Master’s thesis

Construction and evaluation of plasma protein multilayers used for local drug delivery

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LITH-IFM-A-EX--10/2224--SE

Linköping University, 2010
Construction and evaluation of plasma protein multilayers used for local drug delivery

Olof Sandberg, May 2010

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Performed at AddBIO and IFM, 2009-2010
Linköping University, SE 581-83 Linköping

Examiner
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Abstract

With the studies performed in this theses the local drug delivery technique FibMat developed by the biotech company AddBIO, was shown to be applicable to other plasma proteins and drugs than the fibrinogen-bisphosphonate combination that is today being commercialized. Hence the potential for a broader field of application was demonstrated. The application targeted today is as a surface modification giving improved strength to bone around screws used in bone implants.

The effect of changing protein and manufacturing conditions was studied with null ellipsometry. It was demonstrated that with changes in incubation temperature, pH and salinity the fibrinogen could be successfully exchanged for the plasma proteins human serum albumin and immunoglobulin G.

With liquid scintillation counting it was shown that the developed protein multilayers were able to absorb and release the bone strengthening drug alendronic acid in levels comparable to that of the fibrinogen based ditto.

Disk susceptibility tests with the bacteria S. Aureus showed a potential for antibacterial functionalization with gentamicin. The release was, in the case of the fibrinogen multilayer, detectable up to 48 hours. Similar test revealed an inability of silver nanoparticle incorporated protein multilayers to achieve inhibitory levels.

Key words: fibrinogen, albumin, IgG, bisphosphonate, antibiotic, local drug delivery
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Abbreviations

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<th>Definition</th>
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<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
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<tr>
<td>Ag-NPs</td>
<td>silver nano particles</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>FibMat2.0/Z</td>
<td>fibrinogen matrix version 2.0 with zoledronic acid</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>LSC</td>
<td>liquid scintillation counting</td>
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<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>UVO-cleaner</td>
<td>ultra violet ozone cleaner</td>
</tr>
<tr>
<td>wt%</td>
<td>weight percentage</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström, $= 10^{-10}$ m</td>
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Chapter 1

Introduction

1.1 Background

Bone implants are becoming increasingly common in our society. By permanent replacing entire joints or fixating fractures, they help take away pain and restore mobility and quality of life for more and more people every year. For example, a total of 700.000 patients got a knee replacement and another 700.000 a new hip, just in 2002 (Ratner et al., 2004).

Two of the main problem areas with bone implants today are infections, osteomyelitis, and lack of proper tissue/implant integration. These problems are described in greater depth in the next chapter.

Both the problem of osteomyelitis and that of poor tissue/implant integration are today primarily solved through pre-emptive measures. Infections are prevented primarily through working under aseptic conditions and giving antibiotics before operations. As rates of infections are very low these strategies should be considered very successful.

Lack of proper tissue/implant integration is addressed through work on the implant surface of the implant, giving it a topography encouraging integration. Much works is also put on correct matching of implant and tissue, through the use of proper dimensions and material properties such as elasticity. The aim is to minimize destructive force application on the interface.

Local drug delivery, the concept of administering drug treatment locally where needed via a carrier system, is a method that can be used to address both the problem of poor integration and that of osteomyelitis.
This master’s thesis was conducted at the biotech company AddBIO in collaboration with Linköping University, Department of Physics and Measurement Technology, Biology and Chemistry, IFM.

AddBIO is today commercializing a local drug delivery technology named FibMat, short for fibrinogen-matrix. It is a 20-50 nm thick multilayer of the plasma protein fibrinogen that can be loaded with for example the drug bisphosphonate. Together with the bisphosphonate zoledronic acid the product is called FibMat2.0/Z. This product addresses the problem of poor tissue/implant integration by enhancing the bone holding power around the implant, improving the short and long term stability of the implant.

1.2 Aim

The aim of this thesis was to explore whether the FibMat technology could be applied to other proteins and bioactive substances than the fibrinogen / bisphosphonate combination which is under development today. The driving motive is exploration of the potential for a broader application of the technology.

The proteins included in this investigation were human serum albumin, HSA, human immunoglobulin G, IgG, and fibrinogen. The substances investigated were the antibiotic gentamicin, 10 nm spherical uncoated silver nanoparticles, Ag-NPs, and the bisphosphonate alendronic acid. The ambition was to see if multilayers with a loading capacity comparable to that of fibrinogen could be made also with HSA and IgG and whether the incorporation of antibacterial substances could give these multilayers an antibacterial function.

1.3 Methods

The primary model chosen to simulate the implant surface for which the FibMat product is developed was a silicon wafer, 10x5x0.5 mm (length x width x depth) with 2000 Å of titanium vapor deposited onto the top surface. This titanium covered silicon surface will henceforth be referred to as a "surface".

The chosen model has the advantage of enabling the use of ellipsometry as a method for measuring the multilayer thickness.
The laborative work was divided into two phases. In phase one the different conditions identified as potentially influencing the multilayer thickness were investigated. These conditions were then varied and the resulting multilayers were studied with ellipsometry and atomic force microscopy, AFM, techniques.

In phase two, the substance-absorbing qualities of the constructed protein multilayers were investigated. Liquid scintillation counting, LSC, was used to measure the amount of absorbed bisphosphonate. For the antimicrobial substances disk diffusion susceptibility testing was used. To give a measure of the release profile of these antimicrobial substances serial plate transfer tests were made. These techniques have all been used under conditions comparable to these (Aronsson, 2008), (Engström, 2009), (Madhumathi et al., 2009), (Furno et al., 2004).

The bisphosphonate used in FibMat2.0/Z, zoledronic acid, does not allow for detection via liquid scintillation. This since no isotope labeled version is commercially available. All these measurements were instead performed on the bisphosphonate alendronic acid. Alendronic acid and zoledronic acid are very similar in structure and chemical properties, giving merit to the assumption that the incorporated amounts of the two drugs into the multilayer would be comparable. This has not been validated by studies, however.

1.4 Sources

All articles were found via PubMed or Web of Science. Apart from these a number of books, the results from two master’s thesis written at the same company, two non-commercial and two commercial web pages have been cited. All figures used not composed by this author have been freed of all copyright restriction by the original source.
Chapter 2

Theory

2.1 Implant failure, causes and solutions

2.1.1 Implant failure caused by early loss of fixation

Aseptic loosening, i.e. loosening caused by improper interaction of bone and implant, is the most frequent cause of implant failure in bone applications. The potential reasons are many, both mechanical and biological. Examples are micro movement, poor alignment of the implant, and inflammations (Revell, 2008).

Following an implant operation the bone reacts similarly as it would to a normal wound. First, there will be an inflammatory reaction. Damaged or dead bone is resorbed by osteoclasts, bone eating cells. Osteoblasts, bone forming cells, meanwhile forms new bone. This way the wound will be healed and most of the time the implant will be anchored successfully in the new formed bone.

However, before enough new bone has been formed to safely anchor the implant, the resorption process will mean less bone for the implant to anchor to. This can bring worsened implant stability. Sometimes this also brings micro movements of the implant. This can create debris which cause further inflammation and damage to the bone. This in turn translates into increased osteoclastic resorption and counteracts the bone forming process, further worsening the implant stability, thus forming a negative trend (Stadelmann et al., 2008). If the bone suffers from an ailment such as osteoporoses, the risk for this occurring is much increased.

Lacking a proper anchoring, collagenic scar tissue with an insufficient mechanical durability will form, leading to poor bone holding power and most likely failure.
2.1.2 Implant failure caused by infection
When an implant is placed in a human body there is always a risk of bacterial colonization. In orthopedics the frequency of infection is relatively low. However it still represents a substantial problem to the health care system and the afflicted patient. Treatment of osteomyelitis related to an implant is either a long and hard systemic antibiotics treatment with severe side effects, or removal of the implant, which calls for a second implantation with much reduced prognosis. With all the new operations and treatments required, the costs for an infected implant can be enormous. As an example, treatment of osteomyelitis in a prosthetic joint implant in the USA has been estimated to cost above $50,000 per case (Nathwani et al., 2003).

Commercially, drug delivery systems for treatment of osteomyelitis, in the form of PMMA beads has existed for many years. Their high dose delivered locally entails an efficient antibacterial effect. However they also have the disadvantage of having to be removed after a fulfilled treatment (Giuliano et al., 1986).

The bacteria causing the infections often come from the skin of the patient or the medical personnel (Shierholz et al., 2001). Though prophylactic treatment with antibiotics is standard procedure in most implant operations, hopes of creating an entirely sterile wound have proven unrealistic. Hence developing measures to deal with minor bacterial contamination is a rational strategy.

The surface of a clean metallic implants have a high surface free energy and is thus capable of catalyzing various chemical reactions. Together with a release of ions and a mitigated immune response, this makes implant surfaces attractive for bacterial colonization. A term often used in these circumstances is that of "a race to the surface", expressing an idea that whichever succeeds at first populating the surface, bacteria or tissue cells, will have a much improved chance of permanent residence. If tissue cells are the first to populate they will present a wall of living cells to which the bacteria will not be able to adhere. In a matter of days these will then instead be taken care of by the immune system (Gristina, 1987).

