Bioactive Coatings and Antibacterial Approaches for Titanium Medical Implants

OSCAR JANSON
The aim of this thesis was to characterize and manufacture coatings and surfaces with antibacterial properties and retained or enhanced bioactivity and biocompatibility. The aim was also to study the optimal composition and parameters of mixtures for debridement of bacterial biofilms on titanium surfaces. The mixtures contained TiO$_2$ particles and H$_2$O$_2$ and were irradiated by light to activate reactive oxygen species (ROS) formation.

In the first part of the thesis, characterization of a thin, multifunctional hydroxyapatite (HA) coating was performed. The coating was applied to anodized cancellous bone screws with the purpose of stimulating local bone formation without bonding too firmly and providing local antibacterial effect. Specifications of the coating included a thickness of around 1 µm, high crystallinity, Ca/P ratio close to the theoretical value of 1.67 and comprise the functional groups of HA. Additionally, the adhesion of the coating to the substrate should be stronger than the cohesion of the coating. Characterization results showed that the coating met the specification for all criteria.

In the second part of the thesis, titanium discs were soaked in H$_2$O$_2$ and subsequently in NaOH and Ca(OH)$_2$ to acquire an antibacterial surface that at the same time is bioactive and biocompatible. The surface demonstrated bioactive properties, assessed by soaking in phosphate buffered saline for seven days in 37°C and examined in scanning electron microscopy and X-ray diffraction.

The third part of the thesis consisted of studying the ROS generation of TiO$_2$/H$_2$O$_2$ mixtures irradiated with UV-Vis light, and to study the antibacterial effect of these mixtures on *S. epidermidis* Xen 43 and *Pseudomonas aeruginosa* biofilms on titanium surfaces. The generation of ROS from different TiO$_2$ crystalline forms and different H$_2$O$_2$ concentrations under light UV-Vis irradiation was determined by rhodamine B degradation. It showed that rutile and 1-3.5 mM H$_2$O$_2$ resulted in the highest degradation of all combinations with almost 100% degradation under 365 nm light and 77% degradation under 405 nm light after 10 min.

The debridement of the *S. epidermidis* and *P. aeruginosa* biofilm discs showed that 0.95 M (3%) H$_2$O$_2$ was the most effective parameter for disinfection of the discs. The addition of TiO$_2$ particles showed a significant extra effect in one of the three studies.

**Keywords:** Bioactivity, antibacterial, titanium, hydrogen peroxide, hydroxyapatite, biomaterial

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To my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


* These authors contributed equally to this work and are joint first authors

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Author contribution

Paper I  Part of experimental work and part of writing.

Paper II  Part of planning, part of experimental work and major part of writing.

Paper III  Part of planning, part of experimental work and part of writing.

Paper IV  Part of planning, all experimental work (except specific surface area analysis) and part of writing.

Paper V  Major part of planning, all experimental work and major part of writing.
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<tr>
<td>Ca(OH)$_2$</td>
<td>Calcium hydroxide</td>
</tr>
<tr>
<td>Ca/P</td>
<td>Calcium/phosphorous ratio</td>
</tr>
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<td>CP-Ti</td>
<td>Commercially pure titanium</td>
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<td>EDS</td>
<td>Energy dispersive X-ray diffraction</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EPS</td>
<td>Extracellular polymeric substance</td>
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<td>FIB</td>
<td>Focused ion beam</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HDF</td>
<td>Human dermal fibroblast</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HO$_2$.</td>
<td>Hydroperoxyl radical</td>
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<tr>
<td>MAA</td>
<td>Metabolic activity assay</td>
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<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>•OH</td>
<td>Hydroxyl radical</td>
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<tr>
<td>O$_2$.</td>
<td>Superoxide radical</td>
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<tr>
<td>PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>Platelets derived growth factor</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>RhB</td>
<td>Rhodamine B</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>TAT</td>
<td>Thrombin-antithrombin</td>
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<td>TCC</td>
<td>Terminal complement complex</td>
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<tr>
<td>TiO$_2$</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>Ti64</td>
<td>Titanium alloy Ti-6Al-4V</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>UTHA</td>
<td>Ultrathin hydroxyapatite</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
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<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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1. Introduction

The average human life expectancy in the world has increased drastically during the last hundred years. A higher amount of elderly people than ever, live an active lifestyle and the demand of replacing worn out joints or lost teeth is increasing.

One of the first known implants is dated back to the Mayan population 600 AD who used seashells to restore missing teeth. Radiological studies have shown prominent bone formation around the seashell, similar to around modern dental implants [1].

In the end of the 19th century and beginning of the 20th century, Lane and others started to use screws and plates of steel to fixate bone fractures [2]. The first successful hip joint replacement was performed in 1938 by Smith-Petersen with a mold made of vitallium [3].

One of the earliest osseointegrated implants, was an iridio-platinum implant placed in the jaw-bone of a patient by Greenfield in 1913 [1]. In the following decades, a continuous evolution of implant materials and techniques took place, before Bränemark implanted the first osseointegrated titanium implant in 1965 [4]. Titanium has since then been used as an implant material in for example teeth, hip and knee implants and bone screws. The field of biomaterial implants is an emerging field with need for research to meet the demand to counteract the problems that it is facing. One such problem is implant-associated infections.

Peri-implantitis is an infectious disease affecting dental implants; it is caused by bacterial biofilms formed by periodontal pathogen around the dental implant abutment and approaching the screw, leading to inflammation and associated bone loss. A meta-analysis has estimated the prevalence of peri-implantitis to 22% of all patients with dental implants [5]. This has led to large cost for dentistry and much suffering for the patients. The economical and societal demand for an effective treatment solution for peri-implantitis is thus very high. Similar issues can be found with orthopedic implants. For example, the reoperation rate caused by deep infection two years after hip implant surgery is between 1-2% [6]. But the cost and complications of a revision surgery are much higher than for dental implants.

Antibacterial coatings and surfaces can be classified as either passive or active. Passive coatings are coatings that do not release any antimicrobial substances from the surface. Examples can be surfaces with a specific crystal phase, texture or roughness that impede the bacteria from attaching. An ac-
Active coating is a coating that releases an antimicrobial substance. For example, a hydroxyapatite coating loaded with antibiotics, or antibacterial coatings with inorganic ions such as silver and copper can be considered active antibacterial coatings.

Hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radical ($\cdot\text{OH}$) and superoxide radical ($\text{O}_2^-\cdot$), also known as reactive oxygen species (ROS), are predominately used as bactericides in this thesis. Reactive oxygen species are used by the immune system to eliminate pathogens but can also lead to damage to the cells, if not scavenged by antioxidants.

A fast and strong integration of the implant into the bone are crucial for a successful implantation. The cells of the host are competing with bacteria to colonize the surface; therefore it is important to create a surface that supports osteoinduction.

Titanium in itself mediates good osseointegration, however the anchorage is not through a chemical bond, rather a physical bond and often accompanied with a non-mineral layer separating the implant surface from the bone. Therefore titanium implants can be covered by bioactive coatings like hydroxyapatite (HA). Methods to make the surface directly bioactive by modification of the native oxide layer have been extensively studied. In this thesis the oxide layer is modified by soaking in $\text{H}_2\text{O}_2$, $\text{NaOH}$ and $\text{Ca(OH)}_2$.

The disposition of the thesis is as follows: In the first paper, a hydroxyapatite coating on titanium cancellous bone screw was characterized. In paper II, the bioactivity, biocompatibility and antibacterial properties of $\text{H}_2\text{O}_2$ and alkali modified surfaces were studied. The thromboinflammatory responses of these surfaces were studied in paper III. The ROS generation from light irradiated TiO$_2$ and $\text{H}_2\text{O}_2$ mixtures were studied in paper IV, and finally the antibacterial ability against biofilms of these mixtures was studied in paper V.
2. Aims and objectives

The first general aim and objective for this study was to create surfaces or coatings with the ability to prevent bacterial biofilm formation on the implant and at the same time have a proper bioactivity and biocompatibility.

The specific aim of paper I was to characterize a hydroxyapatite coating on cancellous bone screws with respect to thickness, crystallinity, Ca/P ratio, functional groups and adhesion to the substrate.

The specific aim of paper II was to produce a Ti-peroxy gel layer on commercially pure titanium (CP-Ti) grade 2 discs, which on degradation release H₂O₂. The objective was to obtain antibacterial effect without provoking cytotoxicity. The aim was also to incorporate alkali ions in the Ti-peroxy gel layer, which possibly could produce a bioactive surface.

