Antibacterial Strategies for Titanium Biomaterials

ERIK UNOSSON
Titanium and titanium based alloys are widely used in dentistry and orthopedics to replace hard tissue and to mend broken bones. It has become a material of choice due to its low density, high strength, good biocompatibility and its capacity to integrate closely with the bone. Today, modern materials and surgical techniques can enable patients to live longer, and aid in maintaining or regaining mobility for a more fulfilling life. There are, however, instances where implants fail, and one of the primary causes for implant failure is infection.

This thesis deals with two possible ways of reducing or eliminating implant associated infections; TiO$_2$ photocatalysis, where a surface can become antibacterial upon irradiation with UV light; and incorporation of silver, where a subsequent release of silver metal ions result in an antibacterial effect.

For the TiO$_2$ photocatalysis strategy, a simple and cost effective chemical oxidation technique, using hydrogen peroxide (H$_2$O$_2$) and water, was used to create an active TiO$_2$ surface on titanium substrates. This surface was shown to effectively degrade an organic model substance (rhodamine B) by generating reactive oxygen species (ROS) under UV illumination. However, it was shown that Ti-peroxy radical species remaining in the surface after the H$_2$O$_2$-oxidation process, rather than generation of ROS from a heterogeneous photocatalytic process, was responsible for the effect. This discovery was further exploited in a TiO$_2$/H$_2$O$_2$/UV system, which demonstrated synergy effects in both rhodamine B degradation tests and in antibacterial assays.

For the silver ion release strategy, a combinatorial materials science approach was employed. Binary Ag-Ti oxide gradients were co-deposited in a reactive (O$_2$) environment using a custom built physical vapor deposition system, and evaluated for antibacterial properties. The approach enabled synthesis and composition-structure-property evaluation unlikely to have been achieved by traditional means, and the gradient coatings demonstrated antibacterial properties against both S. aureus and S. epidermidis according to silver ion release. The release was shown to depend more on structural features, such as surface area, crystallinity and oxidation state, than on composition.

Ag-Ti oxide gradients were also evaluated under UV illumination, as Ag deposits on crystalline TiO$_2$ can enhance photocatalytic properties. In this work, however, the TiO$_2$ was amorphous and UV illumination caused a slight reduction in the antibacterial effect of silver ions. This was attributed to a UV-induced SOS response in the S. epidermidis bacteria.

The results of this thesis demonstrate that both TiO$_2$ photocatalysis, or UV induced activation of Ti-peroxy radical species, as well as incorporation of silver are viable antibacterial strategies for titanium biomaterials. However, their clinical applications are still pending risk-benefit analyses of potential adverse host tissue responses.

Keywords: Titanium, silver, biomaterial, antibacterial, photocatalysis, hydrogen peroxide, reactive oxygen species, combinatorial materials science
“Truth is stranger than fiction”
Probably Lord Byron

To friends and family
List of Papers

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


V  **Unosson, E., Morgenstern, M., Engqvist, H., Welch, K.** *In vitro* antibacterial properties and UV induced SOS response from *Staphylococcus epidermidis* on Ag/Ti oxide thin films. *Submitted*

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Author’s Contributions

Paper I  Major part of planning and evaluation. All experimental work and major part of writing.

Paper II  Major part of planning and evaluation. All experimental work and major part of writing.

Paper III  Part of planning and experimental work. Major part of evaluation and writing.

Paper IV  Major part of planning, evaluation, experimental work and writing.