However, if the bacteria succeeds at reaching the implant surface first, the prognosis for the implant is worsened significantly. The bacteria will in many cases change their phenotype, going into a defensive stance in which they secrete a
biofilm, an extracellular matrix made up of carbohydrates and proteins, followed by a protracted reproduction. This change in behavior will protect the bacteria from antibacterial countermeasures released by the body as well as those introduced by the doctor (Gristina et al., 1987). Hence, removal of the implant is many times the only way of ending an infection. Evolution has made bacteria adept at colonizing surfaces such as the ones presented by metallic implants and damaged tissue. This is of great benefit for the bacteria in this race to the surface (Gristina, 1987).

*Staphylococcus Aureus* and *Staphylococcus epidermis* are the two strains most closely connected with osteomyelitis. Both are a natural and harmless part of our skin flora. On entering the body however, they change phenotype and become pathogenic. *S. Aureus* is the most frequently occurring bacteria in infections related to metal implants while *S. Epidermis* prefers polymer based implants. It can also be noted that in two out of three infections more than one kind of bacteria is involved (Gristina, 1987).

### 2.1.3 Local drug delivery technology

Systemic treatments have a major problem: in order to achieve a high enough level of the drug locally in the body where the effect is needed, the whole body must be treated with a much higher dose of the same. This often leads to severe side effects.

Local drug delivery is a solution to this problem. Compared to systemic treatment it allows for a dramatically increased portion of the drug to be delivered directly to the intended target. This radically lowers the total amount of drug that the patient has to use. Side effects are reduced proportionally.

One of the main problems with local drug delivery has to do with drug release profile. A specific released dosage per unit time for a specific duration of time is often desirable. In practice, this has proven very difficult to achieve, see figure 1 on the next page. A more likely scenario is that one or more of the following occurs: a short, too high, burst followed by a prolonged, too low, release (Ratner et al., 2004). In the example of antibiotics, this sustained release of sub-bactericidal levels can have serious repercussions for the patient with an increased risks of resistance developing.
A successful drug delivery strategy is concerned with how to store and how to release a drug in a controlled manner. There are several different strategies to achieve this; for example using diffusion regulating membranes regulating the drug diffusion into the surrounding body, systems controlled by water diffusing into the device and pushing out the drug, and biodegradable matrixes with the drug releasing the drug as it is degraded. There are also devices that regulate their drug release after for example temperature and pH (Ratner et al., 2004).

**Figure 1.** Ideal release profile of local drug delivery devices to the right, the opposite to the left.
2.2 Incorporated substances

2.2.1 Silver nanoparticles

Silver has been used to treat bacterial infections for at least 3000 years. As penicillin’s antibacterial properties were discovered and started being used, around world war II, the use of silver receded. Today however, with increasing reports of antibiotic-resistant strains, use and research of silver in these applications is rapidly increasing. Silver nanoparticles, Ag-NPs, are today considered one of the most commercially promising nano application within medicine (Chen et al., 2008).

Silver is attractive as an antibacterial since it attacks a broad spectrum of strains and occurrences of resistance development is rare (Percival et al., 2005). It also shows good effect against bacteria resistant to other treatments (Alt et al., 2004). The effect is more pronounced against gram negative bacteria than against gram positive (Shrivastava et al., 2007), (Madhumathi et al., 2009).

Ionic silver works by binding to the cell membranes of bacteria, disrupting the electron transport chain and forming pits in the cell walls, leading to cell death. In eukaryotic cells corresponding electron transportation structures are found inside the cell, at the mitochondrial membranes. This ensures a higher resistance for eukaryotic cells against Ag-NPs.

Ag-NPs are believed to derive their bactericidal effect partly from their nano structuring, partly from the ionic silver they release (Fabrega et al., 2009). The nano particles interact with sulphur containing proteins, attacking the respiratory chain (Rai et al., 2009). In E. Coli it has been demonstrated how Ag-NPs induce pits in the cell walls, increasing the permeability, leading to cell death (Sondi et al., 2004).

The large surface area of Ag-NPs entails greatly increased chemical reactivity compared to micro sized particles. Ag-NPs also release ionic silver, which further enhances the antibacterial effect (Morones et al., 2005), (Sondi et al., 2004), (Shrivastava et al., 2007).

The size and shape of the Ag-NPs are believed to influence the bactericidal potency. Smaller size means increased surface area and probability of entering the bacterial cell. Ag-NPs around 10 nm are indicated as having the highest activity.
(Morones et al., 2005). Truncated triangles have proven more efficient than spherical or rod shaped dito (Pal et al., 2007). This shape effect can probably at least in part be explained by the effect on surface area.

Ionic silver is thought to have very good biocompatibility, with no side effects locally or systemically (Hardes et al., 2007). However, when very large doses are applied, irreversible gray or black coloring of skin and eyes, so called argyria can occur. This was one of the main reasons to why silver was largely abandoned with the arrival of antibiotics.

Many believe that Ag-NPs, because of their unique properties related to their nanostructure, should have their biocompatibility investigated more before clearing the use of them in medicinal treatment. Studies have indicated that exposure to Ag-NPs entail a significant decrease in mitochondrial activity in amongst others neuroendocrinal and liver cells. Other finds indicate that Ag-NPs target key components of the cells anti oxidative defense mechanisms, leading to oxidative stress in eukaryotic cells (Chen et al., 2008).

2.2.2 Bisphosphonates

Bisphosphonates are a family of biologically active chemicals discovered in the 1880s and used to diagnose and treat bone disease since the 1970s. 90% of all bone diseases are caused by excessive osteoclastic activity and these may all be treated with bisphosphonates (Bartl et al., 2007). An example is postmenopausal osteoporosis.

![Figure 2. The general skeletal formula of a bisphosphonate.](image1)

![Figure 3. A ball-and-stick illustration of zoledronic acid.](image2)

The basic structure for a bisphosphonate is illustrated in figure 2; two phosphate groups bonded to a carbon with two R-groups. The phosphate groups enable the
drug to bind to bone through interactions with calcium in the bone. The $R_1$ group is often an OH-group, which increases the bone binding capability.

Clinically, bisphosphonates inhibit osteoclastic bone resorption. As osteoclasts dissolve bone in which bisphosphate is present, the bisphosphonate is released. This mechanism means that the drug concentration as experienced by the osteoclast is increased (Russel, 2006).

Bisphosphates also inhibits osteoclast recruitment and osteoblast activity. The result is an encouraged bone production and a discouraged bone break down, what is referred to as a positive resorption balance (Rang et al., 2003).

The different bisphosphonates can be classified into two groups, those with and those without nitrogen in their $R_2$ group. These groups have slightly different mechanisms of action. The nitrogen containing bisphosphonates for example, such as zoledronic and alendronic acid, amongst other effects inhibit the mevalonate pathway. This is pathway for synthesizing for example cholesterol. The end result is cell apoptosis (Russel, 2007). As the osteoclast dies the bisphosphate is released back into the body, enabling it to enter more osteoclasts.

Today bisphosphonates are given orally or intravenously, common side effects for which are stomach aches and brief influenza like symptoms. (FASS, 2010A), (FASS, 2010B). Renal damaged and jaw bone necrosis are other mentioned rare to very rare side effects (Bartl et al., 2007).

### 2.2.3 Gentamicin

Gentamicin belongs to the family of antibiotics. Antibiotics have been used to fight infections for thousands of years, the earliest documented use has been dated to 500 B.C with moldy bean curds used as health improving medicine in China. The term antibiotics, however, originates from 1889 and the researcher Paul Vuillemin (Kumar et al., 2008).

![Figure 4. A ball-and-stick representation of gentamicin.](image)
Gentamicin is the most used member of the antibiotic family of amino glycosides (Rang et al., 2003). The amino glycosides are generally attractive antibiotics with low cost, little of side-effects, low allergy rates, and good chemical stability. Amino glycosides resemble a carbohydrate with a ring consisting of six carbon atoms with hydroxyl and amine groups. Gentamicin consists of three such rings, joined by oxygen atoms. It is a highly polar molecule, with a positive charge at physiological pH.

Gentamicin has a fast working bactericidal effect and resistance development is rare compared to other antibiotics. It attacks a broad spectrum of bacteria, penetrates bone well, and is also able to kill non-proliferating organisms (Kumar et al., 2008). These traits are the reasons to why this antibiotic is the most often used antibiotic for bone applications. In the treatment of infections gentamicin is one of the most effective antibiotics.

Gentamicin works by entering the bacterial cell, attaching to the ribosomes and disrupting the protein production, causing abnormal protein production and fatality to the bacteria (Kumar et al., 2008). It also damages the cell wall.