In paper III, the specific aim was to study the thromboinflammatory response in human whole blood to the surface produced in paper II, to assess if the surface may be bioactive.

The second general aim and objective for this study was to explore the optimal parameters for debridement of biofilm-coated CP-Ti grade 2 discs, with H₂O₂ and photocatalysis of TiO₂ particles.

The specific aim of paper IV was to study which parameters with regards to TiO₂ particle type, H₂O₂ concentration and wavelength of light, generated the most reactive oxygen species (ROS). With the departure from the parameters that generated the most ROS in the paper IV study, the aim in paper V was to explore which parameters are most effective to remove biofilms of the bacteria strains Staphylococcus epidermidis Xen 43 and Pseudomonas aeruginosa from sandblasted and acid etched CP-Ti discs.
3. Theoretical background

3.1 Osseointegrated implants

The term osseointegration was coined by P. I. Brånemark after observing that titanium fused very strongly to bone [4]. Osseointegrated implants can be hip implants, knee implants, bone screws and plates, dental implant and other implants that integrate to the bone.

For an implant to be successful it must attain certain criteria. The first criterion is that it must have the appropriate mechanical properties needed to endure the forces required for that application. The second criteria is that it must be corrosion resistant for the time period it is implanted, and the third criteria is that it must lack cytotoxicity, mutagenicity, immunogenicity and carcinogenicity [7]. It is mainly titanium’s ability to osseointegrate, non-corrosive properties, high strength to density ratio and biocompatibility that make titanium such a popular material for implants in almost all parts of the body. The most relevant implants for this thesis are dental implants and bone screws.

Stainless steel and cobalt chrome are two other materials that are used as implants in the body. The drawbacks of these materials are that they emit ions such as Ni, Co and Cr [8], that can hinder integration. They also have a much higher elastic modulus than bone which can lead to stress shielding. No other metal has as high strength to density ratio as titanium.

Titanium exhibits excellent biocompatibility, due to its thin native oxide layer, that is formed when titanium is exposed to air. The oxide layer is roughly 5 nm thick and amorphous and consists of a mixture of TiO, TiO₂, Ti₂O₃ and Ti₃O₄ [9]. It works as a passivating layer that preclude titanium ions from interacting with blood and other bodily fluids which can corrode the material [7].

Titanium exist in two allotropic phases, the α-phase which has a close packed hexagonal structure (cph) and the β-phase which has a body centered cubic structure (bcc). Titanium used in implants can be divided into unalloyed or α-phase, β-phase and α + β-alloys. The α-phase exists below 883°C and above that temperature it transforms to the β-phase [10]. Alloying with aluminum, oxygen and nitrogen can stabilize the α-phase at lower temperatures and molybdenum, iron, vanadium, chromium and manganese stabilizes the β-phase. Commercially pure titanium consists of the α-phase and is mostly used in dental implants.
There exist four grades of CP-Ti with increasing oxygen content, which increase the tensile strength and decrease the ductility. In dental implants the ductility is important for designing implants with screw threads. The strength is not as important as in load bearing orthopedic implants. Titanium alloy Ti-6Al-4V (Ti64), which is an α + β alloy is the most commonly used alloy for load bearing applications. The biocompatibility is better in pure titanium than in alloys [11], which make it desirable to use pure titanium when high forces is not present.

3.2 Osseointegration

Osseointegration is defined as the direct connection between living bone and an implant on the light microscopic level [9]. Osseointegration is accomplished as a result of the immune system responding to the surface by creating a foreign body response. Surface proteins and the complement system activates the process by forming fibrin and engage granulocytes, mesenchymal stem cells and macrophages [12].

The evolution of the osseointegration process has been described by Terheyden et al. as follows [13]. Seconds after an implant surgery, the serum proteins: albumin, fibrinogen and fibronectin, adhere to the implant surface. The bleeding is stopped by platelets, and platelets release thromboxin, platelet-derived growth factor (PDGF), transforming growth factor alpha and transforming growth factor beta (TGF-β) to stimulate cell division of fibroblasts. Fibrin creates a network called the blood clot. Hours after the surgery, the inflammatory phase starts by bradykinin, released from the platelets, which increases the permeability of the blood vessels. Polymorphonuclear leukocytes are diffused through the endothelial cells of the blood vessels and eliminate bacteria through release of ROS. Macrophages dominate the late inflammatory phase by phagocytosis of bacteria and necrotic cells. Days after the surgery, perivascular cells, which are mesenchymal stem cells on blood vessels, differentiate into osteoblasts and fibroblasts. Osteoclasts are large multinucleated cells that dissolve the bone substance through acidic values and proteolytic enzymes. The dissolved bone releases bone morphogenic protein, TGF-β and PDGF which recruit osteoblasts. Osteoblasts form new bone through expression of proteins such as type I collagen as well as release of calcium phosphates and carbonates. The proliferation phase ends with the formation of woven bone. Weeks after surgery osteocytes are remodeling the woven bone to lamellar bone.

Usually osseointegration of implants are successful, but for older patients or patients with diabetes who suffers from poor bone quality or osteoporosis there is a need to increase the fixation of the implant.
3.3 Bioactive surfaces

Bioglass was invented by Dench in 1969. It was a new type of ceramic material with highly negatively charged surfaces that exhibited bioactivity [14]. Kokubo et al. incorporated alkali ions on TiO₂ surfaces to form a bioactive layer by making the surface negatively charged, thereby forming a bioactive titanium surface [15,16]. According to the ESB consensus conference 1987, a bioactive material is “one which has been designed to induce specific biological activity” [17]. However, in the interaction between an implant and bone, the definition has been stated by Kokubo and Takadama to be a material that bonds to bone [18]. More specifically, they state that a material that is able to form apatite when implanted in the body or in a simulated body fluid is able to form bone. Titanium-hydroxyl groups on the surface attracts Ca²⁺ ions and form amorphous calcium titanate, which subsequently form amorphous calcium phosphate and finally crystalline HA [19]. The apatite layer on the bioactive implant interact with collagen fibrils to bond with the host bone [14].

The negatively charged surfaces lead to superhydrophilicity which is favorable for bioactivity, and binds calcium and phosphate ions, creating a HA layer [20]. Negatively charged surfaces can be made by soaking in solutions with alkali ions. Ion exchange between the surface and the bone leads to a chemical bond [21], making the difference between the cement line and the material surface indistinguishable [22]. Bioactive titanium is osteoconductive i.e. it works as a scaffold for osteoblasts, and calcium incorporated into titanium surfaces have been shown to induce differentiation of osteoblasts from mesenchymal stem cells [23]. The bioactive surface can also recruit osteochondral progenitor cells, this is mediated by the coagulation and complement system that trigger growth factors such as TGF-β, and these in turn signaling to the osteochondral progenitor cells to mature into osteoblasts [24].

An untreated titanium surface is bioinert [25], which means that it is non-reactive with foreign bodies and non-biodegradable by tissues [26]. When coated with hydroxyapatite (HA), which is the inorganic component of bone, a chemical bond is established between the coating and the bone [27]. The biggest advantage is that the fixation occurs faster than without coating [28]. The chemical formula of HA is Ca₅(PO₄)₃OH, but is often written as Ca₁₀(PO₄)₆(OH)₂ because the unit cell comprises two HA molecules. The Ca/P ratio of HA is 1.67 and is an important value when assessing the phase purity.

The most common technique used to coat hydroxyapatite on implants is plasma spraying. The drawback of this coating technique is that it creates a dense HA layer with low porosity [29]. Because of the high temperature needed, the coating also has a thickness of up to 50-75 µm [28]. The high thickness can make the hydroxyapatite coating crack. With physical vapor
deposition it is possible to coat a thinner layer. But because of the line-of-sight limitation of this method, it has problems coating complex surfaces geometries such as screw threads.

The biomimetic hydroxyapatite coating procedure enables formation of bonelike apatite crystals with high bioactivity and good resorption [30]. Furthermore, the coating method can coat complex surfaces at low temperature and biological pH [29]. The nucleation of apatite is stimulated by a local supersaturation in the PBS, caused by release of OH⁻ from the oxide layer, which yields a slight increase in pH of the PBS [31]. Amorphous apatite is formed which subsequently transforms to crystalline HA [31].

### 3.4 Platelet-, coagulation- and complement-activation

Blood is the first substance that comes into contact with the implant during implantation.

