy by a gain in entropy, or by bond formation between the protein and the surface (e.g. electrostatic and Van der Waals forces).

Protein solution concentration influences both the amount of adsorbed protein and the amount of structural change of the protein. When protein concentration is low, mass transfer from the solution to the surface is slow and individual proteins can spend longer time for spreading and adapting the lowest energy conformation possible without competing for surface area. When concentration of the protein solution is high, competition for available spots on the surface leaves no time for the individual protein to adjust or flex and the result is instead high surface density of proteins with low binding strength and less change of the proteins conformation [58, 59]. Generally, small and globular proteins exhibit lower deformation and therefore lower adhesion upon

adsorption when compared to intermediate or large size proteins. Adsorption of proteins takes place in a step-wise manner, where loose binding between the surface and the protein first occurs, followed by deformation and more firm binding to the surface. [56, 60]. Recently it has also been suggested that substrate softness plays a role in determining the level of protein deformation upon adsorption [61].

## 3.2 Influence of surface chemistry

Surface hydrophobicity is a major determinant of protein adsorption on biomaterials and has shown to influence biological processes such as blood coagulation and complement activation [62, 63]. The general observation is that proteins adsorb to a larger amount and are more strongly bound on hydrophobic than on hydrophilic surfaces [44, 56, 64]. Most proteins in blood are hydrophilic on their outside while their hydrophobic domains are turned inwards and remain obscure in the center of the protein [65]. Proteins that adsorb on a hydrophobic surface therefore also tend to undergo greater conformational and structural changes [66, 67]. By a surface induced change in conformation, epitopes that are normally hidden in the native state of the protein can be exposed (see **fig 3.1**) and bind to surface receptors on cells. This is for example known for the adhesion of platelets to adsorbed fibrinogen [68, 69].

Protein adsorption to a surface can be viewed as the ability for a protein to remove water from the surface (or for water to expel the protein). Clearly this would require more energy on a hydrophilic than on a hydrophobic surface [70]. On a surface with very low contact angle, hydrogen bonding between the surface and water is strong enough to prevent proteins from breaking the bond and adsorb to the surface, and recently it has been suggested that on surfaces with a contact angle less than  $65^\circ$  protein concentration on the surface will not increase that of the surrounding bulk solution [59].

Intriguingly, in AFM measurements of protein adhesion, the adhesion strength for a number of proteins has been shown to change in a transition point at around  $60-65^\circ$  with little difference in adhesion force within the hydrophilic (low adhesion strength) region below, or the hydrophobic (high adhesion strength) region above this breaking point [56, 60].

### 3.3 Vroman effect

In complex protein solutions where a multitude of proteins are present like e.g. blood or plasma an exchange of proteins occur over time; in 1969 scientist Leo Vroman discovered that antibodies directed towards specific proteins in blood plasma failed to bind sometime after adsorption of the plasma to a surface [71]. From this the conclusion was drawn that certain proteins are replaced over time by others, with higher affinity for the surface, a phenomenon today denoted “Vroman effect”.

The explanation for the effect is that smaller proteins, like e.g. albumin, are more mobile than larger proteins and therefore reaches the surfaces more quickly, and later becomes exchanged by larger proteins with higher affinity for the surface [46, 50]. Certain proteins, like e.g. fibrinogen, IgG, and albumin are readily displaced by other plasma proteins in a Vroman-like process [72, 73], but recent research suggests that the abundance of proteins in the bulk solution is also a crucial determinant of the amount of a certain protein on the surface [59, 73].

### 3.5 Protein adsorption on nanoparticles and nanostructured surfaces

Protein adsorption on nanoparticles and on nanostructured surfaces have been the target for increased research during the last decade, with sometimes contradictive results. Due to the fact that many proteins are in the same size-regime as nanoparticles geometrical as well as physicochemical factors are possibly involved in the interaction process between proteins and nanostructures.

When nanoparticles are subjected to plasma or serum, a corona of adsorbed proteins, develops around the particles. The protein species in the corona largely defines the biological identity of the particles [74, 75]. The proteins compete for the surface and selective adsorption of certain proteins has been reported to depend on both size and chemistry of the particles [76-78]. The thickness of the corona and the binding strength between particle and protein has been found to decrease with decreasing particle size [79]. Interactions with proteins to

nanoparticles have also been shown to stabilize the proteins conformational state [80], but the opposite have also been shown [81].

On nanostructured surfaces, the adsorbed amount have been found to both increase [82] and decrease [36], and currently no consensus exists on the topic [83]. The amount and function of the adsorbed protein and enzymes have been shown to be dependent not only on size, but also on the morphology of the particle, indicating that both the relative size protein/nanostructure and orientation of the adsorbed proteins may play an important role in determining amount and function of the adsorbed proteins [26, 84, 85].

The function of a protein is largely dependent on its conformation and orientation, and altered conformation and orientation of the adsorbed protein, with possible consequences for physiological reactions have been reported [25, 86, 87]. It is also suggested that on nanostructured surfaces, the amount of protein is determined by surface chemistry, whereas supra-molecular structure depends on the topography of the surface [88]. Bridging of proteins (fibrinogen) between nano-sized surface features have also been reported, with possible implications for platelet binding and activation [89].

### 3.4 Quartz crystal microbalance with dissipation monitoring (QCM-D)

In this thesis, protein adsorption was measured using Quartz crystal microbalance with dissipation monitoring (QCM-D).

The QCM-D technique is an acoustic gravimetric method capable of measuring mass in the range of nanograms/cm<sup>2</sup>. It is based on piezo-electric properties of the quartz crystal [90]. When an alternating current corresponding to the thickness of the crystal is applied the crystal starts to oscillate with its resonance frequency. In the setup used in this thesis a 5MHz sensor crystal was used.

The sensor crystal is covered with a thin layer of gold (~150nm) and can easily be functionalized with other chemistries making it a versatile sensor. Mass adsorbed onto the sensor results in a decrease in the resonance frequency ( $\Delta f$ ).

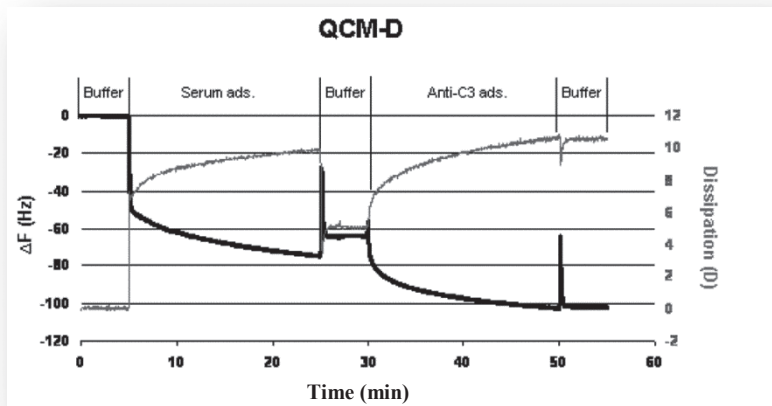
The decrease in frequency is proportional to the adsorbed mass which can be calculated by the Sauerbrey equation:

$$\Delta M = -C \frac{\Delta f}{n}$$

Where  $M$  is the mass in  $\text{ng}/\text{cm}^2$ ,  $C$  is the mass sensitivity constant ( $17,7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ , for a 5 MHz sensor crystal), and  $n$  the overtone number 1, 3, ...  $n$ . The Sauerbrey equation can be used to calculate the mass of adsorbed homogenous and rigid thin layers. If  $\Delta F$  values, when normalized against their respective overtone show large differences the Sauerbrey equation is not valid.

The analytical depth (i.e. how far from the surface mass can be detected) is  $\sim 250\text{nm}$  at the resonance frequency, and decreases with increasing overtone number. The thickness of the adsorbed layer can thus affect the measurement and the Sauerbrey equation is only valid within this region. For thicker layers or large particles (e.g. cells) additional modeling is required.

One of the advantages of QCM-D is that the QCM-D registers water associated within the adsorbing film. Thus, in addition to mass, structural information of e.g. an adsorbed protein film can be retrieved by measuring the dissipation ( $D$ ) of energy in the film (see example in **fig 3.2**). This is in contrast to other surface sensitive methods such as e.g. surface plasmon resonance (SPR), where the “dry” mass of the adsorbed protein is measured. By switching off the alternating current that drives the resonating sensor crystal, the energy lost in the film is calculated from the decay of the oscillation signal and the viscosity of the adsorbed film can be obtained [91]. By combining QCM-D and e.g. SPR, the “real” mass (SPR) along with structural information of the adsorbing protein (QCM-D) can be retrieved [61].