Treatment with gentamicin is done via injections or infusions since it cannot be absorbed from the gut. Potential side effects includes the risk of permanent damage to sensory cells in the ear as well as reversible damage to the liver (Rang et al., 2003).
2.3 Proteins

2.3.1 Human Serum Albumin

With a molecular weight of 66.5 kDa, human serum albumin, HSA, is one of the smallest proteins present in the blood plasma. Nonetheless it constitutes up to 50% of the total mass content of the plasma proteins. It is a heart shaped, easily soluble protein that is stable in pH of 4 to 9 and can be heated at 60°C for up to 10 hours without any detrimental effects. (Kratz et al., 2008). The pI is 4.7. It is synthesized in the liver (Scott et al., 2009).

HSA has a globular structure with dimensions of approximately 8x8.7x6 nm. A ribbon diagram representation is visible in figure 5 below. The structure is made up of six sub domains repeating the same pattern, connected with disulfide bridges.

![Figure 5. A ribbon diagram of HSA. Reprinted with permission, (Sugio et al., 1999).](image)

The primary roles of HSA in the body is to maintain the osmotic pressure and volume of the blood, thus amongst other things preventing the loss of plasma from the capillaries (Guyton et al., 2005). Another main role is transport of a wide range of different compounds, most notably long fatty acid chains but also steroid hormones, vitamins and metal ions. The protein can also be used to transport artificial particles such as antibiotics (Curry, 2002).

2.3.2 Immunoglobulin G

Immunoglobulins make up about 20 % of the total mass of plasma proteins. They are divided into five subclasses, A, D, G, E and M, out of which immunoglobulin class G constitutes about 75%. Being the largest in the group, IgG is further split into four subgroups, named 1-4 after their serum level in healthy westerners (Schroeder et al., 2010).
The primary function of immunoglobulins is to opsonize foreign body particles so these can be eliminated by the immune system. The pI of IgG varies from 6.5 to 8.0.

![Image]

**Figure 6.** A ribbon diagram of IgG. Reprinted with permission (Saphire et al., 2001).

The protein has a molecular weight of about 150 kDa. The structure is made up of two heavy and two light chains. The two light chains is the part where the protein attaches to a foreign body. Each type of receptor bind to a limited set of ligands. Hence this is the more variable part, as adjustments must be made to fit the individual types of antigen. This for the organisms survival very crucial variation is governed through gene rearrangement (Schroeder et al., 2010). The two heavy chains is the effector part which relays information, if the light chains have made contact with a matching antigen, to for example a macrophage (Alberts et al., 2002). The size of the IgG molecule is about 5.9x13.1x14.3 nm. Below, in figure 6, is a ribbon diagram representation.

Aside from identifying antigens for macrophages and neutrophiles to devour, IgG also has methods of liquidating these by itself. E.g. lysis of an invading cells cell wall and neutralization of viruses and toxins (Schroeder et al., 2010), (Guyton et al., 2005).

In the application developed in this thesis, local drug delivery for bone applications, IgG is not a good candidate. This since it is immuno activating. However this can be an asset as it can be used as a positive reference in tests of the protein multilayers immunological profile.
2.3.3 Fibrinogen
Fibrinogen is a plasma protein with a molecular weight of 340 kDa. Together with albumin and globulins it is the main constituent of plasma proteins. It consists of six subunits joined by disulfide bonds (Mosesson et al., 2005), the structure is depicted in figure 7 below. It is elongated with dimension of 45x9x6 nm. The pI is about 5.8.

The protein is synthesized in the liver and is present in the body at concentrations of 2 to 4 mg/ml. Except from directly in blood it is also found in platelets. (Scott et al., 2009).

![Figure 7. The ribbon diagram representation of fibrinogen. Reprinted with permission, (Kollman et al., 2009).](image)

Fibrinogens primary role is related to wound healing, blood clotting and the inflammatory response. As a wound is made, the protein thrombin is activated. This cleaves fibrinogen into fibrin, which is the activated form. The resulting fibrin monomers polymerize and form large networks. These react with platelets and form large, non-solvable structures. These structures clog leaks in the circulatory system (Guyton et al., 2005), (Mosesson, 2005), (Lieberman et al., 2009).

In medical applications layers covered with fibrinogen has shown to have a low complement activation which indicates good biocompatibility.
2.4 Quantification methods

2.4.1 Disk diffusion susceptibility testing

Disk diffusion susceptibility testing is a method for quantification of antibacterial activity of solid surfaces. The technique is based on the diffusion of a substance through the agar and the subsequent inhibition of bacterial growth on the same.

Agar plates are covered with a solution containing a certain density of bacteria. The solution is spread evenly. The surface to be investigated is then placed face down on the plate. After 16-20 hours of incubation at 35-37°C the antibacterial effect is quantified by manual measurement of the zone of inhibited bacterial growth, see figure 10. The diameter measured hence give a value of the critical concentration at which the antibacterial substance fails to inhibit the bacterial growth. By comparing the resulting zones of inhibition to those formed by known quantities of antimicrobial substance, information of the antimicrobial effect of the surfaces is gathered (Lorian, 2005).

![Figure 10. Example of a disk diffusion susceptibility test after a 20 hour incubation](image)

Where applicable, tests are designed so that inhibition zones in the order of 15-35 mm can be expected. Factors effecting the zone diameter are: amount and diffusion characteristics of the antibacterial substance, bacterial susceptibility to the antibacterial substance, density of the bacterial inoculum, time between inoculation and antibacterial substance application (Lorian, 2005).

This technique have the advantages of being easy and cheap. Disadvantages include a slow procedure (over-night) and limited sensitivity.
2.4.2 Liquid scintillation counting

Liquid scintillation counting, LSC, is a technique with which radiation of isotope marked particles in a solution can be measured. This allows for quantification of for example drug concentrations in the solution.

As the isotopes used are radioactive, they undergo radioactive decay. The isotope used in this thesis, $^{14}$C, emits β-particles. These are high energy electrons. As the β-particles travel through the solution this energy is dissipated through heat and excitation of solvent molecules. These molecules have aromatic rings which absorb the energy of the β-particles. This energy is then passed on to a type of phosphor containing molecules called scintillators. Scintillators absorb the energy by inducing a dipole in their solvation shell. The energy is then released in the form of UV-light, often below 408 nm. Since many photomultipliers have a poor response in this band a secondary scintillator is often included. This molecule absorb the emitted photon and releases a photon at a longer wavelength (National Diagnostics, 2004).

The UV-light signal is amplified by photomultipliers and are then recorded by a detector. The intensity of recorded light is thus proportional to the amount of radioactive material, as explained for example by the University of Wisconsin (2009).

This allows for measurement of the amount of radioactive sample through detecting beta particle emissions as light intensity (Lappin et al., 2006).

This technique has the advantage that radioactive isotopes easily can be substituted for their normal equivalent without significant change to the chemical behavior. $^{14}$C has a half-life of 5730 years, hence there is no risk of degradation of the activity during the time of an experiment.

The technique has a high degree of reproducibility and sensitivity and is easy to handle. It allows for close association of the radioactive material and the scintillator, increasing the sensitivity compared to solid scintillation. This since β-particles decay relatively quickly, the mean range in water is 0.28 cm. Disadvantages include the risks and costs associated with the handling of radioactive material (National Diagnostics, 2004).
Since the concentrations to be measured in this thesis are very low, the demands for sensitivity are high. Another method which could offer this level of sensitivity is GC-MS/MS, however this technique has been rejected for reasons such as cost-efficiency and availability.

### 2.4.3 Atomic force microscopy

Atomic force microscopy, AFM, is an imaging technique with a resolution superior to optical techniques. The technology is relatively common and easy to use and images can be made of both insulating and conductive samples (Butt *et al.*, 2005). It can be applied on any surface which is hard enough, in water or in liquid (Ricci *et al.*, 2004).

The principle includes a cantilever, at the head of which sits a very small tip which interacts with the sample. A laser beam is reflected off the head of the tip as it moves up and down, following the topography of the sample. The movement causes the light beam to be reflected in different angles, thus hitting different positions on a detector, see figure 9 (Ricci *et al.*, 2004). The sample is typically mounted on a board controlled by piezoelectricity, giving high precision vertical movement.

![Figure 9](image.png)

*Figure 9*. The principle of an AFM-machine with the laser, cantilever, sample and detector visible. The tip is enlarged.

An AFM can be run in different modes, the one used in this thesis is tapping mode in which the cantilever is kept at its resonance frequency through a feedback loop. The force interaction between sample and cantilever tip, Van der Waal, dipole-dipole or electrostatic interaction, entails a change in the energy of the cantilever,
changing the oscillation frequency. The amount of energy dissipated is related to the tip-sample interaction and the amount and type of features on the sample. As the cantilever is at its resonance frequency very little energy is dissipated save through this interaction, this is a factor increasing the sensitivity of the method. This method does not damage the surface as much as other, contact, modes. Disadvantages to the AFM technique includes that the tip can damage the sample and that the size of the images that can be constructed typically are limited to about 150x150 µm. If the wrong tip or a damaged tip is used this will seriously affect the accuracy of the reading by leading to artifacts, distorting the image.