High molecular weight proteins like albumin, globulin and fibrinogen are the first substances that attach to the surface. The attachment depends on the properties of the surface. These initially attached proteins constitute the surface that platelets, coagulation system and complement system are exposed to. Platelets aggregate on the surface and are later activated by thrombin, generated from the coagulation system. The platelets also augment the coagulation cascade by releasing factor V and fibrinogen. A blood clot is formed that reduces the healing time. The formation of bone goes through the following stages; blood clot formation, inflammation, granulation tissue formation, cartilagous callus formation, cartilage mineralization, and woven bone formation remodeling [32].

Thrombin-antithrombin (TAT) is a complex that is formed to control the rate of coagulation; it consists of thrombin and the anticoagulant antithrombin. It is a common marker for analyzing if the coagulation system is activated.

When the platelets are activated by thrombin, they release granules which consist of growth factors. These growth factors trigger mesenchymal stem cells to differentiate into osteoblasts, osteoclasts and osteocytes. Therefore is platelet activation an important part in bone formation.

The complement system contributes primarily to the recruitment of the immune system and inflammation. C₃a is an anaphylotoxin that is formed from cleavage from C3. It plays an important role in inflammatory processes.

The terminal complement complex (TCC) consists of the C₅b that attaches to C6, C7, C8 and C9. It forms a complex that is very effective in penetrating the membrane of pathogens, leading to cell lysis. If no cell membranes are present, protein S binds to the complex and makes it inactive in
the solution. The TCC has been reported to increase bone fracture healing [33].

3.5 Implant infection

A bacterial biofilm is a bacterial community encased in an extracellular polymeric substance (EPS) [34]. The EPS consists of polysaccharides, proteins and DNA [35]. The formation of a biofilm is advantageous for the bacteria as they obtain a higher resistance against antimicrobials [36]. The biofilm attach to a surface initially by van der Waals forces and hydrophobic effects as the hydrophobic bacteria have reduced repulsion to the surface [37,38]. Subsequently, it anchors with cell adhesion structures. The antibiotic resistance of biofilms has been reported to be up to 100-1000 times higher compared to planktonic bacteria [39]. The reason for this is reduced penetration of the antibiotics, activation of a stress response, physiological heterogeneity of the biofilm population and presence of persister cells [40]. The bacteria in the biofilm are living in a dormant state and are released when it is overpopulated [41]. The formation of biofilms is favored by rough environments such as shearing or by subinhibitory concentrations of antibiotics. Because of the protected environment in the biofilm, implant infections caused by bacterial biofilms can be very difficult to get rid of and can cause chronic infections. On dental implants the biofilm consists of several hundreds of different strains [42], but on joint prostheses the biofilm consists most often of monospecial staphylococcal biofilms [43].

A bacterium can sense the presence of a surface and express the adhesion protein, which glue the bacteria to the surface. The bacteria communicate with each other by a signal system called quorum sensing [44]. This control the nutrient distribution and movement of bacteria within the biofilm community.

Up to 80% of all infections are caused by biofilms [45], and 60-70% of nosocomial infections are associated with implantation of a biomedical device [46]. Biofilm infected implants cause implant loosening and if the bacteria enter the bloodstream, they can cause infections in other parts of the body. For instance, oral bacteria can cause endocardial inflammation, aspiration pneumonitis and diabetes [47,48].

3.6 Antibacterial approaches for titanium implants

The attachment of biofilms on bone anchored medical implants can impair the osseointegration, and therefore it is important to have the implant free from biofilm to achieve good osseointegration. Antibacterial approaches can be designed to either prevent biofilm formation or remove existing biofilms.
The difficulty in achieving a preventive antibacterial surface is that the surface must be able to be biocompatible with the surrounding human tissue, produce a mechanical or chemical bond to the bone and at the same time be able to repel bacteria [49].

Many different implant coatings have been fabricated to kill bacteria or impede biofilm formation. Some examples are; coatings loaded with antibiotics, coatings comprising non-antibiotic organic antimicrobial agents, coatings containing inorganic antimicrobial agents, adhesion resistant coatings, modification of physical and chemical surface properties, anti-adhesive polymer coatings, biofunctionalization with antibacterial bioactive polymers, and coatings delivering nitrogen monoxide [50].

For already attached bacteria the most effective methods used today are mechanical methods like brushing with curettes or mechanical methods combined with chemical methods. Today there does not exist a broad spectrum antimicrobial chemical debridement method [51]. The chemical methods used are sodium hypochlorite, H₂O₂, chlorhexidine, citric acid or essential oils [52]. Drawbacks with mechanical debridement include damage of the implant surface, which can impede re-osseointegration, especially if the debridement tool is made of a harder material than titanium such as stainless steel [53]. The best results are reached by a combination of mechanical and chemical debridement.

Titanium dioxide particles have the advantage that they both have a mechanical effect when used together with, for example, a titanium brush and the ability to generate ROS, both in dark and particularly when exposed to light. In an application they could be embedded in a hydrogel and light could be applied with a dental lamp of appropriate power and wavelength [53]. Hydrogen peroxide, which in itself is an effective disinfectant, has been shown to have synergistic effects when in contact with TiO₂ and to enhance the photocatalytic effect for the rutile crystal phase in particular [54–56]. Photocatalytic TiO₂ particles have been used either as coating of the surface to prevent biofilm formation [57], or immersed in the biofilm [58]. The photocatalytic approach to eliminate bacterial biofilms can be realized either extracellularly by oxidation of peptidoglycan, polysaccharides and phospholipids in the EPS [59], or intracellularly (nucleic acid, enzymes and coenzymes) [60,61]. The main oxidizer of the EPS is •OH, but O₂⁻ and hydroperoxyl radicals (HO₂⁻) can also cause damage to the bacterial cell membrane.

The purpose of the bioactive antibacterial surface for prevention of biofilm formation is to prevent biofilm from forming in the initial weeks after surgery that is most critical for biofilm formation. At this point, the bacteria reaching the surface are still in a planktonic state and more susceptible for antibacterial substances. After this period, with increased host tissue integration, the infection risk is considerably reduced [62].
The best way to prevent biofilm formation on biomedical implants is to have a sterile implant during the surgery. A proper surgical implantation resulting in a fast and complete osseointegration also play a crucial part in maintaining an infection free surface. Having a completely sterile environment during surgery is very difficult to obtain; moreover hematogenous spread of bacteria can result from bacteria originating from infection sites in other parts of the body [62]. Likewise in a revision surgery, remaining bacteria from a previous infection is almost unavoidable. Therefore a great deal of research has been conducted with the aim of finding an implant surface that has antibacterial properties but at the same time exhibits bioactive and biocompatible qualities.

3.6.1 Photocatalytic activity

Electrons are occupied in orbitals. In a single molecule in the ground state the electrons reside in the highest occupied molecular orbital and can be excited to the lowest unoccupied molecular orbital by addition of energy from for example UV light. In bulk metal the electrons can move freely between the atoms. On the other hand, insulators, for example a crystal, have a large band gap between the valence band and the conduction band that is normally impossible for the electrons to overcome. However, in a semiconductor the bandgap is small enough for the electrons of the valence band to overcome, if enough energy is added by, for instance, UV light. When an electron is excited to the conduction band an electron-hole pair is formed (equation 1). The excited electron in the conduction band can react with surface-adsorbed oxygen (O₂) by donating an electron to the O₂ thereby producing O₂•⁻ (equation 2). The corresponding valence band hole can oxidize water (H₂O) or hydroxyl ions (OH⁻) to result in •OH (equations 3 & 4) (for a schematic description, see figure 1.).

\[
\begin{align*}
\text{TiO}_2 + h\nu &\rightarrow e^- + h^+ \quad (1) \\
e^- + O_2 &\rightarrow O_2•^- \quad (2) \\
h^+ + H_2O &\rightarrow •OH \quad (3) \\
h^+ + OH^- &\rightarrow •OH \quad (4)
\end{align*}
\]

The •OH are highly reactive and can attack the lipid layer of the cell membrane of bacteria leading to cell death [63].

Photocatalysis has becoming increasingly interesting for use in applications such as solar cells for energy conversion, self-cleaning surfaces and also water and air purification [64]. Research has furthermore been conducted for potential use in biomedical applications for treating cancer [65] and as an antibacterial agent in dental applications [66].
Titanium dioxide is a semiconductor and has three common allotropes: rutile, anatase and brookite. Anatase and brookite have a bandgap of approximately 3.2 eV and rutile 3.0 eV. Anatase and brookite are more active photocatalysts than rutile because they possess more active sites and have lower recombination rates, while rutile is less photocatalytic but the photocatalytic effect can be increased when rutile is subjected to low concentrations of H$_2$O$_2$ [55,56,67]. A combination of anatase and rutile, such as Evonik P25, has been shown to exhibit a higher photocatalytic effect than pure crystal particles. This is a result of the electrons being transferred between the two crystal structures, thereby utilizing the lower band gap of rutile to extend the photocatalytic effect into the visible region. At the same time, recombination is avoided when the electrons are transferred to the more stable anatase crystal [68]. In biomedical applications it is desirable to use visible light because it induces no harm to the tissue. Rutile has a bandgap of approximately 3.0 eV, which means that light of up to 410 nm can produce a charge separation.