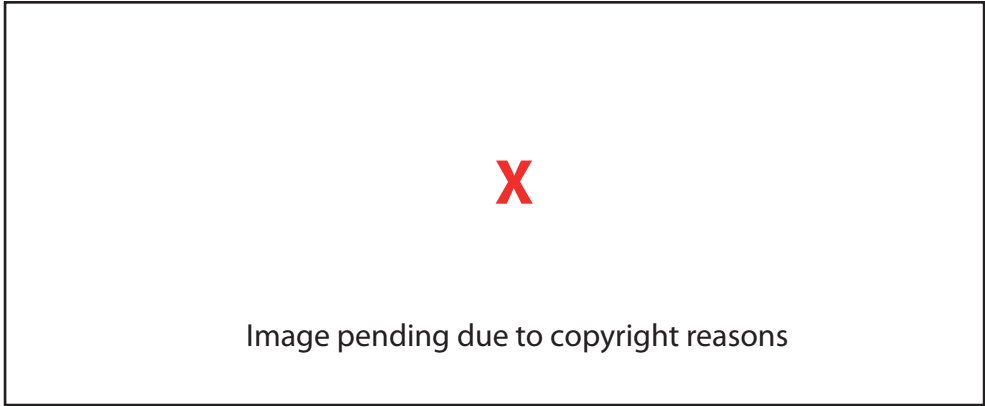
**Figure 3.2** Example of a QCM-D measurement of immune complement; After 5 min baseline check serum is injected and cause a negative frequency shift ( $\Delta F$ ) as proteins adsorb to the surface. Simultaneously, the dissipation (grey curve) increases as the viscosity of the surface bound protein layer increases with increased mass. Loosely bound proteins are washed away and antibodies towards C3 are subsequently injected and add more mass to the sensor as they bind to their target (decreased F).

The QCM-D technique was used to study adsorption of human fibrinogen to model surfaces of Au, Ag, Pd, Ti and a commercial nanostructured biomaterial coating in **paper I**.

In **paper II** it was used to monitor IgG, serum adsorption and subsequent antibody adsorption to the serum layer to the nanostructured model surfaces introduced in that paper (see example in **fig 3.2**).

QCM-D was also used in **paper IV** to study the adsorption of fibrinogen to nanostructured model surfaces with varying amount of nanotopography. When normalized against the effective surface area, it was found that despite the larger surface area of the nanostructured surfaces, the amount of adsorbed fibrinogen was actually lower than on smooth control surfaces (see **table 2**).

Table 2 Fibrinogen adsorption onto different nanostructured QCM crystals. Numbers in parenthesis are SD.





## 4 The immune complement system

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*When a foreign material is introduced into the body, proteins from the immune complement system rapidly adsorb to the surface of the material and recognize it as “non-self”. This will start a series of events aiming to remove or isolate the foreign material. In everyday life, the rapid response to a splinter in your finger exemplifies this process.*

The complement system is an evolutionary ancient protein cascade system found in all multicellular animals (metazoan) that acts as a “first line of defense” towards invading microorganisms and foreign objects [92-94]. It is part of the innate immune system and is presently known to involve over 30 different proteins and cell surface receptors [95, 96]. The immune complement was discovered in the late 1900 century as a heat labile part of the already documented adaptive immune system, and was hence considered a complement to the latter [97]. Primitive animals that lack the adaptive part of the immune system rely solely on the complement for protection of their integrity and only in mammals is the complement actually a “complement” to the adaptive immune system [98].

The main function of the complement is to recognize foreign materials and label them, thereby presenting them to phagocytic cells. or, if bacteria, destroy them through cell lysis [94, 95]. Normally the proteins of the complement system circulate the blood stream in an inactivated state as proenzymes. However, upon activation from the surface of microorganisms, viruses, fungi or non-self-materials, a well-coordinated cascade event is triggered.

Key proteins of the complement assist the adaptive immune system by rapid binding to the surface of the pathogen, thereby presenting them to circulating macrophages for destruction and clearance [99]. Protein fragments that are cleaved off from the larger protein complexes during activation of the cascade can alone act as antimicrobial agents [100], or act as anaphylatoxins that stimulates histamine secretion from mast cells and recruits monocytes to the complement activating site through chemotaxis [95, 100]. Activation of the

immune complement on biomaterials can lead to both local and systemic adverse effects and recently standardized methods for evaluation of complement activation from new biomaterials has been suggested [101, 102]

Originally it was believed that the only function of the immune complement was to protect the host from infection. However, besides creating a link between the innate and adaptive immune system in mammals, complement proteins are involved in such diverse processes as the clearance of apoptotic cells, bone and skeletal development, neural stem cell guidance, angiogenesis and lipid metabolism among others [103-105].

Activation of the immune complement is triggered through three pathways overviewed in **fig. 4.1** and described below. Complement factor 3 (C3) holds a central position in the activation cascade as the three different pathways converge into the activation of this protein

## 4.1 Classical pathway

Central for activation via the classical pathway is the binding of the complement factor C1 to IgG or IgM aggregates present on a surface. Factor C1 is comprised of the serine proteases (C1r-C1s)<sub>2</sub> and the sub-complex C1q. C1q itself consists of six identical globular head groups connected by collagen-like fibrils which gives the molecule the resemblance of a bouquet of six flowers [106, 107] (fig.x). Binding of these head groups to a well conserved position in the hinge region of the FC part of IgG triggers a conformational change of the C1 molecule, releasing it from its activation inhibitor C1-Inh [97]. Association of IgG to C1q is very weak in their soluble state, but increases manifold once IgG is aggregated [94, 105]. Each head group of C1q can bind only one IgG molecule, and in order to trigger the conformational change and activate C1, the heads of the C1q molecule must simultaneously bind to several IgG molecules [97, 99]. Once C1 have been activated, the proteolytic subunit C1r cleaves complement factors C2 and C4 to form C2a, the anaphylatoxin C4a, and the enzyme complex C3 convertase (C4b2a).

The C3 convertase acts upon complement factor 3 (C3) and cleaves C3 to C3b and the soluble anaphylatoxin C3a. When C3 is cleaved, a high energy thioester

moiety is exposed on the C3b fragment that is else obscure in the native C3 protein. The thioester is highly reactive towards nucleophiles such as hydroxyl and amino groups. As these molecules are readily found on the surface of pathogens, C3b binds and thereby presents the pathogen to monocytes and neutrophils for further destruction and clearance [95].

After cleaving C3, the C3 convertase is again ready to act on new C3 molecules, thus acting as an amplification loop that continues to produce C3b and C3a. Consequently, several hundreds of C3b molecules can be found on the surface of an opsonized pathogen REF.

Through its thioester group C3b can also bind to the already formed C3 convertase to form the C5 convertase (C4b3b2a). The C5 convertase splits native C5 molecules into C5b and the powerful anaphylatoxin C5a. The reactive C5b then initiate formation of the so called MAC complex by assembly of the complement factors C5, C6, C7, C8 and C9 REF. The MAC complex is a pore forming protein complex that assembles on the surface of cells and by disruption of the cell membrane cause lysis of the cell.

## 4.2 Alternative pathway

The alternative pathway (see **fig. 1.1**) distinguishes from the other activation pathways by spontaneous activation on surfaces. In contrast to the classical or the lectin pathways no antibodies or antigens are required for its activation.

A small portion of the circulating C3 is continuously hydrolyzed or cleaved by serum proteases at a slow but constant rate. This occurs when the thioester of C3 reacts with H<sub>2</sub>O to yield C3(H<sub>2</sub>O) in the so called “tick over” process [108, 109]. In this process, C3 are conformational changed making it susceptible for reaction with factor B. Additionally, a conformational change can also be induced when C3 spontaneously adsorb to a surface, making it reactive towards factor B. The general view is that this occurs when the thioester group of C3 binds to nucleophiles on the surface to form an ester or amide bond [97]. This is however debated as it has been found that C3 can be eluted by SDS from surfaces with covalently bound nucleophiles [110].

Once factor B has reacted with the surface bound C3 it is cleaved by factor D to soluble Ba and fragment Bb. Together with the stabilizer properdin (P) they form the alternative C3 convertase C3bBb(P) that in the same manner as the classical C3 convertase cleaves C3 into C3a and C3b.

The formed alternative C3 convertase then reacts with C3b to assemble the complex C3b<sub>2</sub>Bb which is the alternative C5 convertase that assembles the pore forming MAC complex similarly to its classical pathway analogue, the classical C5 convertase C4b3b2a.

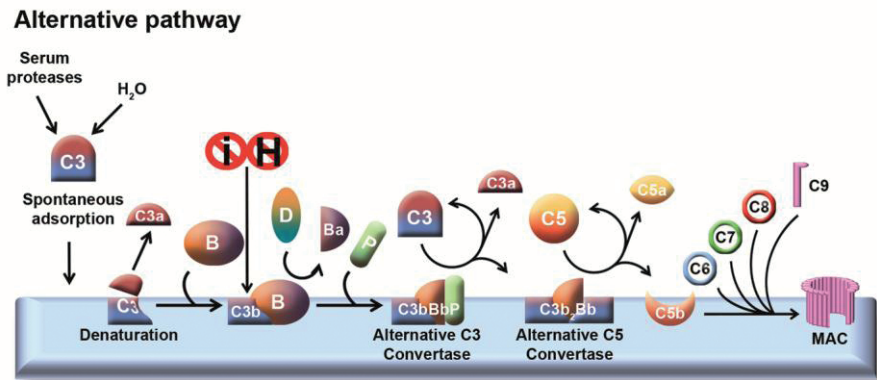
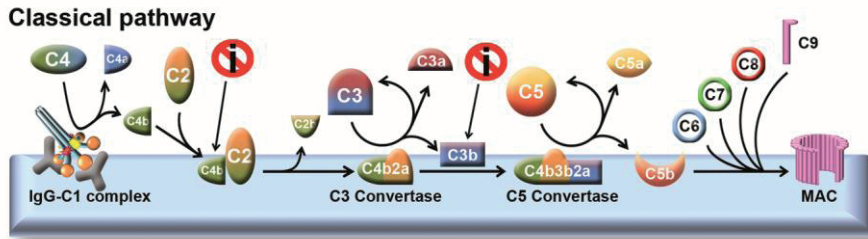
### 4.3 Lectin pathway

Activation via the lectin pathway is considered having little or no importance for immune complement activation on biomaterial surfaces, but has profound importance for the labeling and destruction of invading bacterial cells. The activation is triggered when lectin domains on the C1q structural homologue MBP (mannan binding protein) binds to oligosaccharides present on the cell walls of bacteria. MBP then acts in the same fashion as C1q by cleaving C4 and C2 to eventually form the C3 convertase and eventually assemble the MAC complex.