Paper V  Major part of planning and evaluation. Part of experimental work and major part of writing.
Also published


Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AgNPs</td>
<td>Silver nanoparticles</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<td>cpTi</td>
<td>Commercially pure titanium</td>
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<td>DCT</td>
<td>Direct contact test</td>
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<td>DOE</td>
<td>Design of experiments</td>
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<td>D-PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
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<td>EDS</td>
<td>Energy dispersive X-ray spectroscopy</td>
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<td>GI-XRD</td>
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<td>HA</td>
<td>Hydroxyapatite</td>
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<td>HT</td>
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<td>ICP-AES</td>
<td>Inductively coupled plasma – atomic emission spectroscopy</td>
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<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
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<td>MRSE</td>
<td>Methicillin resistant <em>Staphylococcus epidermidis</em></td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>PVD</td>
<td>Physical vapor deposition</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<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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<td>Ti64</td>
<td>Ti-6Al-4V alloy</td>
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<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
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<td>UV</td>
<td>Ultraviolet</td>
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Introduction

Sometimes, accidents happen and vital parts of our bodies are in need of replacement or restoration. Other times, as increasingly seen on a global scale, the same parts are worn out by a lifelong service to its host. Thanks to the dedicated work of countless medical professionals, scientists and engineers, however, biocompatible materials, surgical procedures, medicines, and antibiotics have been developed to successfully mend this, significantly improving the quality of life for millions of people. Nevertheless, the complexity of our biological system and its intricate symbiotic relationship with bacteria can still cause materials, surgical procedures or treatments to fail. These challenges, along with longer life expectancies and higher demands from society, have resulted in a pressing need for improvements and the development of new advanced products and technologies.

Following surgical placement of an implant, a foreign body reaction occurs in which the human body tries to decide whether to accept or reject this alien object. If chosen wisely and the conditions are right, the material will be accepted and human cells will colonize the surface, integrating it with the surrounding tissue. Unfortunately, competing forces in the form of bacteria may also colonize the surface, prohibiting a proper integration and causing an infection. In such cases, the implant starts to lose its function and a number of countermeasures have to be taken to save the implant. Antibiotic treatment has long been the go-to solution, but two major traits of bacteria make it difficult to beat infection: their ability to form biofilm and their adaptability, resulting in the development of antibiotic resistance.

The biomaterial most commonly used for replacing missing teeth and mending fractured bones is titanium, or titanium based alloys, largely due to its capacity to bind closely with bone in a process called osseointegration. Modern implants are carefully engineered and the surface is usually modified to improve the osseointegrative properties. Now, a critical next step in the evolution of implant coatings is combining osseointegration with antibacterial functionality, which could further help the body accept the implant and potentially reduce the use of antibiotic drugs. But as will be demonstrated in this thesis, solutions to this task are very elusive and currently the topic of much research.

With the aim of contributing to advancing the knowledge surrounding this challenging issue, this thesis focuses on the development of two antibac-
terial strategies potentially applicable on titanium based implant surfaces: TiO$_2$ photocatalysis and the incorporation and release of silver ions. The former can be seen as a latent on-demand strategy, applicable on implants that penetrate soft tissue such as dental implants or bone-anchored prostheses, which is activated by UV light. The latter is a more active approach, applicable also on totally internal orthopedic implants, where antibacterial silver ions will act to defend the implant site from infection.

The more specific Aims and objectives of the thesis are presented in the next section, followed by a more thorough description of Titanium as a biomaterial. The cascade of events that occur during Bacterial colonization and infection is described thereafter.

To provide the reader with sufficient understanding on how bacteria can be killed by UV irradiation on a semiconductor surface, a section describing Photocatalysis on TiO$_2$ is included. The subsequent sections, Chemical oxidation with H$_2$O$_2$ and Synergies in the TiO$_2$/H$_2$O$_2$/UV system describe the surface modification technique utilized for a large portion of the work presented in this thesis, along with key findings related to bacterial killing by reactive oxygen species. The section Silver as an antibacterial agent introduces the second strategy explored in this thesis. This is followed by a brief overview of Combinatorial materials science and Physical vapor deposition, the concept and method by which titanium and silver were combined in this work. In the sections Composition-structure-property relationships and UV-induced SOS response, the main findings related to bacterial killing via silver ion release are presented. Finally, some Concluding remarks are drawn and Future challenges presented. A description of the main Analytical techniques used during the work is also included at the end.
Aims and objectives

The overall purpose of the work presented in this thesis was to explore, evaluate, and propose potential solutions to the problem of implant associated infections. Regarding dental implants, this problem is usually manifested as peri-implantitis, which is a complex and all too common disease where bone resorption gradually leads to implant failure. For orthopedic implants, infections acquired during or after implant insertion can obstruct or disturb proper tissue integration, making it a leading cause for implant failure.