![Figure 1](image.png)

**Figure 1.** A schematic description of the photocatalytic charge separation into an excited electron in the conduction band and hole in the valence band. The conduction band electron reduces oxygen (O$_2$) to generate superoxide radicals (O$_2^{-}$) and the valence hole oxidize water (H$_2$O) and hydroxyl ions (OH$^-$) to generate hydroxyl radicals (•OH). These radicals can attack the bacterial cell wall.

### 3.6.2 H$_2$O$_2$

When titanium is exposed to air, it forms a thin oxide layer, as has been described in Section 3.1. When this layer is oxidized by H$_2$O$_2$ (as shown in figure 2), it grows thicker and forms a nano-porous TiO$_2$ layer on top [69]. Tengvall et al. have comprehensively investigated the interaction between H$_2$O$_2$ and titanium [70–76]. The studies were mainly performed with TiCl
powder which together with H$_2$O$_2$ form a gel [70], but also on titanium surfaces [72]. When H$_2$O$_2$ comes in contact with titanium, a yellow layer which consists of the following titanium-peroxide complex, is formed [70]:

$$
\text{Ti}^{4+}(\text{OH})_x\text{O}_2^x - \text{Ti}^{4+}(\text{OH})_x\text{O}_2 - \text{Ti}^{4+}(\text{OH})_x - \text{TiO}_2 \cdot n\text{HH}_2\text{O}
$$

Where $x = 1$ or 2. The titanium-peroxy radical (Ti$^{4+}$O$_2^-$) is an important component of this gel, therefore it is called a Ti-peroxy gel [75]. The Ti-peroxy gel formation has been associated with the excellent biocompatibility of titanium. Superoxide radicals reduce the oxidized metal (M) ions (in this case titanium ions) of the Fenton reaction (equation 5), leading to a cyclic continuous production of •OH [71]. When O$_2^-$ are trapped inside the TiOOH adduct of the Ti-peroxy gel layer, the •OH formation is quenched.

$$
M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \cdotOH + OH^- \quad (5)
$$

Hydrogen peroxide is released from the Ti-peroxy gel layer by hydroperoxyl anion separation from the Ti-complexes with subsequent proton uptake or by H$_2$O absorption of Ti-peroxides [75].

The bactericidal properties of Ti-peroxy gel has been investigated by Tengvall et al. on Escherichia coli bacteria, but amorphous titania was only shown to be bactericidal in the presence of myeloperoxidase [75]. Myeloperoxidase is released in vivo by neutrophils, and produces the highly reactive hypochlorous acid together with H$_2$O$_2$ [77]. Hypochlorous acid is effective in eliminating pathogens such as bacteria in the human body. It plays also a crucial part in the respiratory burst by forming ammonium chloride and chloramines which are oxidizing thiols both extracellularly and intracellularly. Hydrogen peroxide can act as an antibacterial agent, it is not reactive by itself, but can by oxidation, form highly reactive •OH. This is leading to lipid peroxidation of the bacterial cell wall and subsequently to cell death. Alternatively, H$_2$O$_2$ can diffuse through the cell wall and by the Fenton reaction react with iron from iron sulphur clusters or hemoglobin, thereby forming •OH that can destroy the DNA of the bacterial cell [78–81]. However several bacteria such as S. epidermidis contain catalase which is an enzyme that neutralizes H$_2$O$_2$. Despite this, H$_2$O$_2$ has been shown to be an effective disinfectant against Streptococcus mutans [82], Staphylococcus epidermidis [82–85], and E. coli [86].

Hydrogen peroxide can be photolyzed and form two •OH when exposed to light with wavelengths below 360 nm (equation 6). It can also be formed via catalysis from O$_2^-$ according to equation 7 & 8.

$$
\text{H}_2\text{O}_2 + h\nu \rightarrow 2\cdot\text{OH} \quad (6)
$$

$$
\text{O}_2^- + \text{H}^+ \rightarrow \text{HO}_2\cdot \quad (7)
$$

$$
2\text{HO}_2\cdot + 2\cdot\text{OH} \rightarrow 2\text{H}_2\text{O}_2 + \text{O}_2 \quad (8)
$$
Figure 2. A titanium disc (dark oval near the bottom of the tube) after soaking in 30% H₂O₂ (w/w) for 1 h at 37°C.
4. Methods

4.1 Biomimetic coating

The biomimetic hydroxyapatite coating in paper I was manufactured by pre-treatment in NaOH for 10 min at 70°C, followed by soaking in PBS with added calcium and magnesium ions for 3 days in 70°C. A 4 L container was used for soaking the screws in PBS, and the volume of PBS was adjusted depending on the number of screws to be coated, to ensure that the volume per screw always was the same for all screws.

4.2 Surface modification

Table 1 shows the modifications steps of the sample groups and control group used in paper II. Before the modification steps the surfaces were cleaned by sonication in acetone, ethanol and Milli-Q water for 15 min each. Ti_Control, Ti_H2O2 and TiAuto_Ca were also used for the whole blood study in paper III. TiAuto_Ca was there denoted Ti_Ca.

Table 1. Outline of the surface modification procedures used in papers II and III.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>(\text{H}_2\text{O}_2) (30% w/w)</th>
<th>NaOH (5 M)</th>
<th>(\text{Ca(OH)}_2) (0.1 mM)</th>
<th>Final treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti_Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ti_H2O2</td>
<td>1 h</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ti25</td>
<td>1 h</td>
<td>15 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ti25_Ca</td>
<td>1 h</td>
<td>15 min</td>
<td>15 min</td>
<td>-</td>
</tr>
<tr>
<td>Ti200</td>
<td>1 h</td>
<td>15 min</td>
<td>-</td>
<td>200°C, 1 h</td>
</tr>
<tr>
<td>Ti200_Ca</td>
<td>1 h</td>
<td>15 min</td>
<td>15 min</td>
<td>200°C, 1 h</td>
</tr>
<tr>
<td>TiAuto</td>
<td>1 h</td>
<td>15 min</td>
<td>-</td>
<td>Autoclave (125°C, 1h)</td>
</tr>
<tr>
<td>TiAuto_Ca</td>
<td>1 h</td>
<td>15 min</td>
<td>15 min</td>
<td>Autoclave (125°C, 1h)</td>
</tr>
</tbody>
</table>
4.3 Scanning electron microscopy (SEM)

In contrast to a light microscope an SEM use electrons instead of light to image the surface of a specimen. This enables higher magnification because of the lower wavelength of the electrons compared to visible light.

The electron beam scans the surface and the amount of electrons received by the detector reveals the topography of the surface. There are two types of detectors used in SEM. The secondary electron detector receives electrons that have been released from the K shell after interaction with low energy electrons. These electrons reside from atoms approximately 1 nm from the surface and yield a clear contrast of the topography. A backscatter detector receives electrons of higher energy that have been back scattered against the atoms. Atoms of higher atomic weight scatter more effectively than lighter atoms. The resolution is dependent on the interaction volume and the size of the electron spot, which in turn depends on the wavelength of the electron and the electron-optical system that produce the beam. In papers I, II and IV a LEO 1530 or 1550 SEM (Zeiss, Oberkochen, Germany) was used and operated at 5 or 10 kV and the secondary detector was used. The inspected surfaces were coated with a thin Au/Pd layer to avoid charging effects.

4.4 Energy dispersive X-ray spectroscopy (EDS)

In materials science, EDS is commonly used to evaluate the composition of the surface of a material. The EDS detector is placed in an SEM. Electrons are accelerated to the surface and excite the electrons of the material’s atoms. When the electrons are de-excited an X-ray that has the same energy as the difference between the higher and the lower electron shell is emitted. A higher acceleration voltage produces a deeper interaction volume. If the energy is too low the electron cannot excite an electron to a higher shell. The smallest detection signal is normally defined as three times the standard deviation of the background count. When the X-ray reaches the detector an electron-hole pair is formed. A voltage is applied to separate the charge carriers and the signal is proportional to the energy of the X-ray photon. In papers I and II, EDS was used to characterize the composition of the surfaces.