### 4.4 Regulation of the immune complement

To keep the cascade reactions of the complement under control, a few but important control factors are present in the blood stream to prevent the complement from reaching uncontrollable levels. Factor I acts on all activation pathways by cleaving both C4b and C3b to prevent the C3 and C5 convertases to assemble [99]. Thereby, the generation of the anaphylatoxins C3a and C5a is also inhibited.

Factor H cleaves the surface bound C3b to yield the inactive form iC3b. This inactivated form of C3b is incapable of forming a functioning C3 convertase. Thus factor H effectively inhibits complement activation via the alternative pathway [95].



**Figure 4.1** Outline of reactions in the immune complement cascade. Top image: Activation of the classical pathway is initiated by binding of factor C1 to surface bound IgG. Bottom image: The alternative pathway is activated by spontaneous adsorption or cleavage of factor C3. The alternative pathway is also shown. Inactivators factor I and H are also shown.

## 4.5 Activation of the immune complement on biomaterial surfaces

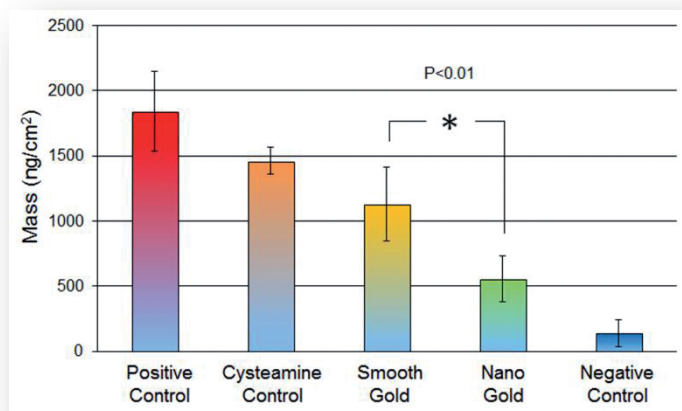
The present view on immune complement activation is that on most biomaterial surfaces, activation starts via the classical pathway e.g. through adsorbed IgG. Over time, convertases of the alternative pathway builds up and continues feeding the loops of the alternative C3 and C5 convertases, producing more C3b molecules and thereby amplifying the response [111]. Surface chemistry and hydrophobicity have been shown to affect the activation and it is suggested that this could derive from surface induced conformational change of the adsorbed complement proteins [62, 112, 113]. The elastic modulus of a substrate is also shown to affect the activation of the immune complement. Possibly by limiting the conformational disturbance of C3 when adsorbed on softer polymers [114]. Perhaps the most striking example of biomaterial induced activation of the complement system is the high systemic levels of C3a and C5a found in patients undergoing dialysis in the late 1970's. This was later found to be caused by complement activation induced by cellulose based dialysis filters [1, 115].

## 4.6 Measurements of immune complement activation

In this thesis immune complement activation was measured in three different ways; in **paper I** measurement of soluble C3a was performed by enzyme linked immune-sorbent assay (ELISA) in serum after whole blood had been exposed to the experimental surfaces. In **paper II** two methods were employed; FITC-labeled antibodies directed against surface bound C3b was visualized using fluorescence microscopy. Additionally, antibodies towards surface bound C3b were also measured with Quartz crystal microbalance with dissipation monitoring (QCM-D). This has previously been shown to correlate well with measurements of soluble C3a with ELISA [63]

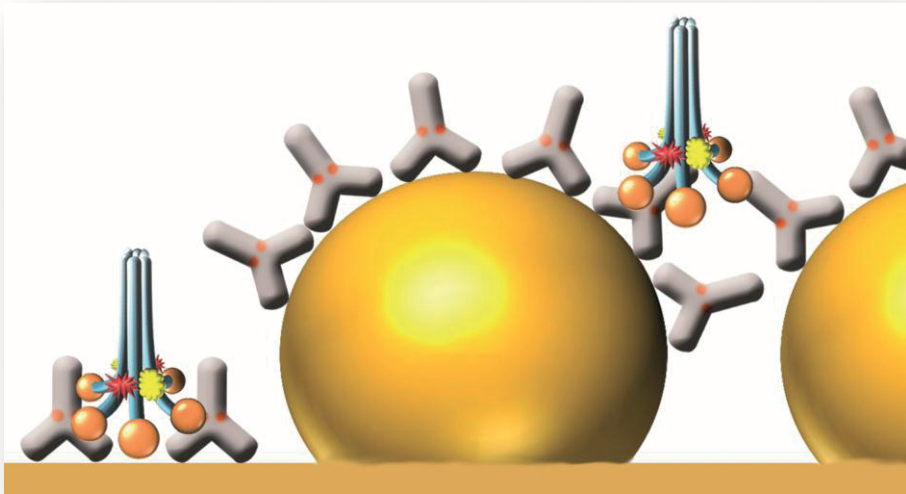
## 4.7 Influence of surface nanotopography

In **paper II** it was found that immune complement activation was attenuated by surface nanotopography (see **fig 4.2** below). This finding led to the suggestion of a novel mechanism that could explain the low activation of the immune complement on nanostructured surfaces by means of geometrical properties of the participating proteins and the curvature of the gold nanoparticles;



**Figure 4.2** Immune complement activation in human serum measured as adsorbed amount of anti-C3 antibodies. Positive control is a surface pre-adsorbed with IgG. Negative control is heat inactivated serum.

As mentioned earlier, C1 must bind to a minimum of two IgG molecules in order to activate C2. The binding occurs between the hinge region of the IgG molecule and the head groups on the C1q molecule. Hence the orientation among the participating molecules must be in concert for binding to occur [107]. If the reaction takes place on a nanostructured surface this may not be accommodated due to altered alignment of the proteins as a consequence of the curvature of the nanoparticles (see **fig 4.3**)



**Figure 4.3** Illustration of a proposed mechanism in **paper II** for the observed attenuation of immune complement activation on nanostructured surfaces. In order to activate the complement cascade, C1 (blue and orange) must simultaneously bind to a minimum of two IgG molecules (grey) on a specific location (red dots) in the hinge region between the Fc and Fab domains on IgG. This is possible on a flat surface, but obstructed on a nanostructured surface by the curvature of the nanoparticle.

In a previous study by Ferraz et.al nano-pits in aluminum substrates was shown to attenuate the immune complement activation when pits of 20nm diameter, but not 200nm was introduced. The explanation was that the limited space in the smaller pits prevented assembly of the complement convertases [35]. This may be an alternative explanation for the results in **paper II** since nano-pits are formed between the gold nanoparticles when they are immobilized on the surface.



# 5 Blood coagulation

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*Activation of the blood coagulation cascade is the body's strategy to prevent blood loss in the event of injury and thus maintain homeostasis. However, the coagulation cascade can also be activated by contact with foreign surfaces. On a blood contacting biomaterial (for example a vascular stent or prosthetic heart valves) this can of course have fatal consequences*

A majority of medical devices and biomaterials come in contact with blood at some time during their life span. Consequently, large effort has been made to develop materials that cause no or little activation of the blood coagulation cascade. Despite this, no true “blood compatible” biomaterial yet exists [116].

When a foreign material encounters blood three major events occur along a timeline;

- i. Adsorption of plasma proteins on the surface
- ii. Adhesion of platelets and recruitment of leukocytes
- iii. Formation of a fibrous capsule

Activation of the blood coagulation cascade is triggered by two pathways; the intrinsic and the extrinsic pathway, briefly summarized below and in **fig 5.1**. The end point of both pathways is the activation of thrombin and its conversion of fibrinogen to a fibrin network where platelets adhere and thus eventually form a plug to prevent hemorrhage [1].