Understanding the cause of infection is instrumental to developing a treatment or a preventive measure, as well as to recognize the limited room to maneuver without inadvertently causing host cell damage or reduced osseointegration. The aims and objectives were thus, from a materials science perspective and with a clear application in mind, to develop TiO$_2$ surfaces with antibacterial functions that could complement the already biocompatible nature of the material. Inherent to the scientific approach, adjacent discoveries relevant to the advancement of knowledge in the field were also pursued.

More specifically, Papers I, II, and III were aimed at developing a cost effective surface modification technique that would result in a photocatalytically active TiO$_2$ surface. Applied on dental implants, abutments, or Ti based dental crowns and bridges, such a surface would add an on-demand antibacterial function activated by UV light.

By using a combinatorial materials science approach, Papers IV and V were aimed at exploring the composition-structure-property relationships in gradient coatings of silver and titanium. Incorporating the right amount, size, and structure of silver species in a TiO$_2$ coating could render an implant surface actively antibacterial while maintaining its biocompatibility.
Titanium as a biomaterial

Depending on the intended biomedical application, certain criteria have to be met by a biomaterial to enable a successful outcome. For hard tissue replacements, mechanical properties such as strength, Young’s modulus and toughness are critical for a good match with the surrounding bone. Further, the material has to be biocompatible, i.e. it should in essence be non-toxic and perform with an appropriate host response in a specific situation. It should also be highly corrosion resistant since leaching ions can provoke allergic and toxic reactions. For articulating joint replacements, a high wear resistance is also essential as wear debris particles are known to cause several adverse responses, including aseptic loosening of the implant.

In respect to the abovementioned criteria, titanium and titanium based alloys are fairly good biomaterials. They are not as cheap as stainless steels or as fatigue and wear resistant as cobalt chromium alloys, but when it comes to interactions with bone, titanium and titanium based alloys have a set of characteristics making it the material of choice for dental implants. First of all, titanium is a low-density element, allowing for strong lightweight constructions. Secondly, it has a comparably low modulus, which reduces the adverse effects of stress shielding when implanted in bone. The term stress shielding refers to a gradual reduction in bone density that can occur around implants when normal loads are transferred from the bone to the implant. Since bone is a living tissue that remodels according to mechanical stimuli, a reduced load will result in a weaker bone. Thirdly and perhaps most importantly, it spontaneously forms a thin passivation film on its surface when exposed to air. This native titanium dioxide (TiO₂) film results in excellent corrosion resistance and has been deemed largely responsible for the outstanding biocompatibility of early titanium implants. The native film, however, is usually too thin (≈5 nm) and non-uniform to meet the specific demands in modern clinical applications. A number of surface modification techniques have therefore been developed to enhance the properties required for, e.g. osseointegration and/or antibacterial properties, some of which will be described and discussed further on. The construct of a dental implant, placed in the bone and equipped with a tooth-mimicking crown, is illustrated in Figure 1.
Figure 1. Illustration of a dental implant with an accompanying crown, placed in the jawbone.

Granted that the bulk properties of a biomaterial are adequate, the fate of an implant or device is decided by the events taking place at the interface with biological tissue. The development of an intimate contact with bone, i.e., osseointegration, is essential for dental and orthopedic implants, and all aspects of surface characteristics, such as surface chemistry, surface roughness and surface topography have to be considered. If the immune system finds the surface hostile or in some way unfavorable, it will respond with inflammation that may become chronic and encapsulate the implant in fibrous tissue rather than facilitating anchorage directly to the bone. Such an outcome usually requires revision surgery, which involves significant costs and an increased risk for infection.