4.5 X-Ray Diffraction (XRD)

A crystal is a solid material that consists of atoms, molecules or ions ordered in a microscopic structure, forming a lattice that extends in all directions. The smallest unit of a crystal is called a unit cell. The lattice planes are usually denoted h, k and l and are perpendicular to the x, y and z axis of the unit cell respectively. X-ray diffraction is applied to reveal the crystal structure of
a crystalline material. The sample is exposed to a monochromatic X-ray beam and the intensities are recorded at every orientation of the crystal. Every compound has a unique diffraction pattern. The scattering of the X-rays is measured and the crystal structure of the material can be calculated from equation 9 which is known as Bragg’s law. In equation 9, \(d\) is the distance between the atomic planes, \(\theta\) is the angle between the incoming X-ray and the crystal lattice, \(\lambda\) is the wavelength of the X-ray and \(n\) is an integer.

\[
2d \sin \theta = n\lambda 
\]

(9)

When the atomic planes have the same distance between them as the wavelength of the incoming X-ray there is an interference that produces a double magnitude of the X-ray wavelength. When this is observed the length and angle of the crystal structure can be identified. A high intensity corresponds to high electron density and thereby a higher possibility to identify the crystal structure. The diffraction angle reveals where in the unit cell the high density area is located. In paper I, the crystallinity of the HA coating was assessed. In paper II, thin film XRD was used to assess if HA was present on the surface or not with a Siemens D5000 grazing incidence X-ray diffractometer (GI-XRD; Bruker AXS GmbH, Karlsruhe, Germany).

4.6 Focused Ion Beam (FIB)

Because of the sputtering capacity of a FIB, it is used as a micro- and nanomachining tool. It can also be used at low beam current to image surfaces. In contrast to SEM that uses electrons as beam source, FIB normally uses gallium ions. A FIB is commonly used for preparation of transmission electron microscopy samples, which have to be very thin. A drawback of FIB is that it is damaging to the surface. In paper I it was used to cut a cross section of the hydroxyapatite coating to enable measurement of the thickness of the coating.

4.7 Fourier Transform Infrared spectroscopy (FTIR)

The bonds of molecules vibrate and rotate with the absorption of certain infrared frequencies that are characteristic for that bond. By analyzing these vibrations the identity of the molecules in a sample can be revealed. In an FTIR measurement, light with a wide range of infrared frequencies are irradiated through the sample. This is repeated several times with the frequencies of the light modified every time. A computer then transforms the signal to sinusoidal frequencies. The algorithm used to calculate the absorption at each wavelength is a Fourier transform. Obtaining spectra from a wide range
of wavelengths results in a higher signal to noise ratio than dispersive spectroscopy. In paper I, FTIR was used to study the functional groups of the hydroxyapatite coating.

**Adhesion assessment**

The adhesion of the ultrathin hydroxyapatite (UTHA) coating in paper I was assessed by three different methods. The first method was a scratch test in which a spherical tip of Al₂O₃ was applied to the surface with increasing load from 0 to 100 N during the movement of the tip. The critical pressure at the point of delamination was obtained from equations 10 and 11, where \( F_N \) is the critical load. \( E' \) is the weighted modulus for the substrate and the tip calculated by equation 11, and \( R \) is the radius of the tip.

\[
p_0 = -0.578 \left[ \frac{F_N \cdot E'^2}{R^2} \right]^{1/3} \tag{10}
\]

\[
\frac{1}{E'} = \frac{1-\nu_{Sub}^2}{E_{Sub}} + \frac{1-\nu_{Tip}^2}{E_{Tip}} \tag{11}
\]

The second method was Rockwell indentation which usually is a method to measure hardness, but can also be used to assess the adhesion of a coating. The adhesion of the hydroxyapatite coating was assessed by examining cracks and/or delamination on and off the hydroxyapatite surface, when subjected to an indentation force of 100 kgf applied with a conical diamond tip (Rockwell test machine, Werzeugmaschinen-Kombinat, Leipzig.)

The last method to assess the coating adhesion was screw insertion. A cancellous screw with the UTHA coating was inserted 10 mm into predrilled holes of polyurethane foam which mimic human cancellous bone. The screw was inserted with a hand driven torque screw driver (Bahco model 6973N). The measurement was performed in both “dry” and “wet” conditions, where the “wet” condition was obtained by soaking the foam in PBS in 37°C for 30 min to mimic *in vivo* conditions.

**4.9 Surface roughness**

Surface roughness is quantified as the deviation of the normal vector of the surface. There are many different roughness parameters but the most common is \( R_a \), which is also used in this thesis. \( R_a \) measures the amplitude of the deviation. The surface roughness influence the bone response strongly, with moderately rough surfaces with a \( R_a = 1-2 \, \mu m \) showing the strongest bone response [87]. The roughness of the surfaces in paper II was measured with
an optical profilometer (WYKO NT1100) in the visible shift interferometry mode.

In paper III the surfaces were ground and polished to exhibit a smooth surface, considering the strong influence of surface roughness on platelet activation. The surface roughness was assessed in a contact profilometer (Dektak XT Advance).

4.10 PBS soaking as bioactivity assessment

Bioactivity assessment by soaking in simulated body fluid was first applied by Kokubo et al [88]. The optimal mineral content of the SBF has been updated several times. Forsgren et al. employed calcium and magnesium enriched PBS to coat titanium plates with hydroxyapatite and obtained excellent results [89].

The containers used for soaking in PBS were 50 ml centrifuge tubes and the surface for assessment or coating was placed facing down. This approach was conducted to secure that the apatite formed was bonded to the surface and not just precipitated on the top of the surface [90]. The soaking time in paper II was 7 days at 37°C and the PBS was replaced once during that time, to secure fresh access of minerals. The mechanism of hydroxyapatite formation is the same as described in Section 4.1.

4.11 Whole blood study

All surfaces in contact with blood except the test and control samples were covered with 0.5 g/cm² heparin. Twenty ml of blood was obtained through heparinized tubes into a centrifuge tube from 5 healthy donors (figure 3 a). A total of 1.4 ml blood was inserted into each well of a slide with the sample on top retained by a clamp (figure 3 b & c), and two samples from each test group was used for each donor. The slide was rotated at 22 revolutions/min for 1 h at 37°C (figure 3 d). A zero blood sample denoted “initial” was set aside without incubation and collected in Eppendorf tubes with a final concentration of 4 mM ethylenediaminetetraacetic acid (EDTA). The other samples were also collected in EDTA-filled Eppendorf tubes after the incubation (final concentration 4 mM). The blood samples were analyzed in a cell counter (XP-300 Hematology Analyzer Sysmex Corporation, Japan). The sample discs were fixated in glutaraldehyde, ethanol and hexamethyldisiloxane (for details see ref. [91]), and analyzed in SEM. The remaining blood after the platelet analysis was analyzed in enzyme-linked immunosorbent assay for TAT, C3a and TCC.
4.12 Rhodamine B (RhB) degradation study

In paper IV, the ROS generation of a TiO$_2$/H$_2$O$_2$ mixture, comprising RhB and irradiated by light during 10 min, was assessed. The amount of degraded RhB was examined by monitoring the decrease in absorbance or fluorescence after the light irradiation. The original objective was to continuously measure the absorbance every 15 s for 60 min to obtain a kinetic reaction coefficient as performed by Unosson et al. [92]. However the TiO$_2$ particles obstructed the light beam of the absorbance spectrometer, making such measurement unattainable. Instead 10 min was chosen as a time point to monitor the organic degradation potential. Although the measurement time is longer than is practical in a clinical setting, it offers an adequate assessment of ROS generation obtained by the photocatalytic effect of the different TiO$_2$/H$_2$O$_2$ mixtures. Rhodamine B is also a fluorescent dye and the fluorescence was measured in the second round of experiments in paper IV.
4.13 Antibacterial tests

4.13.1 Direct contact test
In paper II, two different tests were performed to measure the antibacterial effect of the modified titanium surfaces. The first experiment was a direct contact test. This method is an effective method to assay the antibacterial effect on planktonic bacteria on solid surfaces because it is independent of solubility and diffusion [93]. The test was performed by applying a drop of bacteria solution to the surface of the modified titanium discs, followed by incubation at 37°C for 45 min. The incubated discs were subsequently analyzed with luminescence spectroscopy during 7 h. The luminescence measurement was made possible because of the employment of the gene modified strain S. epidermidis Xen 43, which is bioluminescent. The lux gene inserted in the genome of metabolically active organisms offers a way of real time quantification of the bioluminescent microorganism [94]. Bioluminescent light producing microorganisms are very sensitive for subtle changes in cell viability. They are also a favorable option for high throughput screening and low cost because no extra substances are needed.