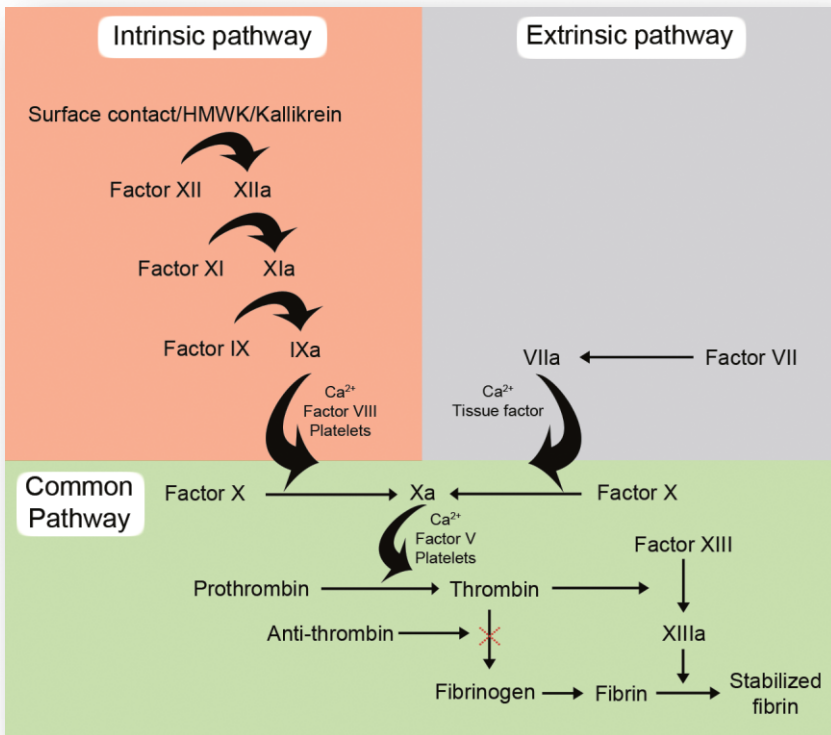
## 5.1 Extrinsic pathway

The extrinsic pathway (see **fig 5.1**) is activated by tissue damage, for example when a blood vessel ruptures as a consequence of injury. The primary activator is the protein tissue factor (TF) that is expressed on the surface of damaged sub-endothelial and leukocyte cells. Factor VII is then activated to VIIa and the

complex VIIa-TF acts upon factor X in the common pathway described below. The extrinsic pathway is probably of little importance for activation on biomaterials, but considering that a surgical procedure is involved in most insertions of biomaterials it could be a possible contribution [12].

## 5.2 Intrinsic pathway

The intrinsic (contact activation) pathway (**fig 5.1**) is activated by contact with foreign surfaces when Factor XII auto-activates and forms a complex together with HMWK and kallikrein upon adsorption to a surface.



**Figure 5.1** Outline of the two activation pathways of the blood coagulation cascade, and their shared common pathway. On biomaterial surfaces activation occurs through the intrinsic pathway. Image inspired and redrawn from ref 12.

[117]. The complex activates factor XI which in turn activates factor IX. In a calcium dependent step factor VIII acts upon factor X in the part of the pathway shared by the extrinsic and intrinsic (common pathway). Factor X is cleaved to Xa and activates prothrombin to thrombin. Thrombin then cleaves of fibrinopeptide A and B from fibrinogen, generating a fibrin monomer. Multiple monomers are then crosslinked to form a fibrin mesh by factor XIII [12]. Subsequently, platelets bind to the fibrin mesh, forming a stable clot.

### 5.3 Platelet binding and activation on biomaterial surfaces

Platelets circulate the blood in a pre-activated state. When they are activated through any of the two pathways they change shape dramatically from spherical to stellate. Their projected area thereby increases manifold, effectively forming a platelet plug at the site of damage to prevent blood loss [118]. Due to their large increase in surface coverage, activated platelets are easy to distinguish from inactivated platelets in a light microscope or SEM as seen in **fig 5.2**.

On biomaterials platelets respond to proteins adsorbed to the biomaterial surface through binding of cell surface integrins. Surface bound fibrinogen is by far the most important protein in mediating platelet adhesion and activation [119]. The integrin  $\alpha_{IIb}\beta_{IIIa}$  is present in high abundance on the surface of platelets and binds to specific epitopes on (adsorbed) fibrinogen (e.g. RGD) and other plasma proteins such as fibronectin and vitronectin. Additionally, platelets can also bind to von Willebrand factor (VWF) through its GPIb receptor that facilitates platelet binding to collagen [119-122]. Upon activation, platelets release additional fibrinogen along with adhesive and cell stimulating proteins such as thrombospondin from their  $\alpha$ -granula [123].

The nature of the adsorbed fibrinogen molecule is known to play an important role for the attachment and activation of platelets. Conformational changes to the molecule upon adsorption have been extensively studied and are linked both to the concentration of fibrinogen as well as the chemistry of the underlying surface [64, 124]. These changes in conformation have shown to affect adhesion of platelets by exposure of epitopes in the protein that are normally obscure in

the proteins native state [68, 69, 119, 125]. Additionally, crosstalk between the complement and coagulation system has recently been described that can affect coagulation and platelet activation [12, 14, 126, 127].

## 5.5 Influence of surface nanotopography

As mentioned above, adsorption of fibrinogen is considered the most important protein for platelet adhesion and activation. The influence of nano-sized surface features and how they could affect the effective amount of fibrinogen that a platelet comes in contact with was highlighted already 20 years ago, when nanostructured surfaces began to appear in biomaterial research [128]. Ligand density is an important factor that influence platelet adhesion and spreading [129].

In **paper V** it was found that platelets responded differently to a nanostructured surface depending on the size and inter-particle spacing of the surface bound gold nanoparticles; activation (spreading) of the platelet cell was attenuated to a higher degree on surfaces with 56nm particles than on surfaces with 36nm particles. Attenuation was also found to be more prominent with decreasing inter-particle distance. Surprisingly, no difference was found when surface hydrophobicity was changed, suggesting that on nanostructured surfaces, topography overrules chemistry in terms of activating platelets. In **fig 5.2** SEM micrographs of platelets seeded on a nanostructured and smooth gold surface pre-adsorbed with fibrinogen are shown.

A suggested mechanism (presented in detail in **paper V**) for the observation is that in order to bind to all fibrinogen present on a nanostructured surface (both *on* and *between* the particles) a certain flexibility of the cell membrane is required. If particles/surface features are close enough and tall enough this will not be accommodated due to the stiffness of the cell membrane. Hence, the actual ligand density that the platelet comes in contact with is therefore lower than the total amount of fibrinogen on the surface.

Similar results were found in a recent study where slightly larger surface protrusions were used [89]. Intriguingly, where the largest attenuation of

platelet adhesion and spreading was found, the ratio between height and spacing resembled the results in this thesis.

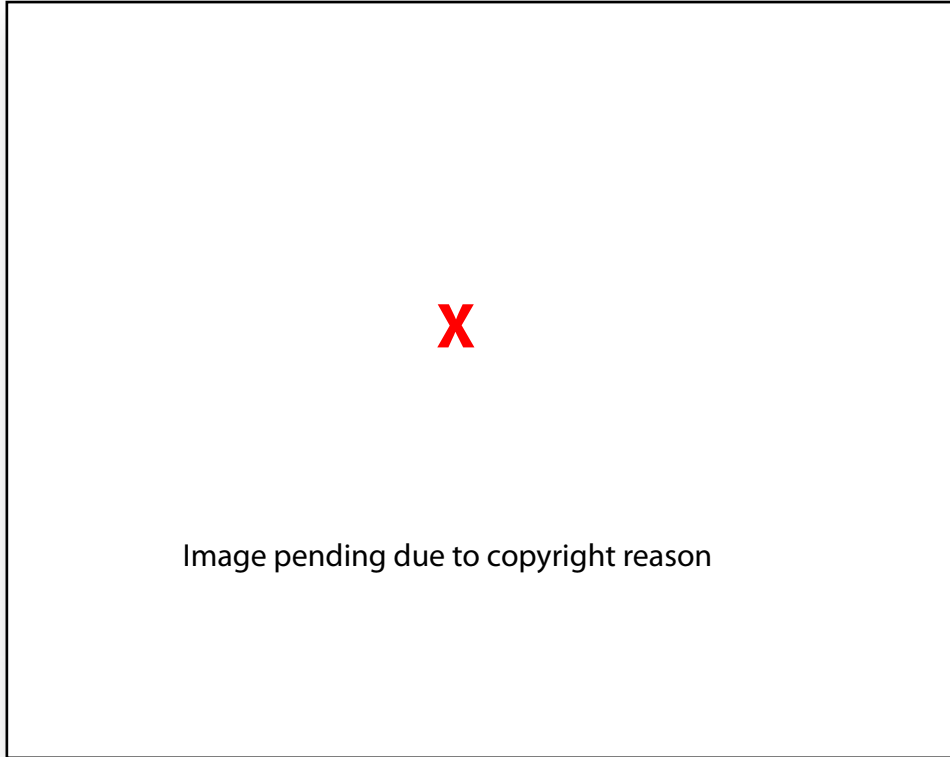


Figure 5.2 SEM micrographs of platelets adsorbed to two surfaces with same surface chemistry but different nanotopography. Non-activated cells are spheroidal in shape and lighter than the activated cells which are dark and clearly flattened. In the images to the left (a) surface nanotopography was introduced by binding gold nanoparticles to the surface, the right hand images (b) platelets are seeded on a smooth gold surface. Both surfaces were pre-adsorbed with human fibrinogen before platelet seeding. Scale bar in original images is  $20\mu\text{m}$ , in magnifications  $2\mu\text{m}$ .