The biocompatible nature of TiO$_2$ allows for osteoprogenitor cells to be recruited to the implant site and differentiate into osteoblasts, which can make new bone at or around the implant surface. Although some bioactive implant coatings, primarily of hydroxyapatite (HA), can facilitate direct chemical bonding between bone and surface, the bond holding an integrated implant in place is primarily of physical nature. This is why surface roughness parameters and topography, both at the nano- and microscale play an important role. Small features or porosities at the surface allows for individual osteoblasts to adhere and lay down new bone, while larger features can allow ingrowth of bone for better interlocking and anchoring.
Surface modifications of titanium for improved osseointegration therefore strive to optimize properties related to surface chemistry, crystallinity, roughness and topography, all of which affect the surface energy. The surface energy in turn dictates the initial adsorption of water molecules and proteins directly upon implant insertion, which is followed by cell adhesion.\textsuperscript{5,10} Some of the more common techniques that are applied to modify or coat titanium surfaces include anodic oxidation,\textsuperscript{11} sol-gel coating,\textsuperscript{12} ion implantation,\textsuperscript{13} chemical or physical vapor deposition,\textsuperscript{14,15} and plasma spraying.\textsuperscript{16} Chemical treatments using acids, alkaline solutions or hydrogen peroxide are also available and present a flexible and cost effective alternative.\textsuperscript{17,18} The same basic techniques can also be applied to equip the surface with additional features such as photocatalytic properties, or more direct antibacterial properties via incorporation of antibiotics or noble metals. Both these approaches will be discussed in further detail ahead.

In summary, titanium and its alloys have been trialed and tested as a biomaterial for the better part of 50 years, and it still presents the most dependable choice for dental and orthopedic implants.\textsuperscript{5} There are, however, many areas in which its properties can and need to be improved. For instance, lower stiffness and improved wear resistance are crucial objectives for better articulating and load bearing joint replacements. More urgently though, as is explained in the next section, strategies for equipping titanium based implants with antibacterial features need to be developed and applied.
Bacterial colonization and infection

Unfortunately, the same surface properties that make an implant or device hospitable for human cells can also provide a welcoming environment for pathogenic bacteria. In an illustrative phrase coined by Gristina in 1987, the events that take place upon insertion of an implant is described as a “race for the surface”, in which the host cells and opportunistic bacteria compete for occupation of the new real estate. An inert, biocompatible material poses no significant threat to bacteria. On the contrary, it presents a habitable substrate for which bacteria have evolved and adapted to for millions of years. Tissue cells are therefore at a significant disadvantage already from the start, and a foreign surface yet unguarded by the immune system can quickly become conquered and covered in biofilm, the stages of which are illustrated in Figure 2. Once a bacterial community is firmly attached to a comparably immense implant, the regular immune responses such as phagocytosis become effectively impossible. For a successful outcome, it is therefore of utmost importance to eliminate as many routes of infection as possible, and then aid the host tissue cells in broadly defending the implant surface from bacterial colonization.

Figure 2. The five stages of biofilm development. Stage 1: Planktonic (free floating) bacteria adhere to the biomaterial surface. Stage 2: Cells aggregate, form micro colonies and excrete extracellular polymeric substances (EPS), i.e. slime. The attachment becomes irreversible. Stage 3: A biofilm is formed. It matures and cells form multi-layered clusters. Stage 4: Three-dimensional growth and further maturation of the biofilm, providing protection against host defense mechanisms and antibiotics. Stage 5: The biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces. Figure adapted from Monroe under the creative commons license. Image credit: D. Davis.
An implant-associated infection can originate from many sources, and one of the principal means of reducing the risk is rigorous precaution during surgery. Proper ventilation, clothing and sterile instruments, along with discipline in the operating theatre can significantly reduce the risk of perioperative contamination, but no surgical site or implant surface is truly sterile. Even after successful surgical placement of totally internal implants, such as hip, knee or heart valve replacements, an infection can arise by hematogenous spread of bacteria having entered the bloodstream elsewhere. Percutaneous or permucosal implants and devices, which penetrate through skin or a mucous membrane, are at constant risk of infection as they are exposed to the external environment. Such implants and devices include various catheters, sutures, fixation pins and screws, voice prostheses, but also bone anchored prostheses and dental implants.