4.13.2 Biofilm inhibition test
Assessment of bacterial biofilm presents a bigger challenge than planktonic bacteria for obvious reasons.

The second antibacterial test, used in paper II, was a biofilm inhibition test where the bacteria were grown on the surface during 16 h. The non-adherent bacteria were then washed away with PBS and the discs were reincubated with fresh tryptic soy broth (TSB) and measured for luminescence once every hour for 9 h. The luminescence signal is proportional to the amount of planktonic bacteria in the solution. The assumption made was that the amount of released bacteria from the biofilm is proportional to the amount of biofilm bacteria.

4.13.3 Biofilm removal
In paper V, biofilms of S. epidermidis Xen 43 were grown on grit blasted and acid etched CP-Ti discs during 8 h at 37°C. The discs were subsequently decontaminated with the treatment conditions displayed in table 2.

After the decontamination step the discs were immersed in TSB and measured once every hour for up to 18 h, and between the measurements the discs were incubated at 37°C. Pseudomonas aeruginosa was chosen as a strain to test the antibacterial activity against gram negative species, and because it is not bioluminescent a metabolic activity assay (MAA) was per-
formed. In the MAA, the blue, non-fluorescent dye resazurin was used as an indicator of the metabolism. When it is metabolized by bacteria it transforms to resorufin which is a pink, fluorescent dye. After the cleaning procedure the discs were immersed in TSB and resazurin and assessed for fluorescence once per hour for 6 h and between the measurements the discs were incubated at 37°C in an orbital shaker with a rotation speed of 250 rpm. The fluorescence excitation and emission peaks of resazurin are 530 and 590 nm respectively. Five calibration curves with planktonic bacteria from OD 0.02, corresponding to approximately 1.6 x 10^7 cells/mL, down to zero were obtained to compare how much bacteria that were removed from the biofilm. The MAA was also performed on *S. epidermidis Xen 43* as a reference. One disc from each test group was fixated according to the description in Section 4.11 and examined with SEM.

Table 2. Description of the treatment conditions for the debridement of the *S. epidermidis Xen 43* or *P. aeruginosa* biofilm-covered CP-Ti discs, used in paper V. Exposure time was 1 min for all treatment conditions.

<table>
<thead>
<tr>
<th>Sample designation</th>
<th>Concentration rutile (g/l)</th>
<th>Concentration H₂O₂ (mM)</th>
<th>Light, intensity: wavelength (mW/cm²:nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (Positive control)</td>
<td>-</td>
<td>-</td>
<td>Ambient light</td>
</tr>
<tr>
<td>PBS (Negative control)</td>
<td>-</td>
<td>-</td>
<td>Ambient light</td>
</tr>
<tr>
<td>0_Hi_Amb</td>
<td>-</td>
<td>950</td>
<td>Ambient light</td>
</tr>
<tr>
<td>R_Hi_Amb</td>
<td>0.5</td>
<td>950</td>
<td>2.1:405</td>
</tr>
<tr>
<td>R_Low_Li</td>
<td>0.5</td>
<td>2</td>
<td>2.1:405</td>
</tr>
</tbody>
</table>

4.14 Biocompatibility

To assess the biocompatibility of the modified surfaces, the viability of two different cell lines were examined. The first one was human dermal fibroblasts (HDF), which simulates the biocompatibility with the soft tissue. The other one was MC3T3 preosteoblasts which simulates the biocompatibility with the bone. The discs were immersed in cell culture and incubated for 1 h at 37°C. The medium was subsequently removed and 50 μl of cell suspension was added dropwise to each disc. The cells were allowed to attach to the discs during 1 h at room temperature, before 450 μl of cell culture was added to the disc-containing wells. The samples were incubated at 37°C and measurements of the viability were made after 24 h and 72 h. One hour before the viability measurements at 24 h and 72 h, the culture media was replaced with 350 μl fresh media, containing 5% alamarBlue® and incubated for 1 h at 37°C. After the measurement at 24 h, the discs were washed with PBS to remove alamarBlue®, and 500 μl of fresh media was added to the cell-cultured discs. The fluorescence measurements were made with 100 μl of the
solution in a black well plate in a Tecan Infinite M200 plate reader (570 nm excitation, 590 nm emission).
5. Results and discussion

5.1 Characterization of HA coating

In paper I, an UTHA coating was manufactured and characterized. The thickness was in the order of 1 µm and the coating has the ability to be loaded with pharmaceutics for increased implant fixation or antibiotics. It can also be removed without damaging the bone. The UTHA coating was manufactured with a biomimetic coating method, i.e., the HA crystals were formed by nucleation from a PBS solution supersaturated in respect to calcium and phosphate ions. A FIB was used to reveal the thickness of the coating.

As seen in the SEM images in figure 4 the coating was approximately 1 µm thick.

![Figure 4. a & b) Scanning electron micrographs of flaked hydroxyapatite. c & d) Focused ion beam cut hydroxyapatite revealing the thickness of the ultrathin hydroxyapatite coating. Note that the images are taken at a 52° incidence angle.](image)

The XRD spectra in figure 4 showed peaks characteristic for HA.
Figure 5. The X-ray diffraction patterns for UTHA, thick HA and a reference powder. The UTHA coating was too thin to obtain an HA XRD signal. Therefore a thick HA coating was also manufactured by soaking in PBS for 9 days instead of the 3 days for the UTHA coating.

Energy dispersive X-ray spectroscopy showed a Ca/P ratio of the UTHA coating of 1.68 ± 0.15 which is very close to the theoretical value of 1.67. Precipitated powder filtered from the PBS solution showed a Ca/P ratio of 2.03 ± 0.25.

As seen from the FTIR spectra in figure 6, only the precipitated powder displayed an OH peak, PO₄ peaks were observed with all specimens.
Figure 6. The FTIR spectra of the UTHA coating, a thick HA coating on CP-Ti discs, and precipitated powder filtered from the PBS soaking solution after three days. Hydroxyl groups are clearly observed for the precipitated powder and phosphate groups are observed for all three specimens.

A scratch test was performed to measure the adhesion of the HA coating to the implant. The HA coating was smeared out and flattened which indicates that the adhesion to the implant was stronger than the cohesive forces within the coating, see figure 7.
Figure 7. Scanning electron micrograph of the UTHA coating after scratch tested by an alumina sphere. Smearing and flattening can be seen.

A Rockwell indentation test was also performed to test the adhesion of the UTHA coating. As can be seen in figure 8a, no damage was made to the coating at or near the indentation crater which indicates good adhesion. Scanning electron microscopy examination revealed a similar flattening of the coating as was observed with scratch testing, see figure 8b.

Figure 8. a) Optical image of a Rockwell indentation on UTHA coating, b) scanning electron micrograph of an edge of an indentation.

The SEM images of screw insertion in polyurethane foam, displayed in figure 9, showed no detachment of the coating from the threads. This result additionally supports the strong adhesion displayed in the Rockwell and scratch tests.
5.2 Bioactivity

Titanium has a thin native oxide layer. By soaking in 30% H₂O₂ this layer can be made thicker and O₂⁻ can be trapped in the amorphous oxide. When implanted in the body, this layer can slowly leak out H₂O₂ which reduces the
risk for infections. If the patient has impeded bone quality, for example osteoporosis, the osseointegration needs to be enhanced by soaking in alkali ions to yield a bioactive surface.

In paper II the oxide surface was sequentially soaked in NaOH and Ca(OH)$_2$ for 15 min each and, as can be seen in figure 11, yielded a hydroxyapatite layer when subsequently soaked in PBS for 7 days. Soaking in NaOH, with treatment times of 24 h and subsequent heat treatment is a common procedure to acquire crystalline sodium titanate [19]. Forsgren et al. performed a much shorter NaOH soaking of only 10 min on an anodized titanium surface and still managed to precipitate hydroxyapatite on the surface when subsequently soaking the sample in PBS [89]. This anodized titanium had a thick titanium oxide layer and therefore it was assumed that it would be possible to acquire the same bioactivity with similarly short soaking times also with a titania layer formed from H$_2$O$_2$ soaking. Furthermore, Ca$^{2+}$ ions have been shown to additionally increase the bioactivity by its double positive charge, which increases the ionic activity potential of the PBS solution more than Na$^+$ ions do [95]. The applications for this bioactive and antibacterial layer are mainly dental implants, spinal cages and bone screws [19].