# 6 Gold nanoparticles

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*In the era of nanotechnology it is tempting to think that nanoparticles are new features, realized by recent advances in the nano-workshop. However, nanoparticles are constantly produced in for example the smoke from volcanos and in rivers, where clay nanoparticles are formed by degrading minerals. Perhaps a more esthetical example is the beautiful red colors of stained glass in medieval church windows, which originates from man-made gold nanoparticles incorporated in the glass.*

Solutions of gold nanoparticles display vivid colors ranging from red to deep purple depending on their size. In contrast to larger particles, where the color is simply a reflection of specific wavelengths from incident light, colors from nanoparticles derive instead from surface plasmons. The use of gold nanoparticles have received great attention in very diverse areas during the last decades due to their chemical and optical properties; Surface plasmon resonance of gold particles are readily utilized especially in molecular and biological sensing, electronics and cosmetics [130-134]. In medicine, functional gold nanoparticles are used in e.g. thermal treatment of tumors [135].

Colloidal gold nanoparticles have an absorption maxima in the visible spectrum around 500 nm which makes them suitable for characterization with standard spectrophotometric procedures such as absorbance spectrometry and light scattering spectroscopy [136]. However, the shape of nanoparticles is also known to influence the plasmon wavelength and should be of consideration when making absorbance measurements for optical size determination [137, 138]. In this thesis, size of the gold nanoparticles was determined from multiple SEM images using image analysis.

## 6.1 Turkevich synthesis of gold nanoparticles

By far the most used process to synthesize gold nanoparticles is the so called Turkevich metod. This is also the method of synthesis for particles used in the experiments presented in this thesis. The process is based on reduction of  $\text{Au}^{3+}$  ions to  $\text{Au}^0$  using a weak reducing agent [139, 140]. Typically an aqueous

solution of sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ) is used to reduce gold ions in an aqueous solution of the gold salt  $\text{HAuCl}_4$ .

This method of synthesis yields particles up to around 100nm in diameter with size distribution of the particles increasing as the mean particle size increases. Particle size can be varied from ~20nm to 100nm by varying the ratio between  $\text{Au}^{3+}$  ions and sodium citrate. The choice of this method for the experiments in this thesis was the polymer free synthesis along with relatively simple easy control of particle size.

## 6.2 Other methods of gold nanoparticle synthesis

In the Brust-Schiffrin method, particles are synthesized in a two phase system (water-toluene) using  $\text{NaBH}_4$  as the electron donor to reduce  $\text{HAuCl}_4$  transferred from the aqueous phase by coupling to a transport agent. Through this protocol nanoparticles of small size (1-5nm) can be produced. Additionally Ag and Cu particles have also been synthesized using this method [141, 142]. The synthesis of gold nanoparticles via laser ablation has also been described. Shortly, a beam of high energy laser is fired at a thin gold foil immersed in a detergent solvent. The result is gold nanoparticles that are protected from aggregation by a layer of organic molecules at their interface [143]. This method of course has limitations in its obvious high cost. Also, for subsequent functionalization with e.g. bio-reactive ligands capping of the gold nanoparticles with organic molecules may interfere with the binding between ligand and nanoparticle. Synthesis of gold nanoparticles by certain bacteria have also been described [144].

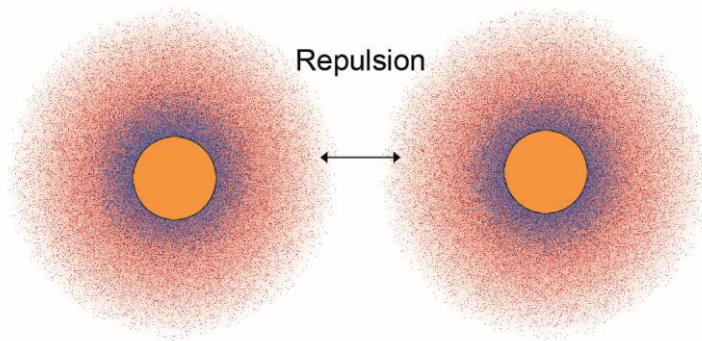
## 6.3 Stability of colloidal solutions

Colloid particles are in constant Brownian motion in their surrounding medium due to thermal energy. This would naturally cause the particles to eventually collide, possibly bind to each other and aggregate, and over time all particles would have aggregated and forced by gravity to sedimentation [145, 146].

However, two forces are working in opposition to each other to keep major contributors that keeps this from happening, the major reason to why nanoparticle solutions can be kept stable for

The driving forces for aggregation of particles are the weak, but attractive Van der Waal's forces. Van der Waal's forces are short range (in the order of a few nanometers) electrostatic forces derived from induced dipole moment of electrons and are attractive between particles in solutions, given that the Hamaker constant are similar for the two interacting materials [146].

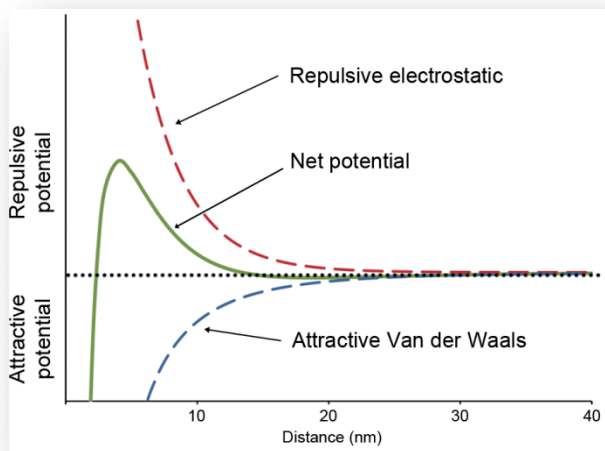
Counteracting the attractive Van der Waal's forces are the repulsive forces from the electric double layer formed by ions from the surrounding solution that accumulate around the nanoparticle. In the synthesis of gold nanoparticles through the Turkevich method, the sodium citrate used as a reducing agent in the preparation accumulate on the surface of the formed nanoparticle to give the particle a negative net charge shell of ions. Positive ions in the surrounding solution are attracted to the high charge density of negative ions in the shell and in turn accumulate outside the negative "particle", thereby changing the surface charge. The result is particles that are shielded from each other by electrostatic repulsion as illustrated in **fig 6.1** [145, 147]. Although not related, the analogy of two permanent magnets repelling each other when their magnetic poles with same polarity meet can illustrate the phenomenon.



**Figure 6.1** Illustration of the repulsive electric double layer formed from counter ions attracted to the negative surface of colloidal particles in an electrolyte.



For interactions of similar colloid particles in an electrolyte, the relation between attractive and repulsive forces acting on colloid particles can be described by the DLVO theory (Derjaguin, Landau, Verwey and Overbeek) that summarizes the forces as a function of distance between particles [146]. An example of the sum of contributing potentials is seen in **fig 6.2** below.



**Figure 6.2** Fundamentals of the components in the DLVO-theory; Interaction potentials between colloidal particles in an electrolytic medium as a function of distance between the particles.

# 7 Nano-functionalization of surfaces

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*Bottom up or top down? These two approaches are common to choose from when preparing surfaces with nano-sized features. Today a handful of techniques are available for the preparation of surfaces with nanotopography. Each method has of course its advantages and disadvantages. In this thesis gold nanoparticles were immobilized on a surface to create surface nanotopography.*

**F**abrication of nanostructured surfaces can be divided in two different approaches; either surface nanoscopic features are realized by removing material from a bulk of material (top down), or by adding material to the surface (bottom up).

For the top down approach, variations of lithographic methods are often employed. The basis of conventional lithography is to cover a substrate (usually Si) with a protective polymer (photoresist). The desired pattern is then projected on the protective polymer and cured with e.g. UV-light to crosslink the polymer to make it insoluble. Washing the surface with a solvent removes the uncured photoresist, and subsequent etching is performed to etch parts of the substrate that is not protected by the cured polymer. With this technique surface features of a couple of tens of nanometers can be produced. Drawbacks however, are high cost and time consuming procedures [148].

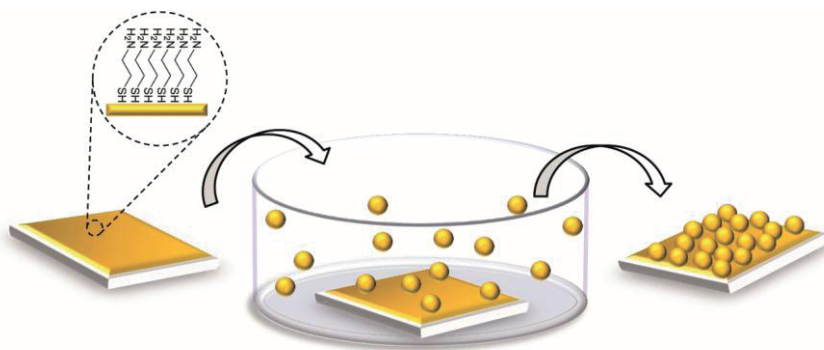
In this thesis I have used a bottom up approach by introducing gold nanoparticles on a flat surface and thereby achieving surface nanotopography.

## 7.1 Self-assembly of gold nanoparticles

In all experiments presented in this thesis, particles were synthesized by the Turkevich method in order to have full control over the surface chemistry of the particles. The immobilization of particles to a gold substrate is extremely sensitive and polymers or other residuals from the synthesis are therefor likely

to interfere with the binding process. In this thesis, surface nanotopography was introduced by binding gold nanoparticles to a supporting substrate of gold sputtered silica wafers ( $\text{SiO}_2$ ).