According to the Swedish Hip Arthroplasty Register (2013), the infection incidence after primary hip replacement surgery can be as low as 0.5-1%. Other complications such as aseptic loosening, however, contribute to a failure rate of 5% within 10 years. Revision surgeries are generally associated with higher risks of infection and other complications, and require significantly more resources than the primary surgery. A revision hip arthroplasty for infection can take five hours instead of three, result in two liters of blood loss instead of one half, require a month in the hospital instead of one week, and cost $100,000 instead of $20,000, all without guaranteeing an implant free of infection.

The primary causative agents responsible for orthopedic implant-associated infections have been identified as *Staphylococcus aureus* and *Staphylococcus epidermidis*, reported present in up to 70% of clinical isolates. Both of these species are common around the human body, with *S. aureus* often residing in the nares and *S. epidermidis* living on the skin, not posing any significant threat but rather keeping other pathogens away. Trouble begins first when the skin barrier is broken, or when the immune system is compromised. Both of these species are strong biofilm formers and have developed resistance to a range of antibiotic drugs. Unfortunately, the methicillin resistant strains of *S. aureus* (MRSA) and *S. epidermidis* (MRSE) are often acquired at the hospital, causing difficult-to-treat infections that add costs and patient discomfort, or worse.

Concerning dental implants, which are currently being placed at an annual rate of ten million globally, an estimated 7% suffer complete failure within 10 years. A majority of these failures are related to peri-implantitis, a disease generally caused by biofilm-producing oral pathogens that lead to inflammation and a progressive bone loss around the implant. Peri-implantitis has been reported to affect around 10% of implants and 20% of patients (thus having multiple implants), and is a major source of concern for patients and dentists alike. Although *S. aureus* and *S. epidermidis* are
causative agents also for peri-implantitis, the etiology of biofilms formed in the oral cavity and on dental implants is usually different from biofilms found on orthopedic implants. Species that cause periodontitis (*Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* etc.), which is a common reason for tooth loss and prescription of a dental implant in the first place, are often also involved in the development of peri-implantitis. Streptococcus mutans, which is another common species around teeth and responsible for caries and plaque formation, have also been found to colonize dental implant surfaces. Peri-implantitis, as well as other implant-associated infections, do not only pose an immediate threat to the implant and the surrounding bone, but can also cause bacteremia if viable bacteria enter the bloodstream. This can induce sepsis, or cause infection and biofilm formation on other, perhaps more sensitive implanted devices such as artificial heart valves.

Monitoring and controlling the events that occur on the surface of an implant is thus of great importance, as downstream consequences of microbial colonization may result in serious complications. As implied in Figure 2, the further a biofilm is allowed to develop, the more difficult it becomes to eliminate. A primary strategy to avoid implant-associated infection is thus to inhibit planktonic bacteria from adhering in the first place. This can be realized by applying hydrophilic anti-adhesive coatings, based on, e.g. polyethylene oxide (or polyethylene glycol). However, such surfaces tend to also inhibit protein adhesion, which limits cellular attachment and makes for poor tissue integration. On the other hand, optimizing the nanotopography of a titanium surface has been shown to reduce bacterial adhesion, while still promoting certain protein adhesion. Another strategy is to equip the surface with its own active defenses, which can be done by doping or loading it with antimicrobial substances such as antibiotics, chitosan, or silver. These substances can either be leaching from the surface to kill bacteria preemptively, or be more or less immobilized on the surface to kill bacteria upon contact, prior to forming a biofilm. For antibiotic loading, however, the risk of eliciting bacterial resistance has to be considered. Silver ions, which will be discussed in further detail ahead, have a lower propensity to develop bacterial resistance, and can also be effective against methicillin-resistant strains. A more passive approach, which can be viewed as an *on-demand* antibacterial strategy, is arming the surface with photocatalytic properties. This approach requires a certain set of surface characteristics, and is only applicable for UV-light accessible areas, i.e. percutaneous or permucosal implants. The theoretical background and basis for this approach is developed in the next section.
Photocatalysis on TiO₂