Figure 10 shows the amorphous sodium and calcium titanate layers on top of the H$_2$O$_2$ oxidized layer. There is a clear trend that the coverage of the layer increases with Ca$^{2+}$ and heat treatment and heat and pressure (autoclaving).
Figure 10. Scanning electron micrographs of CP-Ti discs after the different treatments. a) Ti_Control, b) Ti_H₂O₂, c) Ti25, d) Ti25_Ca, e) Ti200, f) Ti200_Ca, g) TiAuto, h) TiAuto_Ca.

In figure 11, the XRD patterns show the strongest HA peak for TiAuto_Ca, and there is a clear trend that Ca²⁺ and heat treatment/autoclaving promote bioactivity.
When soaked in whole human blood and incubated for 1 h at 37°C, the modified titanium discs Ti_Ca and Ti_H2O2 displayed coagulation and adhesion of blood to the surface that was similar to the Ti control surface as can be seen from the SEM images in figure 12. The polyvinyl chloride (PVC) surface that functioned as a negative control exhibited distinctly less adhesion and coagulation. As displayed in figure 13, the platelet aggregation, coagulation and complement activation were similar for the modified surfaces and the Ti control, which displayed a significant increase compared to the initial value. The PVC surface demonstrated a significant increase in complement activation, but no coagulation activation which resulted in the absence of platelet activation.
Figure 12. Scanning electron micrographs of the test surfaces after incubation in whole blood for 1 h at 37°C. a & b) Ti_Ca, c & d) Ti_H2O2, e & f) Ti, g & h) PVC. The panel to the right shows a magnification of the framed area to the left. The titanium surfaces showed similar amount of adhered blood cells in contrast to the PVC surface which displayed very limited adhesion.
Figure 13. Amount of cells or proteins left after 1.4 ml blood comprising 0.50 IU heparin/ml was incubated in PVC wells with the test or control specimens on top and rotated for 60 min at 37°C. a) Platelets as percentage of value in blood before incubation, b) amount thrombin-antithrombin (TAT) µg/L, c) amount C3a µg/L, d) amount terminal complement complex (TCC) AU/mL. (*p <0.05, **p < 0.01; ANOVA with Tukey multiple comparison test)

5.3 Antibacterial effect
To assess the antibacterial effect of the modified surfaces, a direct contact test and a biofilm inhibition test were performed with *S. epidermidis Xen 43*, which is a bioluminescent strain. The bacterial growth was assessed with a luminescence spectrometer. As can be seen in figure 14, bacterial cells on TiAuto_Ca and Ti_H2O2 exhibited a 70% and 93%, respectively, decrease in bacterial viability compared to bacterial cells on Ti_Control, in the direct contact test. The biofilm inhibition test yielded a 65% and 83% decrease in bacterial viability for biofilms grown on TiAuto_Ca and Ti_H2O2, respectively, compared to Ti_Control.
**Figure 14.** Antibacterial assessment showing the luminescence of the bioluminescent strain *S. epidermidis* Xen 43 on sample discs, corresponding to the bacterial viability. a) After a 45 min direct contact test, b) after 5 h of incubation following the 45 min direct contact test. Different letters denote statistically significant difference (one-way ANOVA, Tukey post hoc, p < 0.05). c) Biofilm inhibition during 9 h, d) biofilm inhibition after 6 h incubation (one-way ANOVA, Tukey post hoc, p < 0.05).

In **paper IV** we monitored the ROS generation to find the optimal parameters with regards to TiO₂ particle type, H₂O₂ concentration and energy (wavelength) of irradiated light. The optimal parameters were used in **paper V** for debridement of bacterial biofilms. Figure 15 shows that TiO₂ particles and light irradiation of 365 and 405 nm, had a large positive influence on the ROS generation.
Figure 15. Degradation of RhB after 10 min irradiation with light of 365, 405 and 470 nm wavelength, or in darkness, for solutions of 0 or 0.5 g/l TiO$_2$ and 0 or 1.9 M H$_2$O$_2$. Error bars denotes standard deviation for three measurements. Groups with different letters are significantly different at $p < 0.05$ within the same H$_2$O$_2$ and TiO$_2$ concentration. Groups with different numbers are significantly different at $p < 0.05$ within the same irradiation wavelength.

The RhB experiments displayed in figure 16 and 17 confirmed the increase in ROS generation of rutile in combination with H$_2$O$_2$ at concentrations between 1 and 3.5 mM. At H$_2$O$_2$ concentrations of 19.6 mM and higher, the organic degradation was decreased because H$_2$O$_2$ reacts with •OH, forming the less reactive HO$_2$• according to equation 12.

$$\text{H}_2\text{O}_2 + \cdot\text{OH} \rightarrow \text{H}_2\text{O} + \text{HO}_2\cdot$$  \hspace{1cm} (12)
Figure 16. Degradation potential of four TiO$_2$ particle types, measured by the degradation of RhB after 10 min irradiation with light of 365 nm wavelength. Different letters denote significant difference within the same particle type and different numbers denote significant difference within the same H$_2$O$_2$ concentration at p < 0.05.
Figure 17. Degradation potential of four TiO₂ particle types, measured by the degradation of RhB after 10 min irradiation with light of 405 nm wavelength. Different letters denote significant difference within the same particle type and different numbers denote significant difference within the same H₂O₂ concentration at p < 0.05.

In paper V we used as a starting point the results from paper IV which showed that rutile combined with 2 mM of H₂O₂ generated the highest amount of ROS. We also included 0.95 M H₂O₂ because it has been shown in earlier studies to be effective against bacteria and is part of a common treatment against peri-implantitis [52]. The results in paper V showed that 0.95 M was the most effective parameter against both S. epidermidis Xen 43 and P. aeruginosa. Particles only produced an additional effect in one of the experiments, the luminescence experiment with S. epidermidis Xen 43, as shown in figure 18. Light irradiation did not show any statistically significant extra effect at the p < 0.05 level. As can be seen in figure 19, the positive control (Ethanol) did not remove the bacterial biofilm but merely killed the bacteria.
Figure 18. Regrowth of *S. epidermidis* Xen 43 after decontamination treatment. A) Luminescence intensity during 18 h incubation for 4 test groups, positive (Ethanol) and negative (PBS) control. B) Time to reach a luminescence intensity of 2 000 for 3 test groups and Ethanol. (PBS and R_Low_Li reached an intensity of 2 000 before the first measurement after 8 h, and were thereby excluded). Groups with different letters are significantly different at p < 0.05.

Figure 19. Scanning electron micrographs of *S. epidermidis* Xen 43 on discs after decontamination treatments of the 4 test groups, positive (Ethanol) and negative (PBS) control.

The MAA with *S. epidermidis*, as showed in figure 20, indicated that R_Hi_Li was the most effective debridement treatment. As can be seen in figure 21, due to the poor adhesion of the *S. epidermidis* biofilm, a large portion of the biofilm was removed already at the washing step, which could contribute to the large variability in the recorded data.
Figure 20. Regrowth of *S. epidermidis* Xen 43 after decontamination treatments. A) Fluorescence during 6 h incubation for 4 test groups and positive (Ethanol) and negative (PBS) control. B) Fluorescence after 3 h incubation, different letters indicate significant difference (p < 0.05). Groups with an asterisk are significantly different (p < 0.05), from the negative control (PBS).

Figure 21. Scanning electron micrographs of *S. epidermidis* Xen 43 on discs after decontamination treatments of the 4 test groups, positive (Ethanol) and negative (PBS) control.

The MAA with *P. aeruginosa* indicated that the debridement treatments comprising 3% H₂O₂ were the most effective (figure 22). Figure 23 shows that a substantial amount of rutile particles are left after the debridement treatment.
Figure 22. Regrowth of *P. aeruginosa* after decontamination treatment. A) Fluorescence during 6 h incubation for 4 test groups and positive (Ethanol) and negative (PBS) control. B) Fluorescence after 6 h incubation, different letters indicate significant difference and asterisk indicate significant difference from PBS, both at p < 0.05.

Figure 23. Scanning electron micrographs of *P. aeruginosa* on discs after decontamination treatments of the 4 test groups, positive (Ethanol) and negative (PBS) control.