2.4.4 Null ellipsometry

Ellipsometry is a precise and convenient method for quantification of the thickness of thin films (Ohlídal et al., 2000). It is a nondestructive and label-free method and does not require the use of a reference. Under well organized conditions the precision is down to individual Å.

The technique utilizes the polarization of light. A laser beam is generated and directed at a sample. Before hitting the sample the light is given a specific polarization. At the moment of reflection the light induces a local current which interacts with the surrounding material. This way the film influence the polarization of the light (Harland et al., 2005). By emitting light of a certain known polarization and measuring the polarization after reflection, the thickness can be calculated, see figure 8 below.

![Figure 8. The measurement principle of ellipsometry, change of light polarization.](image)
The ellipsometer estimates the change in polarization through two values, $\Delta$ and $\Psi$. $\Psi$ involves the change in wave amplitude. $\Delta$ is the phase shift, the change in angle of the polarization, illustrated by how the ellipse points in the x/y plane in the figure above. The equations relating $\Delta$ and $\Psi$ to $\rho$ and to $R_p$ and $R_s$, the parallel and perpendicular polarized parts of the light is as follows, (Harland et al., 2005):

$$\rho = \Re(\rho) + \Im(\rho) = \tan(\Psi) \cos(\Delta) + i \tan(\Psi) \sin(\Delta) = \tan(\Psi) e^{i\Delta}$$

$$= \frac{|R_p|}{|R_s|} e^{i(\delta_p - \delta_s)} = \frac{R_p}{R_s}$$

After a measurement the $\Delta$ and $\Psi$ are run through mathematical models, and depending on the assumptions made regarding optical properties of the measured film, these will give a corresponding value of the thickness. The algorithm used in this work is the McCrackin algorithm (McCrackin, 1969).

In the null ellipsometry set up used in this thesis the emitted light has its polarization shifted until the light reflected from the surface is linearly polarized. By using the angle required to do this together with the angle at which the detector records nothing of this light, the machine can calculate the $\Delta$ and $\Psi$ values needed (Harland et al., 2005).

The name null ellipsometry comes from the term nulling, i.e. when the light reflecting of the surface has a linear polarity, and the observation that light often is elliptic in its polarization.

Limitations to the technique include requirements for low surface roughness and high surface reflectivity. Also, without knowledge of the optical properties of the layer, calculating the thickness becomes very complex.
Chapter 3

Materials

3.1 Protein buffers and solutions

- The protein buffers used were, unless otherwise stated, 10 mM acetic buffers prepared from sodium acetate and acetic acid. Protein concentration was 2 mgml⁻¹.
- Scintillation cocktail by the name of Optiphase Supermix was purchased from Perkin Elmer, USA.

3.2 Proteins

- IgG was from Octapharma, Sweden. Article number: 008565.
- Fibrinogen was from CalBioChem, Germany. Article number: 341587.
- HSA was from Sigma, USA. Article number: A-3782.

3.3 Incorporated substances

- Gentamicin was purchased from Sigma Aldrich, USA. Article number: G1397.
- Alendronic acid was purchased from LKT laboratories, USA. Article number: A4515.
- Isotope labeled alendronic acid was purchased from Moravek, USA. Article number: MC 2238.
- Uncoated Ag-NPs with 10 nm diameter and concentrations of 0.02 mgml⁻¹ and 1 mgml⁻¹, stabilized in 6 mM citric acid, were purchased from Nanocomposix, USA.
3.4 Surfaces
The surfaces were 10x5x0.5 mm (length x width x depth) hydrophilic silicon wafers with crystal direction (100) and 2000 Å of titanium deposited on one side through vapor deposition. The vapor deposition was handled by research engineer Agneta Askendal at IFM. For LSC studies titanium disks 6.4x1 mm were used.

3.5 Bacteria and agar plates
The bacteria used was *S. Aureus*, strain ATCC 29213 designated Wichita, isolated from a wound. The bacteria was purchased from ATCC, USA. The plates used were of the Müller-Hinton type.

3.6 Hardware and software
- The ellipsometer was from Rudolf Research, USA. Designation Auto-E1 III.
- The AFM machine was from Veeco, USA. Designation DI Dimension 3100.
- The LSC machine was from Wallac, USA. Designation Micro-Beta 1450.
- Minitab 15 from Microsoft, USA, was used to handle statistics.
- NanoScope from Veeco, USA, was used to process the AFM data.
- The UVO-cleaner was from Jelight Company Inc, USA. Designation 42.
- Microsoft Works was used to process and present data.
Chapter 4

Methods

4.1 Method overview and tested parameters

The method used in this thesis to produce protein multilayers with functionalization on surfaces can be divided into two principal steps, visualized in figure 11. This is a brief summary:

In the first step a surface was placed in an eppendorf tube along with protein incubation solution, and incubated at a specific temperature for a specific time. Ellipsometry was the main method used to quantify the results of this step.

In the second step a protein covered surface was placed in a new eppendorf tube containing functionalization solution with either gentamicin, alendronic acid or Ag-NPs. Here LSC and disk diffusion susceptibility tests were used for quantification of the incorporated levels.

For the multilayer production step tested parameters were: pH and salinity of the incubation solution, buffer concentration, incubation temperature, and stability in elution fluids. Parameters studied for the functionalization with alendronic acid were: total absorbed amount and eluded amount at one and four hours. For gentamicin and Ag-NP parameters tested were: released amount at day 1 and 2, effect of rinsing, and effect of merging step 1 and 2.

Figure 11. A schematic over the production/quantification process.
4.2 Producing the multilayer

4.2.1 Preparing the surfaces for the multilayer production

The surface which was to be covered with a protein multilayer, a 10x5x0.5 mm (length x width x depth) hydrophilic silicon wafer with crystal direction (100) and 2000 Å of Ti deposited on one side through vapor deposition, was rinsed in deionized water, dried with N₂ gas and then put in a UV-ozone-cleaner, UVO-cleaner, for four minutes.

The UVO-cleaning step removes contamination through the exposure to short wavelength UV-light. The light is absorbed by contaminating molecules, who then form more volatile particles, combine with atomic oxygen also formed by the UV-light, and desorb from the surface (Jelight Company Inc, 2009).

4.2.2 Preparing the protein incubation solution

A buffer with a certain salinity and pH, depending on the test series in question, was prepared by mixing sodium acetate, acetic acid, deionized water and NaCl in certain proportions. The solution was given a temperature of 22°C. By measuring the mass of protein and then adding the correct amount of solution a concentration of 2 mg/ml⁻¹ was achieved. The exact wt% NaCl used has been classified throughout this rapport for patent reasons.

4.2.3 Producing the protein multilayer

- The cleaned surface was rinsed in a gentle stream of deionized water for two seconds and then carefully dried with N₂ gas.
- 0.5 ml of protein solution; 10 mM acetic buffer with 2 mg/ml⁻¹ of protein concentration, was transferred to an eppendorf tube and the surface added. The protein solution was used within four hours of making the solution.
- The tube was incubated at a given temperature for 10 minutes. The lid of the tubes were kept open. The exact temperatures used have been classified.
- The surface was rinsed for two seconds in deionized water, dried with N₂ gas and stored awaiting quantification by ellipsometry. If the surface was to be given functionalization the drying step was omitted, instead the
surface was directly after the rinsing step transferred to the functionalization incubation solution.

4.3 Characterizing the multilayers

4.3.1 Measuring the multilayer thickness with null ellipsometry
Five points on every surface was measured. The machine gave a $\Delta$ and a $\Psi$ value for every point and these were put through the McCrackin evaluation algorithm. The refractive index of the protein layer was assumed to be 1.465 (Stenberg et al., 1983). This produced values of the multilayer thickness. The data was then processed using Microsoft Works.

4.3.2 Atomic force microscopy measurement
Protein multilayer covered surfaces were evaluated in the AFM machine using tapping mode. The data was analyzed with NanoScope software. The hardware and software was handled by research engineer Jörgen Bengtsson.

4.3.3 Stability studies
For the long term stability tests multilayer-covered surfaces were incubated in eppendorf tubes with 0.5 ml of deionized water with 0.9 wt% NaCl at 37 °C for 1 hour, 1 day or 1 week. After this they were dried with N₂ gas and had the thickness evaluated with ellipsometry.

For the tests of various buffers the surfaces were incubated in 0.5 ml of the buffers in question at 37 °C for 1 hour and then had their thickness measured with ellipsometry.

4.4 Functionalization

4.4.1 Preparing functionalization solutions
Alendronic acid was added to 2.5 mM acetic buffer at pH 4.9 to a final concentration of 0.5 mg/ml. $^{14}$C labeled alendronic acid was added, to a final concentration of 1 wt% of the total alendronic acid concentration.
Gentamicin was diluted in deionized water to a final concentration of 10 mg ml$^{-1}$ while Ag-NP was used in the original solution, a 6 mM citric acid buffer of pH 7.4.