For a heterogeneous photocatalytic reaction to take place, two principal things are needed: (i) a material having a band gap, and (ii) a photon with sufficient energy. In essence, this translates to (i) a material where electrons cannot move freely without an input of energy, i.e. a semiconductor, and (ii) a lamp that emits light at short enough wavelengths.

All elements and materials consist of atoms, which are built on protons and neutrons that constitute the nucleus. Orbiting the nucleus is a set of negatively charged electrons, leveling the positive charge of the protons. Every orbital (there can be several) represents a specific energy level on part of the electron. Depending on the interatomic spacing in a material, neighboring atoms can either share electrons by having overlapping orbitals/electron states, as in metals, or be separated so an energy gap appears between the electron states, as in insulators and semiconductors. This energy level separation is called a band gap, and constitutes the forbidden energy region that extends from the top of the electron filled valence band to the bottom of the empty conduction band. In insulators, the band gap is wide, whereas in semiconductors it is narrower. This means that if electrons in a semiconductor are provided with a little energy, they can jump the band gap to occupy the conduction band, making the material conductive. Compared to pure silicon (Si), which has a band gap of 1.1 eV, crystalline anatase TiO₂ is considered a wide band gap (3.2 eV) semiconductor, meaning that more energy is required to excite its electrons to the conduction band.

In TiO₂ photocatalysis, the objective is not to conduct electricity, but to generate electron-hole pairs. When generated, the electron-hole pair is at an energetically very unfavorable state, making it short lived (on the order of a nanosecond). In this short lifetime it can either recombine, to release the excess energy as heat, or transfer its charge to molecules adsorbed on the surface of the semiconductor, primarily oxygen (O₂), water (H₂O), or hydroxyl groups (OH⁻). For efficient photocatalysis, the recombination rate should be low, and the charge transfer to adsorbed species should be high. Primary reactions in this process are given in Equations 1-5, and the products superoxide (O₂⁻), hydroxyl radicals (•OH), and hydrogen peroxide (H₂O₂) are classified as reactive oxygen species (ROS), which can exert oxidative stress on nearby organisms, such as bacteria.
TiO$_2$ + h$\nu$ → e$^-$ + h$^+$ \hspace{1cm} (1)

O$_2$ + e$^-$ → O$_2^-$ \hspace{1cm} (2)

H$_2$O + h$^+$ → •OH + H$^+$ \hspace{1cm} (3)

OH$^-$ + h$^+$ → •OH \hspace{1cm} (4)

•OH + •OH → H$_2$O$_2$ \hspace{1cm} (5)

The entire process, which is illustrated by a simple model in Figure 3, is catalyzed by an input of energy in the form of UV light. The anatase form of TiO$_2$ has a band gap of 3.2 eV, which corresponds to the photon energy of light at 385 nm. This wavelength is considered near-UV light, in the UV-A range, and lies close to the visible spectrum (400-700 nm).

The bactericidal activity of photocatalytic TiO$_2$ has been attributed to lipid peroxidation that damages the bacterial cell membrane, leading to lost respiratory activity and ultimately cell death. The lipid peroxidation, itself a free radical chain reaction, is initiated by •OH and propagates by reacting with polyunsaturated phospholipids, which are essential components of the cell membrane. Out of the ROS produced in the photocatalytic reaction, •OH is the most reactive, and therefore the most lethal for bacteria. However, due to its reactivity, it can never travel far from the surface where it was produced, and the bactericidal effect due to •OH production becomes very local. Other ROS, such as H$_2$O$_2$, are less reactive but have been shown to complement •OH by acting on bacteria at a distance.