The effective removal of biofilms with 3% H₂O₂ is also supported by the literature [83–85]. A study by Kim et al. [58], used rutile particles under 405 nm irradiation and managed to obtain a 4-log reduction after 20 min. In that study the particles were immersed in the biofilm, enabling the generated radicals to disrupt the biofilm. Visible light on its own, has been shown to be bactericidal against *P. aeruginosa* and *S. aureus* [96], and to be able to reduce biofilms of *Listeria monocytogenes* [97], and *Streptococcus mutans* [98].
The results of the different cleaning solutions in paper V, where 3% H$_2$O$_2$ exhibited the strongest antibacterial effect were not unexpected considering the ability of H$_2$O$_2$ to penetrate the EPS of bacterial biofilms. However considering the strong ROS generation of rutile combined with 2 mM of H$_2$O$_2$ seen in paper IV, a stronger effect with these cleaning parameters was expected. The weak effect on the biofilm with these cleaning parameters can be explained by the short lifetime of •OH, which likely only resulted in oxidation of the bacteria directly in contact with the catalyst. In the RhB degradation study in paper IV, the RhB dye adhered to the surface, thus enabling the short lived radicals to oxidize the dye and thus perhaps overestimating the antibacterial effect one could expect on a biofilm.

A limitation in this study was that the adhesion of the biofilm was relatively weak and thus a significant portion of the biofilm was detached just through washing by pipetting.

Another limitation with this experiment was that the radical generation is limited to the close proximity of the particle surface. In the bacteria studies in paper V it is likely that very few of the particles came in close contact with the biofilm so that they would be able to oxidize the biofilm. In an application where the particles and H$_2$O$_2$ are incorporated or mixed in a hydrogel, the effect might be better if the hydrogel is applied in a very thin layer. This would enable the particles to be in close contact with the bacterial biofilm, thereby facilitating oxidation of the EPS by •OH. Both the surface of the particles and the surface of the bacterial biofilm are negatively charged which further complicates a close contact between particles and biofilm.

5.4 Biocompatibility

The Ti$_2$H$_2$O$_2$ surfaces were toxic against both cell types because the cells had a proliferation of less than 70% compared to the control at both time points, as can be seen in figure 24. The Ti_CaAuto surfaces elicited a decrease in proliferation on HDF cells after 72 h, but further studies need to be performed to evaluate if the proliferation is statistically significantly below the cytotoxicity level, which is 70% of the control specimen [99].
Figure 24. Viability assessment of human dermal fibroblast and MC3T3 pre-osteoblasts on surface modified titanium discs after 24 h and 72 h incubation. The control group was a well containing no sample disc. Different letters denotes significant difference across the time points within each cell type (p < 0.05), analyzed with one-way ANOVA and Tukey post hoc (n=6).
6. Conclusions

The UTHA coating showed characteristics with regards to thickness, composition and adhesion which make it suitable as a coating that can obtain strong fixation and with the possibility to be removed at a later point in time without damaging the bone. It also has excellent properties due to its porosity to be loaded with pharmaceuticals like antibiotics or bisphosphonate.

The titanium surface soaked in a sequence of solutions of H$_2$O$_2$, NaOH and Ca(OH)$_2$ with subsequent autoclaving, showed bioactivity by exhibiting apatite formation on the surface after soaking in PBS for 7 days at 37°C as displayed by XRD.

The surface also induced a platelet, coagulation and complement activation similar to non-modified titanium surfaces. This further supports the bioactive properties of the surface.

Furthermore, it displayed a 70% and 65% decrease in bacterial viability of *S. epidermidis Xen 43* on a direct contact test and biofilm inhibition test respectively. In biocompatibility testing, MC3T3 cells proliferated at the same rate as the control while human dermal fibroblasts decreased with 28% compared to the control after 72 h incubation, so further testing is required to more definitely ascertain biocompatibility.

A mixture of rutile particles and 2 mM H$_2$O$_2$ produced a 77% RhB degradation after 10 min of 405 nm light irradiation, which suggested a possibly effective cleaning or debridement protocol for dental applications using light irradiation not harmful to sensitive tissues. However, the low concentration of H$_2$O$_2$ was however not as successful against bacterial biofilms of *S. epidermidis Xen 43* and *P. aeruginosa* where instead 3% H$_2$O$_2$ displayed the best effect with little or no enhancement with the addition of TiO$_2$ particles or light irradiation.
7. Future work

The adhesion of the putative calcium titanate layer on the surface modified discs should be examined in further studies as should the amount of H$_2$O$_2$ or other ROS released from the surface. A future step in the development of this implant surface is to perform an *in vivo* study. Screws with this modified titanium surface could be implanted in an animal model such as a rat model to evaluate if the bioactivity and antibacterial effect also is present *in vivo*.

In paper V, 3 % H$_2$O$_2$ was shown to be the most effective debridement solution. However, there were several limitations in this study such as non-ideal attachment of the biofilm. Therefore it would be interesting to grow the biofilm in a bioreactor during a longer time to ensure a more representative and well attached biofilm. This may reveal if the •OH created by rutile and low concentrations of H$_2$O$_2$ are able to remove the biofilm or kill the bacteria within the biofilm.
Titan är ett av de vanligaste materialen i medicinska implantat för personer som skadat leder eller blivit av med tänder. Implantaten ger en bra minskning av smärta och ökad rörelsefunktion. Titan existerar i olika faser, α och β, eller i en kombination av de bågge faserna, α + β. Alfa-fas används i tandimplantat, medan α + β eller β-fas används i ortopediska implantat. Anledningen till att just titan är så vanlig som implantatmaterial är att det är biokompatibelt och att det har bra mekaniska egenskaper. Framför allt har titan en elasticitet som är nära bens elasticitet och styrkan i förhållande till densiteten är hög.

Bioaktivitet definieras som förmågan hos ett material eller en medicin att interagera med kroppen. Ett bioaktivt implantat har förmågan att bilda en kemisk bindning mellan implantatytan och benvävnaden. Normalt sett skapar titanimplantat en bra fixering till ben, men hos äldre patienter, diabetespatienter eller andra med nedsatt benhälsa kan implantatets förmåga att binda till ben behöva förbättras.


Ett vanligt problem med implantat är att det kan uppstå infektioner. För att undvika att infektioner inträffar kan implantatytan beläggas med antibakteriella medel. Vi etsade titandiskar av grad 2 med väteperoxid (H₂O₂) för att öka tjockleken på det mycket tunna naturliga oxidlagret som finns på titan. Det bildades då ett amorft lager av titanperoxidkomplex som har visat sig kunna släppa ut H₂O₂ när det långsamt degraderas under förhållanden liknande de som finns i kroppen. Detta lager kan också användas till att ladda natrium- och kalciumjoner i, för att skapa en superhydrofil yta som är bioaktiv och kemiskt kan binda till ben.
Studien visade att en yta med H$_2$O$_2$, natrium och kalcium samt autoklavering under 125 °C, uppvissade bioaktivitet in vitro genom att kristallisera hydroxyapatit på ytan vid dopning i PBS. Den visade också antibakteriell förmåga mot *S. epidermidis Xen 43* vid ett direktkontakttest och biofilminhiberings test. Cytotoxicitetstest visade ingen cytotoxisk effekt mot MC3T3 pre-osteoblaster, mot mänskliga hudfibroblaster var spridningen ganska stor och fler test behöver göras för att klargöra om cytotoxisk effekt föreligger eller inte.

För att mäta hur väl ytan kan växa in i ben mättes även trombocytinbindning, koagulations- och komplementaktivering till ytan efter att ha inkuberats i människoblod från friska donatorer. Trombocyter som blir aktiverade vid ytan skickar ut tillväxtfaktorer som aktiverar benenertering, aktivering sker med hjälp av det intrinsiska koagulationssystemet och komplementsystemet. Vi såg en signifikant ökning av trombocytinbindning, koagulations- och komplementaktivering vilket styrker att den modifierade ytan har en god bioaktivitet.


Detta arbete visade genom mätning av degraderad rhodamin B, att TiO$_2$ av kristallfasen rutill tillsammans med låga koncentrationer H$_2$O$_2$ (1-3.5 mM) och belysning med ljus av 365 eller 405 nm våglängd, genererade betydligt fler RSF än andra kombinationer. I test med bakteriearterna *S. epidermidis Xen 43* och *P. aeruginosa* i biofilm växt på titandiskar av grad 2, visade sig 0.9 M H$_2$O$_2$ vara mest effektivt för dekontaminering. Vi såg delvis en viss ytterligare förbättring med TiO$_2$-partiklar, medan ljusbelysning inte gav någon ytterligare dekontaminerande effekt.
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10. References


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