4.4.2 Incubation of a surface in functionalization solution

- A surface or disk was first given a protein multilayer through the incubation step described under chapter 4.2.
- 0.5 ml of the functionalization incubation solution was added to an eppendorf tube.
- Directly after the protein multilayer incubation the surface was rinsed in deionized water and transferred to the new tube. It was then incubated for five minutes for alendronic acid or an hour for gentamicin or Ag-NPs.
- After the final incubation step the surface was either rinsed, by dipping it for one second in deionized water, or not, depending on the experiment.
- The surface was dried with N$_2$ gas.

4.4.3 Combined protein / functionalization incubation

Attempts were made with including antibacterial substance directly in the protein multilayer incubation step. Gentamicin or Ag-NP was added to the protein incubation solution with a final concentration of 10 mg ml$^{-1}$ or 0.002 mg ml$^{-1}$ respectively.

4.5 Quantification of functionalization

4.5.1 Liquid scintillation to measure alendronic acid levels

For testing the alendronic acid levels with liquid scintillation, 6.4x1 mm disks were used instead of surfaces. This was to ensure that the entire surface presented to the elution liquid was of titanium. The surfaces used for ellipsometry only have titanium on the top, not on the sides or the bottom.

The disk was incubated with protein solution and then alendronic acid solution as described earlier. If the elution profile was to be investigated the disk was put in 6 ml of deionized water for 1 or 4 hours, after which a 1 ml sample of the liquid was recovered. If the total amount of incorporated alendronic acid was to be studied this step was omitted.
Either a disk or an elution liquid sample was then added to 10 ml of scintillation cocktail and left for two hours of incubation on a mixing table. This time is enough to ensure that all protein and alendronate has been removed from the disk and is transferred to the solution.

Thereafter the levels were measured in an LSC machine and collated using Microsoft Works.

4.5.2 Disk diffusion to measure antibacterial activity
For testing the antibacterial activity first bacteria of a concentration giving $10^8$ colony forming units, CFU, per ml was added to an agar plate and spread evenly. Thereafter the surface was added, one agar plate was typically used for three to four surfaces.

The plates were left in room temperature for 30 to 60 minutes after which they were placed in a 35°C environment over night. Inhibition zones were measured the next day, two diameters being measured for every zone.

4.5.3 Serial plate transfer test
For studying the release of absorbed substance for day 2 surfaces were, after 24 hours on the first agar plate, moved to new freshly inoculated agar disks. These new disks were treated in the same fashion as the day 1 disks.

4.6 Statistics
For every point in the graphs depicting multilayer thickness three surfaces were produced. With ellipsometry, the thickness of these surfaces were measured at five points. Hence the interval for multilayer thickness in every point of the graphs is the average, ± the standard deviation, of these fifteen values. Thus these three surfaces are, for statistical purposes, regarded as a single surface, as variations between the three surfaces are regarded as negligible. This is a simplification that allows for easier data representation.

For the agar diffusion tests accounted for in table 1 two measurements of the diameter was made on every surface. The mean and standard deviation of three
surfaces were then combined to give the values in table 1. For the values of figure 22 in the appendix the mean of the individual surfaces are presented.

For the values of alendronic acid levels in figure 19 and 20, one reading each was made for three surfaces, for every unique combination. The mean value and standard deviation are derived from this.

To test for statistically confirmed differences between two points, a two tailed two-sample t test was used. P-values of 0.05 and 0.01 were used. Where statistically confirmed differences between two adjacent points could be shown, this is marked with an asterisk on the line tying these two points together.

Test for differences in variance was performed with an ANOVA F-test, P-value 0.01
Chapter 5

Results and Discussion

5.1 Conditions for multilayer formation

5.1.1 Changing incubation pH at room temperature
The pH of the incubation solution was changed while keeping all other factors constant. The buffer solution was a 10 mM acetic buffer, salt content was 0.9 wt%, the incubation temperature 22°C and the pH range investigated went from 3.0 to 6.0. Surfaces were incubated in the solutions and the thickness of the formed protein layer was measured with ellipsometry. Figure 12 show the results.

Figure 12. Thickness of formed protein multilayer as a function of solution pH at 22°C. Differences between two points statistically significant at p = 0.01 is marked with an asterisk on the line tying these points together.
The HSA protein layer has a thickness of about 36 Å from pH 3.0 to 5.0. At pH 6.0 this goes down to 20 Å. The IgG layer is around 35 Å thick for the entire pH span. For fibrinogen the layer is 22 Å thick at pH 3 but for pH 4.0 and 5.0 the protein layer goes up to 90 Å, going down again to 60 Å at pH 6.0.

The results show that for all three protein, adsorption onto the titanium surface has occurred. Changing the pH effects how the proteins interacts with, and form monolayers on, the surface. Depending on the structural and chemical properties of the respective protein, the nature of this effect will differ and hence the thickness of the resulting monolayer. According to figure 12 fibrinogen adsorbs in thicker layers than HSA and IgG and changing the pH had the clearest effect on the fibrinogen protein monolayer thickness. For HSA and IgG the effect was much smaller, though still statistically significant at p = 0.01. A pH of 4 or 5 gave thicker layers for all three proteins, compared to pH outside of this interval.

In a study on IgG and HSA adsorption to various titanium surfaces, Jansson and Tenvall, 2004, produced results indicating that the thickest protein adsorption to hydrophilic titanium occurred between pH 4.2 and 7.4 for HSA and somewhere below pH 6.5, (the lowest reading point was 5.2) for IgG. However these series were conducted in DMGA-buffer solution without NaCl.

In a study on HSA, fibrinogen and IgG adsorption to hydrophilic silicon, Ortega et al. demonstrated a different pH-dependence than the one illustrated above in figure 12 (1997); HSA here shows a much more pronounced dependence of multilayer thickness on pH, as does IgG. IgG shows a trend of increasing thickness with increasing pH which is absent from figure 12. The maximal thicknesses correspond quite well with those measured in this work: about 25-30 Å for IgG and HSA and 80 for fibrinogen.

Measuring the amount of protein on the fibrinogen, HSA and IgG surfaces, for example with florescamine as described by Engström (2009), could together will null ellipsometry give interesting insight into possible differences in protein concentration for the layers resulting from various protein and production parameters. This would give increased understanding the multilayer forming process and the results thereof.
5.1.2 Changing incubation pH at elevated temperature

The pH of the incubation solution was changed while keeping all other factors constant. The buffer solution was a 10 mM acetic buffer. The exact salt content used is classified and will be referred to as s1, s2 and s3 for HSA, fibrinogen and IgG respectively. The exact incubation temperature is classified for patent reasons and will be referred to as t1, t2 and t3 for HSA, fibrinogen and IgG respectively. The pH range investigated went from 3.4 to 5.8. Surfaces were incubated in the solutions and the resulting multilayer thickness was measured with ellipsometry. The results are displayed in figure 13.

Changing the pH of the incubation solution had an effect on the thickness of the resulting multilayer. This applied to all three proteins studied.

At pH 3.4, the HSA multilayer has a thickness of 414 Å, this increases with increasing pH up to 531 Å at pH 4.3. Thereafter the thickness declines, reaching 36 Å at pH 5.7. Fibrinogen shows a multilayer thickness of 90-100 Å for pH 3.4 to 4.4, thereafter it increases to maximum 266 at pH 5.3. At pH 5.8 it goes down again to 167 Å. The IgG multilayer starts out at 134 Å at pH 3.4. It then climbs slowly, reaching a maximum of 399 Å at pH 5.3.

Figure 13. Thickness of the protein multilayer as a function of solution pH at elevated temperature. Differences statistically significant are marked with an asterisk, * p = 0.01. The approximate pl of the proteins are indicated with a black circle. For IgG the value is 6.5 and above and hence not visible in the diagram.
One observation is that HSA, having a lower pI compared to the other two proteins, 4.7 compared to 5.8 and 6.5 to 7.8, also has maximum in layer thickness at a lower pH. Hence the effect seem to be related in part to the pI of the protein, which is logical since a pH close to the pI of the protein ensures lower electric charge and therefore lower electrostatic repulsion between the protein molecules. However it is interesting to note that for all three proteins the optimal pH is somewhat lower than the respective pI.

5.1.3 Effect of changing the salt concentration of the incubation buffer

The effect of changing wt% of sodium chloride in the incubation buffer was studied. Surfaces were incubated in solutions with different salt content. Other factors were kept constant. The pH and incubation temperature used were: pH 4.4 and t1°C for HSA; pH 5.0 and t2°C for fibrinogen; pH 4.5 and t3°C for IgG. The resulting multilayer thickness of the surfaces was measured with ellipsometry. The exact results are classified for patent reasons.