A convenient method used to functionalize noble metal surfaces is by formation of a self-assembled monolayer (SAM) on the surface using alkane-thiols [149-151]. In the experiments in this thesis the short amine terminated alkane-thiol cysteamine was used to functionalize the gold substrate [152]. Gold nanoparticles were then immobilized on the cysteamine layer through electrostatic binding between the net positive amine groups on the cysteamine molecule and the negatively charged gold nanoparticles from a buffered solution [133, 153, 154]. A schematic overview of the process is shown in fig 7.1.



**Figure 7.1** Schematic of the fabrication of nanostructured surfaces. A cysteamine functionalized surface is immersed in a colloidal solution of charge stabilized gold nanoparticles. The particles bind to the amine terminated cysteamine via electrostatic interaction (image redrawn with permission from Anders Lundgren).

The above method was used in **paper II** and **IV** to bind particles of 36 or 56nm in size to achieve a surface with controlled nanotopography.

## 7.2 Controlling surface chemistry

Any chemistry used to bind the particles to the gold substrate was effectively removed by a simple washing step where the surfaces are immersed in basic piranha solution (5:1:1 H<sub>2</sub>O, Ammonium hydroxide, Hydrogen peroxide) at 70 degrees Celsius for 15 minutes. During the washing process particles also become sintered into the underlying gold substrate as seen in fig 7.2, ensuring that the particles are not washed away or detach from the surface in any of the steps in the procedure.

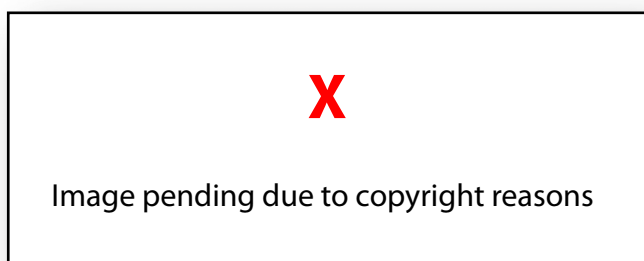


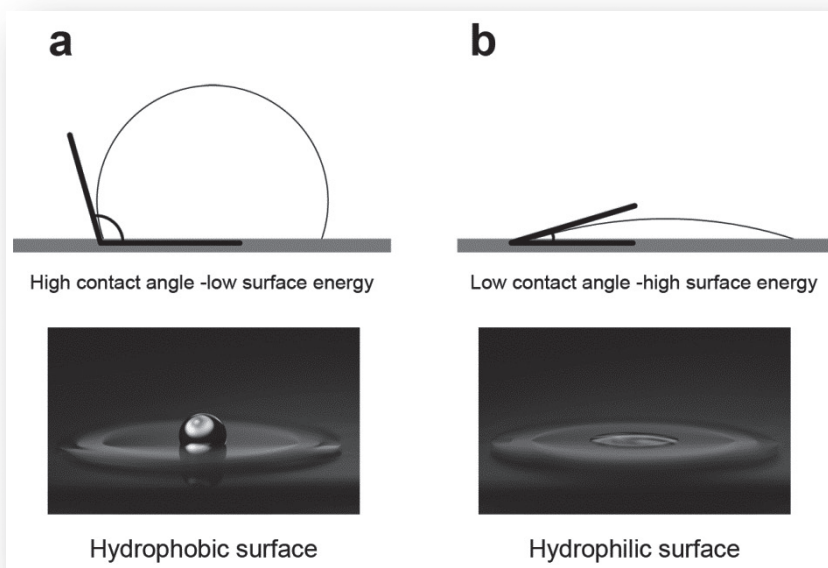
Figure 7.2 Gold nanoparticles immobilized on a smooth gold substrate using cysteamine. Left image is before washing in basic piranha and right image after washing. Note the sintering effect of the washing procedure where particles are partly melted into the gold substrate. Scale bar is 100nm.

Subsequent functionalization of the prepared nanostructured surface is easily done by alkanethiols where a vast amount of different functional groups can be introduced on the surface. In this thesis 1-propanethiol and octanethiol was used to hydrophobize the surface in paper II and paper IV respectively.

Surface energy can conveniently be characterized by measuring the water contact angle of a water droplet placed on the surface. Low energy surfaces exhibit a high contact angle (hydrophobic), and high energy surfaces exhibit a low angle (hydrophilic). The term "wettability" is often used to describe the hydrophobicity of a surface and describes how successfully a water droplet can wet the surface. Hence, hydrophilic surfaces are highly "wetable" as opposed

to hydrophobic ones. For most hard metals, the pristine surface has high surface energy, thus exhibiting a low contact angle (high wettability).

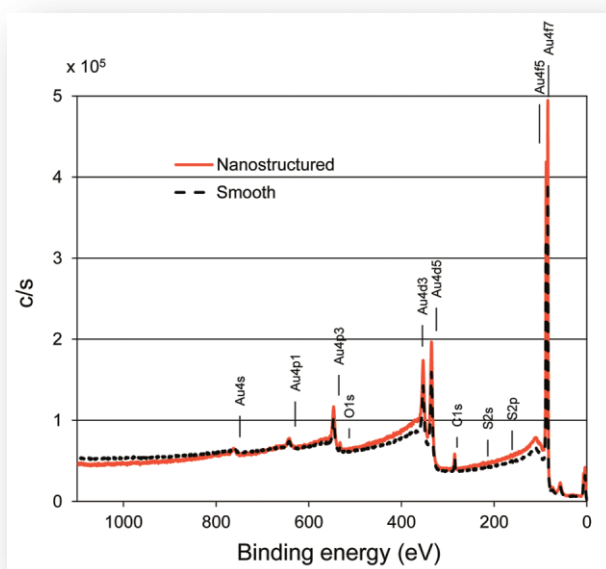
The difference can be quite dramatic as exemplified in **fig 7.3a**, where a gold surface functionalized with octanethiol exhibits a high contact angle, and **7.3b** represents a gold surface washed in basic piranha. In this thesis contact angles were measured using the sessile drop method to ensure that the washing procedure or hydrophobization of the experimental surfaces was successful. Briefly, a 5  $\mu$ l droplet of ultra-pure water (18.2 M $\Omega$ ) was placed on the surface and the contact angle was measured using image analysis of macro photographs of the droplet.



**Figure 7.3** Example of the influence of surface energy on the contact angle of a water droplet.

A comparison of surface chemistry using X-ray photoelectron spectroscopy (XPS) (also known as electron spectroscopy for chemical analysis, ESCA) was performed in **paper II** to ensure similar surface chemistries between smooth

and nanostructured surfaces after the washing procedure. XPS is a surface sensitive quantitative method used to measure the chemical composition of the uppermost few nanometers of a substrate. It is based on the photoelectrical effect described by Einstein in 1905 [155]. In short, by irradiation of the surface by x-ray photons, electrons from molecules on the surface are ejected. The energy of the emitted electrons is specific for specific molecules and can thus be used as a fingerprint to identify and quantify the chemical composition of the surface [156, 157]. In **fig 7.4** XPS measurements of the surface chemistry of a native clean gold surface and a gold surface with immobilized gold nanoparticles after the washing procedure are compared.



**Figure 7.4** Overlay of two XPS measurements of chemical surface composition where a nanostructured gold surface is compared with a pristine smooth gold surface. Essentially no difference was found between the surfaces.

Results from XPS measurements were complemented with time of flight secondary ion mass spectrometry (TOF-SIMS) measurements. The measurements confirmed that only very small differences in chemical composition between nanostructured and smooth samples could be found. In

TOF-SIMS the surface is bombarded with heavy ions from a primary ion source. This causes fragmentation of molecules in the top 1-10nm layer on the surface of the analyzed substrate. The fragments contain both positive and negative species and are detected with mass spectrometry. The flight time of the molecular fragments through the detector is dependent on the mass to charge ratio ( $m/z$ ). Smaller fragments travel faster than larger ones and the fragments can thus be sorted and depending on their ( $m/z$ ) the native molecule from which the fragment descends can thus be determined [158].

### 7.3 Controlling inter-particle distance

As described in the previous chapter, the inter-particle distance between charge stabilized nanoparticles is highly dependent the ionic strength of the colloid solution and thus screening of the particles from their electric double layer. Consequently, a particle suspended in a high ionic strength solution will have shorter distance to its neighboring particles than a particle suspended in a low or non-ionic solution.

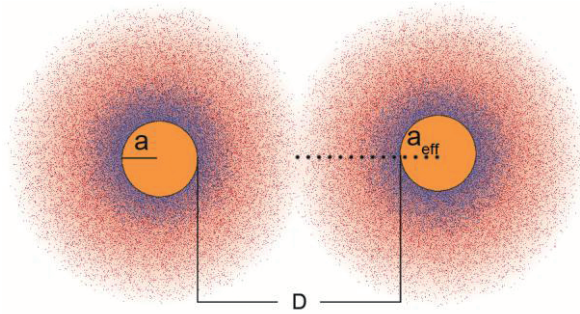
The above also applies when charge stabilized particles adsorb to a surface; If a charged particle is adsorbed to e.g. a cysteamine functionalized surface, there is a probability that, due to Brownian motion, another particle will arrive at the exact same location after some time. However, due to repulsion forces from the electric double layers surrounding the particles, the incoming particle will move out of range of the double layer force before it finally binds to the surface. This technique has previously been used to control distance between particles on a variety of substrates and particles of different sizes and material, and are central in e.g. colloidal lithography [147, 159-161].