![Figure 3](image-url)  
*Figure 3.* A simplified illustration of the photocatalytic disinfection process on anatase TiO$_2$. Incoming UV irradiation promotes an electron (e$^-$) to the conduction band, leaving a hole (h$^+$) behind in the valence band. Adsorbed O$_2$ can accept an electron and become reduced to O$_2^-$, while H$_2$O can donate an electron to fill the hole, thereby becoming oxidized to •OH. These, and other potential ROS, can react with organic molecules of nearby bacteria, mineralizing them to CO$_2$ and H$_2$O.
Chemical oxidation with H$_2$O$_2$

As previously mentioned, many surface modification techniques have been developed to enhance the osseointegrative properties of implants, and/or to deter implant-associated infections. In many instances, photocatalytic properties can coincide with bioactivity. Anatase TiO$_2$ coatings have been shown to promote initial osteoblast adhesion, but also to reduce bacterial adhesion when compared to native TiO$_2$. Among the more cost effective methods to modify the surface of titanium is chemical oxidation, which is primarily done by treatment in H$_2$O$_2$ or in alkali solutions, resulting in an amorphous titania gel coating. Such wet chemical treatments also permit a uniform, controlled coating of implants or devices with complex shapes, where other techniques are often limited to a “line-of-sight” deposition or oxidation.

Chemical oxidation with H$_2$O$_2$ was used to modify the surface of commercially pure titanium (cpTi) and/or Ti-6Al-4V alloy (Ti64) in Papers I, II, and III. Previous work had shown that treatments with H$_2$O$_2$, followed by either heat treatments or aging in H$_2$O could induce crystallization of the amorphous titania gel into anatase, resulting in improved bioactivity. For Paper I, it was therefore hypothesized that such treatments could also be used to produce a photocatalytic surface. The purpose of the study was to investigate if a low temperature (80°C) H$_2$O$_2$-oxidation and H$_2$O-aging process of Ti64 could be optimized for adequate photocatalytic activity within a limited treatment time. In Paper II, cpTi and Ti64 substrates were again H$_2$O$_2$-oxidized and H$_2$O-aged according to previous optimization, but were also subjected to heat treatments to improve crystallinity and stability of the photocatalytic films. In Paper III, the optimized TiO$_2$ coating was tested against bacteria in conjunction with UV light and a small addition of H$_2$O$_2$.

The simple steps of the H$_2$O$_2$-oxidation and H$_2$O-aging process, as well as an illustrative image of the resulting surface morphology, are given in Figure 4.
Photocatalytic activity

The optimization of the process, carried out in Paper I, was guided by a design of experiments (DOE) software package (MODDE, Umetrics AB, Umeå, Sweden), where the input variables were limited to oxidation time in $\text{H}_2\text{O}_2$ ($t_1$) and aging time in $\text{H}_2\text{O}$ ($t_2$). The output was a quantification of each resulting surfaces’ photocatalytic activity (PCA). The quantification method is elaborated on in the Analytical techniques section, under Rhodamine B degradation. Briefly, the photocatalytically-induced degradation of an organic dye was monitored over time, yielding a degradation rate constant ($k$) reflecting the PCA of each surface. From the DOE, 16 experimental runs were carried out, varying $t_1$ from 0.5 to 18 h, and $t_2$ from 1.5 to 54 h. Untreated samples were used as control, while samples with $t_1 = 24$ h and $t_2 = 72$ h were used to validate the statistical model.

As time is always of the essence, an explicit goal of the study in Paper I was to develop a simple yet quick modification method of Ti64 for high PCA. One of the intended areas where photocatalytic disinfection can be of use is on Ti-based dental crowns and bridges, which are always custom made to fit a specific patient. Extended oxidation and aging was therefore unwanted, as it would result in longer production lead times. As was shown, however, the PCA depended almost linearly on the total treatment time.