It was demonstrated that increased salt content meant increased multilayer thickness, up to a specific level individual for the three proteins studied. These levels are coded s1 wt%, s2 wt% and s3 wt% for fibrinogen, IgG and HSA, respectively.

At 0.0 wt% salt the HSA layer thickness was 29 Å and at s1 wt% 476 Å. The IgG multilayer thickness at 0.0 wt% salt was 31 Å, and at s3 wt% the thickness stopped increasing, at 300 Å. The fibrinogen multilayer was 258 Å at s2 wt% and after this salt concentration multilayer thickness did not increase with increasing concentration.

A higher NaCl content will mean higher charge shielding, as counter ions will screen the proteins from each other's charge. Differences between the proteins could be caused by differences in charge and structure and the effect this has on the multilayer forming process.

5.1.4 Buffer concentration, effect on multilayer thickness

The impact of buffer concentration on the thickness of the resulting multilayer was studied. Surfaces were incubated in solution of varied buffer concentration. Salt wt%, pH and incubation temperature were: s1 wt%, pH 4.4 and t1°C for HSA; s2
wt%, pH 5.0 and t2°C for fibrinogen; s3 wt%, pH 4.4 and t3°C for IgG. The buffer concentration range investigated went from 1 to 100 mM. The thickness of the multilayers formed on the surfaces were measured with ellipsometry. The results are shown in figure 14.

At 1.4 mM buffer concentration the HSA layer was 87 Å thick. At 6.8 mM this had increased to 314 Å. The maximal thickness was found at 50 mM with 440 Å. Fibrinogen had a thickness of 68 Å at 1.0 mM, this increased with increasing buffer concentration up to 16 mM, after which it lay constant at 120-130 Å. IgG had the maximum multilayer thickness, 360 Å, at the minimal buffer concentration, 1.4 mM. The thickness then decreased until at 50 mM it leveled out at 280 Å.

It was shown that multilayers formed by IgG went thicker with lower concentrations. For HSA and fibrinogen lower buffer concentration meant thinner multilayers. After a concentration of 51 mM for HSA and 16 mM for fibrinogen no statistically significant change in thickness could be detected, at p = 0.01.

![Figure 14](image.png)

**Figure 14.** Thickness of multilayer as a function of the buffer concentration. Differences statistically significant are marked with an asterisk, p = 0.01.
Reasons for the inverse dependency of the IgG multilayer thickness to buffer concentration, compared to that of the HSA and fibrinogen multilayers, are unclear. The buffer concentration used in this paper, 10 mM, is a reasonable compromise between these opposite trends for IgG, HSA and fibrinogen. It is noteworthy that a lower buffer concentration also means a lowered ability to buffer.

Interestingly, in a study performed at 22°C on hydrophilic silicon, increasing the buffer concentration was demonstrated to entail thinner adsorption layers for not only IgG but also for HSA and fibrinogen, at the relevant pH (Ortega-Vinuesa, et al., 1997). The difference in temperature, salinity, choice of buffer and surface most likely explain this difference through their effect on the forces governing the protein adsorption.

5.1.5 Effect of changing the incubation temperature

The incubation temperature was varied whilst other factors were kept constant. Surfaces were incubated in standard incubation solution at different temperatures. Salt wt% and pH for the buffers used were: s1 wt% and pH 4.3 for HSA; s2 wt% and pH 5.4 for fibrinogen; s3 wt% and pH 5.3 for IgG. The resulting multilayer thickness was measured with ellipsometry. The exact results have been classified.

The HSA multilayer was 515 Å at t1°C. For fibrinogen the multilayer formed was 268 Å at t2°C. IgG was 379 Å at t3°C. It was found that changing the incubation temperature gave significant changes in multilayer thickness, regardless of the protein.

Fibrinogen formed multilayers at a much lower temperature than IgG and HSA. Since fibrinogen is a protein, which naturally form networks and attach to surfaces this characteristic is not illogical.

The results shows there is an optimal temperature for all three proteins when it comes to maximal multilayer thickness. This makes sense from a thermodynamic viewpoint, since with higher enthalpy the proteins are more likely to break free of the surface while, with too little kinetic energy, the energy barriers for forming the multilayers are too high.

The fibrinogen multilayer results are comparable to those in a study by Engström, (2009). Under similar protein concentration, temperature and wt% NaCl but in pH
5.5 PBS buffer, she recorded a thickness of 250-270 Å. The small difference compared to the 267 Å measured in this study would seem to indicate a robust production technology.

The question arises what effect the heating of proteins can have on the immunological response to a surface covered with the same protein. It is possible that the denatured proteins will be regarded as something other than their non-denatured counterpart. Hence even though HSA and fibrinogen-covered surfaces induce little or no immunological response, these heat-treated surfaces might. In a study, fibrinogen was heated to 60 °C for ten hours, and then injected into rabbits. The results showed no new antigen structures were formed (Ronneberger, 1986). A closer study of the immunological response to the surfaces designed in this thesis is under way.

**5.2 Further characterization of multilayer**

**5.2.1 Protein multilayer stability up to a week**

Multilayered surfaces were produced according to standard procedure. Salt wt%, pH and incubation temperature used for the proteins were: s1 wt%, pH 4.3 and t1°C for HSA; s2 wt%, pH 5.4 and t2°C for fibrinogen; s3 wt%, pH 5.3 and t3°C for IgG.

Half of the surfaces had their thickness measured with ellipsometry directly after the protein multilayer incubation step, half were after the protein multilayer incubation step incubated in deionized water with 0.9 wt% NaCl at 37°C for one hour, one day or one week, then had their thickness measured. The results are noted in figure 15. This study was used as a very simple model to assess possible behavior in body fluid.
Figure 15. The amount of protein lost from the surface with 1 hour, 1 day and 1 week of incubation in deionized water. The bars with less saturation indicates thickness after incubation. Bars marked with an asterisk show a thickness significantly different from before incubation, $p=0.05$.

A clear difference in stability was demonstrated between the different proteins. The HSA multilayers lost 5%, 43% and 66% of the thickness when incubated for 1 hour, 1 day and 1 week, respectively. The only other statistically significant difference was for IgG with 3% loss in thickness after 1 week, $p = 0.05$. Fibrinogen did not show any loss of multilayer thickness. These results will have an impact on the applications for which the respective protein multilayers could be considered relevant.

Both fibrinogen and IgG are proteins designed for attachment to each other or to surfaces meaning that the multilayers formed by these proteins could be more stable, compared to those out of HSA. Also, the structure of fibrinogen which is elongated, could be better suited for forming more stable protein multilayers compared to the globular HSA. These factors could explain the lower stability of HSA in deionized water.

5.2.2 Protein multilayer stability in different buffers

Multilayer covered surfaces were produced from the incubation solutions for the three proteins. The salt wt%, pH and incubation temperature used were: s1 wt%, pH 4.3 and t1°C for HSA; s2 wt%, pH 5.5 and t2°C for fibrinogen; s3 wt%, pH 5.5 and t3°C for IgG.
Half of these multilayer covered surfaces had their thickness measured directly, half were incubated at 37°C for one hour in a specific solution after which the thickness was measured with ellipsometry. The solutions used were: PBS buffer pH 7.4, 0 and 0.9 wt% NaCl, acetic buffer pH 4.3 and s1 wt% NaCl, deionized water with 0 and 0.9 wt% NaCl.

In the test described in 5.2.1, protein stability up to a week, HSA was found to form the least stable multilayer, hence this protein was studied more extensively in this test, the results of which are seen in figure 16 below.

The results showed that multilayers of HSA lost 42% and 34% of their thickness after 1 hour incubation in PBS buffer of pH 7.4 and salinity 0.0 and 0.9 wt%, respectively. The HSA multilayer did not lose thickness after incubation in the acetic buffer of pH 4.3 and s1 wt% NaCl, nor in the deionized water, with or without NaCl. The fibrinogen multilayers lost 14% of thickness after one hour of incubation in PBS buffer of pH 7.4. Thickness of the IgG multilayers was not affected by the PBS incubation.

There is a notable difference for HSA when incubated in PBS buffer pH 7.4 and acetic buffer pH 4.3. A high electrostatic repulsion between the protein molecules, caused by the higher pH and the low pI of HSA could explain this loss of thickness. That the multilayer structure of IgG and fibrinogen is better at resisting

![Figure 16](image-url)

**Figure 16.** The stability of the different proteins matrices in different buffers during one hour incubation. Before and after represented with strong and weak color respectively. Bars marked with an asterisk show a thickness significantly different from before incubation, p=0.05.
the same pH could then be partly explained by their higher pI as well as the potentially more beneficial structure compared to the globular HSA.

5.2.3 AFM pictures
Surfaces were coated with protein multilayers. Three surfaces for every protein had the thickness measured with ellipsometry and one surface was studied with an AFM microscope. The salt wt%, pH and incubation temperature used were: s1 wt%, pH 4.3 and t1°C for HSA; s2 wt%, pH 5.2 and t2°C for fibrinogen; s3 wt%, pH 5.2 and t3°C IgG. The AFM results are seen in figure 17 below. The recorded ellipsometry thickness was 510 Å, 240 Å and 390 Å for HSA, fibrinogen and IgG, respectively.