Adsorption of charge stabilized nanoparticles from an electrolyte to a functionalized substrate can be described through the theory of random sequential adsorption (RSA). The RSA theory describes the random adhesion of hard spheres or discs to a surface. Under the condition that the particles are irreversibly attached once they reach the surface and that no particles or discs can occupy the same space or overlap each other, a maximum surface coverage of 54.7% (jamming limit) cannot be exceeded [147, 162].

The thickness of the electric double layer surrounding the particles (commonly denoted the Debye length,  $\kappa^{-1}$ ), can be regarded as a hard sphere and RSA modeling can be applied to calculate inter particle distance using the relation below:

$$\theta_{eff} = \theta_{jam}(a/a_{eff})^2$$

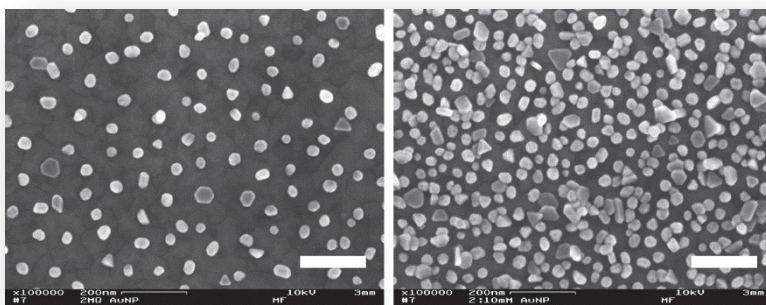
where  $\theta_{jam}=0.547$  (i.e the maximum surface coverage for hard spheres according to RSA theory),  $a$  is the particle radius and  $a_{eff} \approx a + \kappa^{-1}$  (Debye screening length) [163].



**Figure 7.5** Illustration of how the electric double layer increases the particle radius “a” to the effective radius of the Debye screening length “a<sub>eff</sub>” of a colloid in an electrolyte.

In **fig 7.6** the effect of solution ion strength on inter-particle distance is exemplified. In the left hand image gold nanoparticles are suspended in a solution of ultra-pure water (18.2M $\Omega$ ), and inter-particle distance is large due to substantial screening from the electric double layer. In the right hand image particles are instead suspended in 10mM sodium citrate buffer, resulting in a thin shell of counter ions around the particles, and hence shorter inter-particle distance.





**Figure 7.6** SEM-image of gold nanoparticles immobilized on a smooth gold substrate illustrating the significance of ion strength in controlling inter-particle distance. In left hand image particles were suspended in ultra-pure water, and in the right hand image in 10mM buffer. Scale bar is 200nm.

## 7.4 Preparation of gradients in nanotopography

To prepare surfaces with a gradual amount of bound nanoparticles, repulsion of the individual particles by their electric double layer is utilized in a procedure where diffusion of ions is utilized to change inter-particle distance gradually. The process is overviewed in **fig 7.7a** and described in detail in **paper III**. Briefly, a solution of charge stabilized nanoparticles was centrifuged to obtain a pellet. On the final gradient surface, the largest inter-particle distance is determined by the ion strength of the initial particle solution. Hence the pellet was re-suspended in ultra-pure (18.2 M $\Omega$ ) MilliQ water. Due to the low ionic strength, the particles “sense” each other through the repulsive forces from their electric double layer at a maximum distance, and are hence distributed in the solution as far as possible from each other.

The particle solution is injected into a cuvette where a cysteamine functionalized gold surface is placed. Subsequently a buffer is injected in the bottom of the container. Diffusion then progresses upwards in the container and, according to Fick's 2<sup>nd</sup> law of diffusion, a gradual decrease in ionic strength in the solution is created from bottom to top in the container.

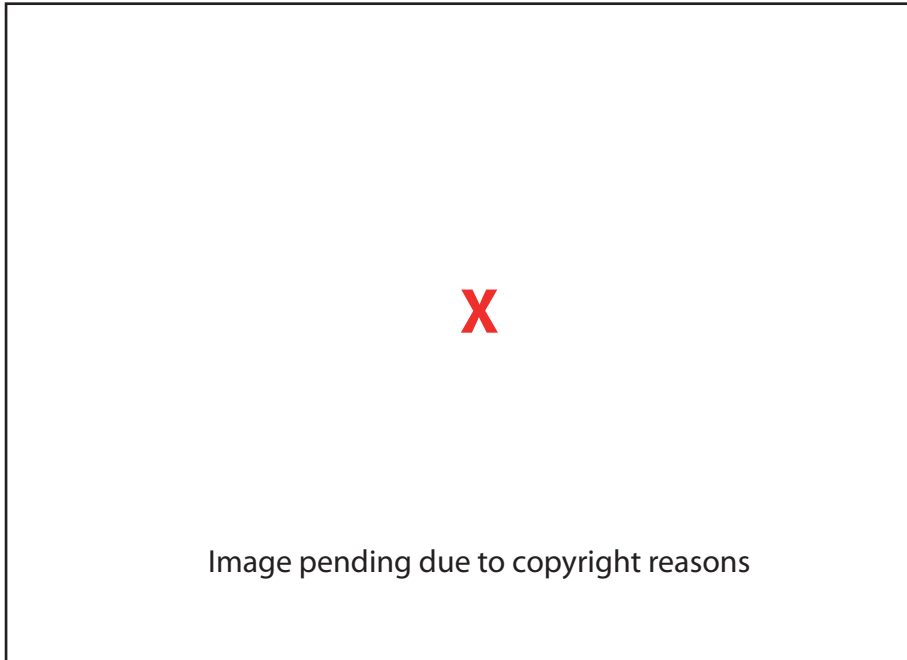


Figure 7.7 Overview of the fabrication of surfaces with gradual nanotopography. Top: A cysteamine functionalized gold surface is placed in a cuvette and gold nanoparticles suspended in ultra-pure water (18.2M $\Omega$ ) are injected. A high molar buffer is then carefully injected below the particle solution. As diffusion propagates upward in the cuvette, particles are immobilized on the surface according to the current ion strength in their surroundings. b and c the projected surface area coverage and inter-particle distance for two different particle sizes is plotted against distance along the gradient.

Consequently, as inter-particle distance is dependent on ionic strength of the colloid solution, particles will bind to the cysteamine functionalized gold surface in accordance with the prevailing ionic strength for a specific position in the diffusion gradient, thus creating a gradient in nanotopography. The gradient stretches over approximately 8mm and was evaluated using SEM by analyzing multiple images taken with 0.5mm spacing along the entire gradient. The surface coverage of gold nanoparticles and inter particle distance for two particle sizes (56 and 36nm) are shown in fig 7.7 b and c respectively.

In **paper III**, binding of gold nanoparticles of a smaller size (~10nm) was done using octane di-thiol in order to further functionalize the gold particles or/and the surface in-between the particles with specific chemistry to study the influence of surface chemical nano-patterning on bacterial adhesion.

In **paper IV** this method was used to prepare surfaces with a gradient in nanotopography with two different particle sizes to study the influence of particle size and inter-particle distance on adhesion and activation of human platelets (also described in chapter 5).

# 8 Summary of papers

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## Paper I

Mats Hulander, Jaan Hong, Marcus Andersson, Frida Gervén, Mattias Ohrlander, Pentti Tengvall and Hans Elwing

*Blood interactions with noble metals: Coagulation and immune complement activation*

Applied material & interfaces (1) **2009**, 1053-1062

## Contribution

I planned the study and carried out the experiments and participated in all blood related work with the slide chamber model together with **J.H.** I also performed all QCM-D measurements. I analyzed the data and wrote the manuscript draft.

## Summary

This study was performed in collaboration with the Swedish med-tech company Bactiguard AB. Bactiguard's surface coating consists of nano-sized deposits of gold, silver and palladium and has been shown to reduce infection on urinary and central venous catheters as well as endotracheal ventilation tubes without compromising biocompatibility [31, 32].

To evaluate the contribution from the different noble metals and the nanostructured Bactiguard coating, we prepared smooth surfaces with the respective noble metal present in the coating and compared different aspects of blood compatibility. The well characterized and well used biomaterial metal titanium was also compared as a reference. Great effort was put in ensuring that the surfaces were properly cleaned in order to make certain that any response in the experiments truly derived from the metal chemistry.

Briefly, surfaces were exposed to fresh human whole blood using the "slide chamber model" for 60 minutes and platelet consumption was measured. Levels of thrombin-anti-thrombin complex (TAT) and complement factor C3a was measured with ELISA in the supernatant after centrifugation. Adsorption of human fibrinogen was studied in real time using QCM-D.

It was found that generation of C3a was highest on Ag and low on Ti and the Bactiguard coating. Platelet depletion was very high on Ti and Au and low (<20% of blank) on Ag, Pd and the Bactiguard coating. Formation of TAT-complexes was extremely high on Au and Ti and markedly low on the Bactiguard coating. The amount of TAT did not correspond to the adsorption of fibrinogen other in the case of the Bactiguard coating. Hence the conclusion was drawn that the amount of adsorbed fibrinogen cannot be used to predict blood compatibility of noble metal surfaces. The contribution from the nanotopography for the overall low response of the Bactiguard coating could not be excluded.

## Paper II

Mats Hulander, Anders Lundgren, Mattias Berglin, Mattias Ohrlander, Jukka Lausmaa and Hans Elwing

*Immune complement activation is attenuated by surface nanotopography*  
International journal of nanomedicine (6) **2011**, 2653-2666

### Contribution

I designed the study together with **H.E** and developed the nanostructured surfaces together with **A.L**. I performed all surface fabrications and experiments except the ESCA/XPS and ToF-SIMS analysis. I analyzed the data and wrote the manuscript draft.

### Summary

The immune complement (IC) is a protein cascade system that recognizes foreign materials and bacteria when they enter the body. Adsorption of proteins from the immune complement system is one of the first events that take place on a biomaterial when introduced to the physiological milieu and activation of the cascade system can result in both local and systemic inflammatory reactions. Here we studied how surface nanotopography affected the activation of the immune complement system.

A protocol for fabrication of nanostructured surfaces was developed that allowed immobilization of gold nanoparticles to a gold substrate without changing the surface chemistry. Thus any biological observation found when comparing a pristine, smooth gold substrate with a nanostructured one was sure to derive from the difference in nanotopography and not surface chemistry.

Briefly, human pooled serum was incubated on smooth and nanostructured surfaces, and antibody binding to complement factor C3 was subsequently measured using QCM-D. Additionally, FITC-labeled antibodies were used to confirm the results with fluorescence microscopy. It was found that activation of the immune complement was attenuated by the nanostructured surface, even when provoked with pre-adsorption of IgG, a powerful activator of the IC. The attenuating effect was blunted by hydrophobization of the surfaces. A mechanism, where the curvature of the nanoparticles prevents proper alignment of the proteins involved is suggested.

## Paper III

Anders Lundgren, Mats Hulander, Joakim Brorsson, Malte Hermansson, Hans Elwing, Olle Andersson, Bo Liedberg and Mattias Berglin

*Adsorption of gold nanoparticles and its application towards chemically functionalized gradient nanopatterns*

Submitted

## Contribution

This is mainly the work of Anders Lundgren. I contributed to some of the experimental planning and performance and to some extent with the analysis of the data.

## Summary

To study interactions between biology and substrates at the molecular level, extreme precision in nano-functionalization is needed. This can be achieved by various lithographic techniques where surface features in the sub-100nm regime can be realized. However, drawbacks of these methods are high cost and need for highly specialized equipment. In this paper a non-lithographic method was developed where gold nanoparticles can be self-assembled in a gradient fashion, creating surfaces where particles are densely attached in one end of the surface and sparsely in the other. In this way, a continuum of conditions can be tested on one single sample.

Nanoparticle patterns were formed by electrostatically tuned self-assembly of charge stabilized gold nanoparticles, and the formation of gradients was induced by ion diffusion, yielding smooth and highly controllable patterns that follow the diffusion profile of the diffusing buffer. The surfaces could be used as versatile templates for the preparation of gradient nanopatterns of chemical entities or nano morphology, allowing high control of molecular compartmentalization. The applicability of these patterns for research related to nanoscopic control of biological adhesion was demonstrated by two classical examples; adsorption of fibrinogen and adhesion of bacteria. Most interestingly, binding of fimbriae carrying *E. coli* bacteria was shown to critically depend on molecular arrangement and specifically on the size of the hydrophobic adhesive domains. The use of gradient surfaces significantly facilitated analysis of non-linear binding (cut-off-phenomena).

## Paper IV

Mats Hulander, Anders Lundgren, Lars Faxälv, Tomas L. Lindahl, Mattias Berglin, Anders Palmqvist and Hans Elwing

*The use of a gradient in surface nanotopography to study influence of nanoparticle size and inter-particle distance on platelet adhesion and activation*

Submitted

### Contribution

I planned the study and prepared the experimental surfaces. I performed all experiments and participated in the evaluation of the FLM results. I analyzed the data together with LF and MB and wrote the manuscript draft.

### Summary

Unintentional blood coagulation on biomaterials (e.g. vascular stents or prosthetic heart valves) can have fatal consequences for the function of the biomaterial and ultimately lead to loss of function with life threatening conditions for the patient. A crucial step in coagulation is the activation of blood platelets. Platelets are known to bind to adsorbed fibrinogen on biomaterial surfaces through specific membrane proteins.

In this paper the significance of nanoparticle size and inter-particle spacing for the adsorption of fibrinogen and subsequent adhesion and activation of platelets on a surface was studied. To achieve this, a surface gradient in nanotopography was prepared based on the development of nanostructured gradient surfaces in paper III. Gradient surfaces with 56 or 35nm size particles were either used after thorough cleaning (hydrophilic surfaces) or made hydrophobic using a methyl terminated alkanethiol. All surfaces were pre-adsorbed with human fibrinogen prior to the experiments. Platelets were then cultured in PBS buffer on the gradient surfaces for 30 minutes and stained for analysis with fluorescent light microscopy (FLM) or evaluation in SEM. Activated platelets (well spread) and non-activated cells (spherical) were then counted manually (SEM) or using image analysis (FLM). Fibrinogen adsorption was measured using QCM-D on non-gradient surfaces corresponding to two positions on the gradients with respect to inter particle distance.

Platelets responded differently depending on size and inter-particle spacing of the surface bound gold nanoparticles. More platelets were found to adhere to



surfaces with 56nm particles, with a peak adhesion where inter-particle distance was around 30nm. This peak was not found on 36nm particles. Activation (spreading) of the platelet cells did not correspond to the number of cells attached, but was found to correspond to inter-particle distance on the 56nm particles and was highest on the smooth control part without particles and lowest where inter-particle distance was low. No influence of particle distance was found on the 36nm particles. Surprisingly, no difference was found when surface hydrophobicity was changed, suggesting that on nanostructured surfaces, topography overrules chemistry in terms of activating platelets. When normalized for effective surface, it was found that fibrinogen adsorption was lower on nanostructured surfaces than on smooth. A suggested mechanism for the observation is that in order to bind to all fibrinogen present on a nanostructured surface (both *on* and *between* the particles) a certain flexibility of the cell membrane is required. If particles/surface features are close enough and tall enough this will not be accommodated due to the stiffness of the cell membrane.

## 9 Conclusions & future outlook

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In this thesis I have shown that experimental surfaces with well-defined nanotopography and chemistry can be fabricated with ordinary laboratory equipment through a straightforward non-lithographic process. By using these nanostructured surfaces to study the impact of nanotopography on the Immune complement and coagulation cascade two main conclusions from the biological experiments in this thesis can be drawn:

- I            Activation of the immune complement system is attenuated by surface nanotopography
  
- II           Activation of human platelets is suppressed by surface nanotopography and is sensitive to both size and inter-particle distance.

In addition, it is concluded that for the protein cascade reactions in the immune complement system an increase in surface chemistry plays a significant role on the attenuating effect of substrate nanotopography, whereas for the activation of platelets no difference was found between hydrophilic and hydrophobic surfaces, suggesting that for platelets, nanotopography of the surface over-rules surface chemistry in terms of activation.

The question of how proteins interact with surfaces when surface features are in the same size range as the proteins themselves is far from understood. Hopefully future use of these nanostructured surfaces could be used to shed light over for example the disputed Vroman effect or other complex interactions where proteins meet surfaces.

The interaction with eukaryotic and prokaryotic cells with nanostructured surfaces are also areas where these surfaces perhaps can facilitate the understanding of mechanisms behind surface interactions and cell functions. It is also of interest to create functionalized nanotopographies for improved control of cell behavior in various applications.

# 10 Acknowledgements

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No man is an island [164]. The research performed and summarized in this thesis would not have been possible without the help of fellow researchers, friends, and family to whom I owe a great debt of gratitude

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Firstly I would heartily like to thank Professor **Hans Elwing** for creating a truly creative and open minded atmosphere in your group. We've had quite a few good laughs along the way! Without my supervisors **Mattias Berglin** and **Jukka Lausmaa** this project would never have landed and I am very grateful for your support. I especially would like to thank my colleague **Anders "partikelpappan" Lundgren** for your support and generous sharing of your knowledge, forcing me to think like an engineer!

My colleague **Emiliano Pinori** for your great sense of humor and openhearted attitude. All the great people at the Lundberg laboratory. **Marcus Andersson**. **Pär Lydmark** and **Robert Almstrand** for getting me in to the "butterfly business". **Lars Nordvall** for your unlimited patience and will to always help. **Bruno Källebring** for keeping IT all together. **Agneta Danielsson** for always having answers. **Peter Thomsen** and all the nice people at the department of biomaterials. **Frank Persson** for answering all my questions. All personnel at the SEM facility. **Jonas Carlsson** for unreal skills in DOS-programming and saving the SEM (and me). **Pia Kärn** and the wild bunch for unconditional love and support. My master students **Frida Gervén** and **Neda Najafinobar**. **Julia Hedlund** and **Stina Brask-Bilén** for convincing me to join the group. **Mattias Ohrlander** for trusting in me when this project started. **Helen Karlholm** and **Linda Persson** and Everyone at **Bactiguard** for always being open and welcoming. All of my fantastic friends that make life worth living outside research, there is no room to name all of you, but you know who you are.

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