The pictures in figure 17 confirms that a layer has been formed on the titanium surface. There are differences between the proteins regarding the homogenicity of the layer. For HSA there are larger bulbs formed while fibrinogen is the more even surface. The resolution is too poor to allow for identification of individual proteins, further conclusions are hard to draw from these pictures. Note that the lateral scale differs between the different protein pictures.

5.2.4 Reproducibility
The multilayer thickness from the investigations performed under similar conditions are collected below, to give an estimation of the reproducibility of the technique. The general production parameters used were: s1 wt%, pH 4.3 ±0.1 and t1°C for HSA; s2 wt%, pH 5.3±0.2 and t2°C for fibrinogen; s3 wt%, pH 5.3±0.2 and t3°C for IgG. The results are seen in figure 18.

The HSA multilayer thicknesses range from 400 to 531 Å, with an average of 486 Å and a standard deviation of 42 Å. For fibrinogen the range is 210 to 268, with an average of 246 Å and a standard deviation of 20 Å. IgG: 300 to 399 Å, average 366 Å, standard deviation 31Å.
Figure 17. AFM pictures of protein multilayers constructed under optimal incubation conditions. 1, 2, 3 and 4 translates into HSA, fibrinogen, IgG and reference. a and b gives the lateral scale of 1000 nm and 10 µm, respectively.
Variations in protein concentration of the protein solution and placement of the surface in the eppendorf tube during incubation are other possible sources of error. An automated production line should increase the reproducibility considerably, as protein concentration and placement of the surface in the tubes will be easier to keep constant. For a lab assistant the method does require some experience for good reproducibility. This, however is not more than what can be expected.

5.3 Alendronic acid levels

5.3.1 Measurement of the total amount of alendronic acid absorbed
Disks and surfaces were incubated in protein solution. The salt wt%, pH and incubation temperature used were: s1 wt%, pH 4.3 and t1°C for HSA; s2 wt%, pH 5.3 and t2°C for fibrinogen; s3 wt%, pH 5.3 and t3°C for IgG.

The surfaces had their thickness measured with ellipsometry and the disks were incubated in alendronic acid solution. The buffer was 2.5 mM, pH 4.0 acetic buffer with a concentration of 0.5 mg ml⁻¹ alendronic acid added, 1 wt% of this ¹⁴C labeled.
The disks were then measured in a liquid scintillation counting machine to measure the total amount of alendronic acid absorbed in the multilayer. The results can be seen below in figure 19.

The thickness for the HSA, fibrinogen and IgG surfaces was 510 Å, 258 Å and 376 Å. The surface concentration of alendronic acid was 294 ng cm⁻², 201 ng cm⁻² and 288 ng cm⁻², respectively.

Partly because of the low number of readings, three for each protein, no statistically significant differences could be established between the different proteins regarding the alendronic acid absorption.

The alendronic acid solution used for incubation has a pH of 4.0. At this pH the proteins are positively charged (pI of 4.7, 5.8 and 6.5-8.0), whereas the alendronic acid is negatively charged. The electrostatic attraction should cause better drug loading into the multilayer. The lower pI of HSA means a lower electrostatic attraction and can hence explain why HSA seem to be the least efficient at storing alendronic acid, per unit of multilayer thickness.

There is probably a significant difference between the amount of protein multilayer formed on the disks compared to the surfaces, since the disks have a higher surface roughness than the surfaces. A higher surface roughness brings larger surface area and more protein multilayer. However, the absolute values for the multilayer thickness formed on the surfaces are not of interest per se. The surface multilayer thicknesses are only used to reveal relative trends in multilayer formation. Thus the difference in surface roughness between the model surface and the implant screws is of little practical significance.

Compared to the results of Engström 2009 for fibrinogen multilayers of approximately 270 Å with alendronic acid functionalization, prepared under similar conditions as in figure 19, she recorded a surface concentration of 160 ng cm⁻², compared to the 200ng cm⁻² noted below in the results of this study. An explanation could be that the surfaces by Engström were dried between the protein multilayer production step and the alendronic acid incubation step. This dehydration of the multilayer slowed down the process as the multilayer had to be rehydrated before uptake of the drug into the matrix was possible.
Figure 19. Surface concentration of alendronic acid. The dots show the multilayer thickness, the bars give the alendronic acid concentration. None of the concentrations were significantly separate from each other, \( p = 0.01 \).

5.3.2 Amount of alendronic acid eluded at one and four hours

Disks and surfaces were incubated in protein solution. The salt wt\%, pH and incubation temperature used were: s1 wt\%, pH 4.3 and t1°C for HSA; s2 wt\%, pH 5.2 and t2°C for fibrinogen; s3 wt\%, pH 5.2 and t3°C for IgG. The surfaces had the thickness measured with ellipsometry. The disks were incubated in alendronic acid solution, acetic buffer of 2.5 mM, pH 4.0, with a concentration of 0.5 mgml\(^{-1}\) alendronic acid added, 1 wt\% of this \(^{14}\)C labeled.

The disks were put in deionized water with physiological wt\% salt, for one or four hours. A sample was then retrieved from each elution liquid, and measured with LSC. The results are summarized in figure 20 below.

HSA, fibrinogen and IgG multilayers formed were of thickness 513 Å, 238 Å and 393 Å. The eluded amounts of alendronic acid at 1 and 4 hours were: 171 ngcm\(^{-2}\) and 165 ngcm\(^{-2}\) for HSA; 112 ngcm\(^{-2}\) and 121 ngcm\(^{-2}\) for fibrinogen; 184 ngcm\(^{-2}\) and 170 ngcm\(^{-2}\) for IgG. The only statistically significant difference was that of the IgG surfaces eluting more alendronic acid than the fibrinogen surfaces, \( p = 0.01 \).

That there is no difference between amounts eluted after one hour compared to four hours implies that for all three multilayers the total amount of alendronic acid available for elution has already been eluted after one hour. When compared to the
amounts in figure 19, 294 ng/cm², 201 ng/cm² and 288 ng/cm² for HSA, fibrinogen and IgG respectively, this amount of alendronic acid available for elution seems to be about half of the total amount of alendronic acid present in each multilayer. Interestingly this fraction is similar to the amount of released bisphosphonate, 30-50%, recorded from chemically immobilized fibrinogen multilayers during overnight incubation in distilled water, as studied by Tengvall et al. 2004. The used bisphosphate, ibandronate, had been spontaneously adsorbed to the multilayer in a similar fashion as in this thesis. In another study by Wermelin et al. 2007, a system with covalently bonded bisphosphonate and fibrinogen, also studied with LSC, gave an approximate release of 60% of the bonded bisphosphonate after 8 hours of incubation in PBS-buffer. However in this study there was a notable difference between the amount eluded at 1 and 4 hours.

It is as of today not known which levels of zoledronic acid that are optimal for improving the fixation of orthopedic implants. It has, however, been demonstrated that a pamidronate/ibandronate surface concentration of 216 ng/cm² improved the mechanical fixation of stainless steel screws in a rat model (Tengvall et al., 2004). Ibandronate and pamidronate have a potency of 1/2 and 1/200, respectively, of that of zoledronate (Bartl et al., 2007). Hence it is possible that the levels demonstrated in figure 20 and 21 have a comparable effect as in Tengvall et al's study.
5.4 Antibacterial functionalization

5.4.1 Serial plate transfer test of gentamicin coated surfaces

Surfaces were coated with a protein multilayer. The salt wt%, pH and incubation temperature used were: s1 wt%, pH 4.3 and t1°C for HSA; s2 wt%, pH 5.3 and t2°C for fibrinogen; s3 wt%, pH 5.3 and t3°C for IgG.

The surfaces were then incubated with gentamicin and had their inhibition zones measured via disk diffusion testing. Half of the surfaces were coated with a protein multilayer and then incubated in a gentamicin solution, the other half were coated with a protein multilayer where the gentamicin was added to the protein incubation solution. After the last incubation step half of the surfaces in each group were rinsed in deionized water, half were not. The surfaces were moved to new agar plates after day 1. The mean and standard deviation of the resulting inhibition zones are summarized in table 1. Example pictures from the study are seen in figure 21. See figure 22 in the appendix for table 1 in chart form.

Table 1 shows that the protein multilayers are able to release enough gentamicin to have a bactericidal effect the first 24 hours, for all variations of gentamicin incorporation. In a study by Kazimoglu et al. zones over 15 mm were evaluated as sensitive (2008).

Comparing the procedure of adding gentamicin during the multilayer construction with that of incubating the protein multilayer in gentamicin afterwards, table 1 indicates that adding the gentamicin during the multilayer construction gives a higher dosage released on the agar plates for all combinations. The exception is for HSA with rinsing after the functionalization incubation step, p=0.01.

Rinsing after the last step made the inhibition zones smaller for all surfaces, p=0.01. That such a large portion of the gentamicin is removed after a brief immersion in liquid indicates that much gentamicin is very loosely associated to the protein multilayer. Hence a fast release of the absorbed substance can be expected.

While the rinsing procedure seemed to introduce a higher variation compared to the results when not rinsing, this was not statistically significant at p=0.01. However,
since rinsing resulted in a lower gentamicin level and a non-improved variation, rinsing should be considered as a contra productive measure.

Figure 21. An example of surfaces with a fibrinogen multilayer where gentamicin has been added at the protein incubation step. Disk one is without rinsing after the last step, disk two is with rinsing and disk three shows the same surfaces as in disk one, but after day two on a new agar plate. The pictures were taken with a standard camera.

Table 1. Recorded inhibition zones for gentamicin coated surfaces, in mm.

<table>
<thead>
<tr>
<th>Protein incubation separate from gentamicin incubation</th>
<th>Rinsing</th>
<th>No rinsing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>HSA</td>
<td>13,0±2,6</td>
<td>0</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>16,1±1,4</td>
<td>0</td>
</tr>
<tr>
<td>IgG</td>
<td>14,9±1,3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein incubation and gentamicin incubation simultaneously</th>
<th>Rinsing</th>
<th>No rinsing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>HSA</td>
<td>16,7±1,5</td>
<td>0</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>14,0±1,4</td>
<td>0</td>
</tr>
<tr>
<td>IgG</td>
<td>14,0±0,8</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls</th>
<th>Rinsing</th>
<th>No rinsing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Reference, 10 µg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reference , 20 µg</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein free surface</td>
<td>11,3±0,2</td>
<td>0</td>
</tr>
</tbody>
</table>

Bactericidal levels for day two were only achieved by those surfaces made from fibrinogen without the last rinsing stage. The reference surface, which totally lacked protein multilayer, showed an increased sensitivity towards rinsing and a
lower amount of gentamicin eluting from it the first 24 hours. It also showed the largest antibacterial effect during hours 24-48.

The results in table 1 indicates that protein multilayer covered surfaces will release between 10 and 20 µg of gentamicin within the first 24 hours. The surfaces used had a surface area of 0.25 cm². This gives an eluted mass of gentamicin the first 24 hours of between 40 and 80 µgcm⁻². If this amount and the release profile are appropriate for an antibacterial functionalization of implants in vivo is uncertain and further testing will be needed. Engström showed that repeated incubations steps could be used for increased multilayer thickness. If increased gentamicin levels are needed, this could be achieved through multiple protein incubation steps or with increased gentamicin concentration in the gentamicin incubation solution. Thicker protein multilayers could mean an elongated release, as the gentamicin will have to diffuse a longer distance to leave the multilayer.

The release profile during the first 24 hours could be closer studied, with readings of every hour rather than for the whole 24 hour period. This will ensure better understanding of the release profile.

As bacteria unable to colonize an implant surface within a couple of days will be taken care of by the immune system, (Gristina, 1987), this gives an indication of what duration an antibacterial functionalization should have to give adequate protection. As such a longer antibacterial effect than the 1-2 days demonstrated in this study could be desirable, depending on the application.

5.4.2 Disk diffusion test of Ag-NP coated surfaces

Surfaces were coated with Ag-NPs and then had their inhibition zones measured via disk diffusion susceptibility. Half of the surfaces were coated with a protein multilayer and then incubated in a Ag-NP solution, the other half were coated with a protein multilayer where the Ag-NPs were added to the protein incubation solution. After the last incubation step half of the surfaces were rinsed in deionized water, half were not.

The results revealed a total lack of antibacterial effect. This included for the pure incubation solution of 0.02 mgml⁻¹ Ag-NPs. In the literature many report gram positive bacteria, such as S. Aureus, to be less susceptible to Ag-NP treatment than
gram negatives ones (Madhumathi et al., 2009), (Shrivastava et al., 2007), (Jain et al., 2009), (Kim et al., 2007). However, at the same time groups such as Alt et al. report complete inhibition of *S. Aureus* by the use of Ag-NPs (2004).

A possible reason for the total lack of antimicrobial effect is that the 10 nm silver nanoparticles did not diffuse out into the agar plate. However, the agar disk diffusion method has been used successfully with 13 nm Ag-NPs from other suppliers, against other strains (Kim et al., 2007), (Pal et al., 2007).

In a study using a similar method of disk diffusion as in this theses, a total CFU of $10^7$ had been inoculated onto an agar plate, and 20 µL of 33nM Ag-NPs, diameter 13.4 nm, was showed to be below the MIC of *S. Aureus* bacteria while 3.3 nM was recorded as the MIC of *E. Coli* and yeast (Kim et al., 2007). This result is not in conflict with the results in this thesis, where 20 µL of 55 nM Ag-NPs of size 10 nm was below the MIC for *S. Aureus* with a CFU count of $10^8$. This congruence indicates that the lack of inhibition is caused by something else then a faulty method. It indicates, as Kim *et al* hypothesizes, that *S. Aureus* requires higher levels of Ag-NPs to be inhibited.

Another possible reason could be that the Ag-NPs used in this thesis for some reason have a weaker effect compared to those used in other studies. Another possibility is that too few Ag-NPs attached to the protein multilayer to have an effect on the bacteria. New test series will have to be attempted before the accuracy of these possibilities can be assessed.
Chapter 6

Conclusion

6.1 Summary of main results
The aim of this thesis was to study if the local drug delivery technology FibMat, developed by the biotech company AddBIO, could be applied to other combinations of plasma proteins and bioactive substances than the fibrinogen and bisphosphonate combination which is today being commercialized.

In the work conducted in this thesis it has been demonstrated that HSA and IgG could be made to form multilayers similar to those made out of fibrinogen. This was achieved by adjusting primarily pH and temperature but also the salinity of the incubation solution. The multilayers show varying degree of stability in solution, a factor which will be important if they are to be used in biomedical applications.

The results demonstrate that multilayers out of HSA and IgG can be made to incorporate and elute the bisphosphonate alendronic acid in similar surface concentrations as those achieved for fibrinogen multilayers.

An antimicrobial functionalization was demonstrated for all three local drug delivery protein multilayers, through incorporation of the antibiotic gentamicin. Incorporation of Ag-NPs proved unsuccessful for giving a similar functionalization.

6.2 Future work
- A commercial medical product needs to have a high degree of reproducibility. Further test series will have to be made to ensure that the reproducibility of the HSA and IgG protein multilayers lie within these regulated parameters.
• If the antibacterial effect will be suitable for commercialization is as of yet, unknown. Repetition of the initial test performed in this thesis will be needed. Also a more in depth study of the antibacterial effect will be needed, with for example analysis of stress levels and ratios of dead/alive bacteria on the surfaces. Investigation of whether the duration of the effect is adequate or if it has to be elongated will also be required.

• A release profile study needs to be conducted to get a satisfactory characterization of the different protein/bio active substance combinations.

• A characterization of the different multilayer’s immunological profile is already under way in another master’s thesis. This will provide central information regarding the product’s biocompatibility.

• The protein multilayer drug delivery technology in this thesis has been discussed with bone applications in mind. Applications such as antibacterial functionalization of catheters is another possibility which could be worth exploring.

• It could be interesting to investigate other proteins and bioactive substances, to further broaden the product potential.

• Investigating the antibacterial effect against strains more susceptible to Ag-NPs such as \textit{E. Coli}, or with Ag-NPs from another source, would be the next logical step in exploring the potential of this application.

• Methods to measure the levels of incorporated gentamicin or Ag-NPs quantitatively need to be worked before further development of these applications can be attempted.
Chapter 7

Acknowledgments

First I would like to thank all the people around me: family, friends, colleagues and combinations of these, who shared their time and positive energy with me during this project, both on and off working hours. They all have a big part in making this an in every way constructive experience. In particular:

- I appreciated the opportunity of working with MSc. Henrik Aronson and MSc. Elin Engström, who gave me the day to day support I needed.
- Dr. Trine Vikinge, CEO of AddBIO, gave me the opportunity of working with this project and was a most supportive and enthusiastic supervisor and CEO. For this I am most grateful.
- I thank my examiner Prof. Pentti Tengvall for his engagement in this project.
- A big thank you goes to Prof. Lennart Nilsson and research engineer Maud Nilsson at Klinisk mikrobiologi for sharing some of their time and resources, making it possible for me to conduct the bacterial studies.
- My colleagues MSc. Anna Nilsson, MSc. Emma Gundersen and MSc. student Maja Richter proved to be valuable comrades and counselors.
- Senior lecturer Eva Enqvist at the Mathematical Statistics Institution helped me sort out the statistics.
- Research engineer Agneta Askendal offered invaluable help in the lab.

My love, Maria Magdalena.
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Figure 22. Inhibition zone for the various combinations of gentamicin incubated protein multilayers.