Figure 5 shows the degradation curves of rhodamine B in presence of selected samples under UV illumination. Quantification of the slope of each curve gives the rate constant ($k$), which was used to plot the contour graph in Figure 6a, as well as the relationship with total treatment time in Figure 6b. It was shown that PCA increased with longer $\text{H}_2\text{O}_2$-oxidation times, as well as with longer $\text{H}_2\text{O}$-aging times, but neither direct ($t_1$ and $t_2$) nor interactive ($t_1 \times t_2$) effects were shown statistically significant at $p < 0.05$. However,
when adding $t_1$ and $t_2$ together for a total treatment time, the PCA showed a statistically validated linear dependence. In conclusion, an extended treatment was needed for a satisfactory result.

**Figure 5.** Graph showing the photocatalytic degradation of organic dye rhodamine B in presence of Ti-6Al-4V samples, oxidized in H$_2$O$_2$ and aged in H$_2$O for durations (hours) indicated in the legend.

**Figure 6.** The contour plot in (a) shows a qualitative increase in PCA with both increasing time in H$_2$O$_2$ ($t_1$) and in H$_2$O ($t_2$). However, the direct ($t_1$ and $t_2$) and interactive ($t_1 \times t_2$) effects on PCA could not be statistically validated at $p < 0.05$. On the other hand, plotting PCA against the total treatment time ($t_1 + t_2$), as done in (b), yielded a linear regression with $p < 0.05$. 
Surface characteristics

The structure and morphology of a Ti surface after extended H$_2$O$_2$-oxidation and H$_2$O-aging, compared to the native oxide is illustrated in Figure 7. Thickness of the titania gel layer has been shown to depend almost linearly on treatment time, which results in an increasingly porous structure with greater surface area. The coatings produced in Paper I were found to be amorphous or poorly crystalline, even after extended aging in H$_2$O. This led to the conclusion that an increase in porosity and surface area, rather than anatase content, was responsible for the increase in PCA. The true nature of the coatings in relation with PCA would be further revealed in Paper II.

![Figure 7. Illustration of the native oxide film formed naturally on Ti (above), and the thicker, more porous film formed after 24 h chemical oxidation in H$_2$O$_2$ and aging for 72 h in H$_2$O (below). The Ti/O ratio and density of the oxide increases with proximity to the Ti substrate. Illustration adapted from Variola et al.](image-url)
Synergies in the TiO$_2$/H$_2$O$_2$/UV system

As was noticed in Paper I, the H$_2$O$_2$-oxidized and H$_2$O-aged Ti64 samples showed photocatalytic properties even though they were essentially amorphous. From the Photocatalysis on TiO$_2$ section, we know that for a true photocatalytic reaction to take place, there has to be a band gap and generation of electron-hole pairs, which in turn requires a crystalline structure. What then, caused the PCA on these samples?

Some experiments, where the samples were tested multiple times to check repeatability, also revealed a staggering decline in PCA with repeated use. This decline is displayed in Figure 8, where four consecutive curves represent the samples’ capacity to degrade rhodamine B in a cyclic fashion. The inset in Figure 8 shows the rate constant $k$ for each repetitive run, which demonstrated that the PCA of the same samples was quickly reduced to approximately one tenth of its initial value. Considering the samples were not crystalline, and that longer H$_2$O$_2$-oxidation times had yielded higher PCA, a reasonable explanation was that Ti-peroxy species left from the H$_2$O$_2$ treatment contributed to the initial activity, but eventually became consumed. The details of this photo-activated reaction, however, were not known.

![Figure 8](image_url)

*Figure 8.* Repeated tests on the same samples, H$_2$O$_2$-oxidized and H$_2$O-aged for 24 h and 72 h respectively, revealed a gradual decline in PCA due to consumption of titanium superoxide radical species associated to the surface.
Added benefits with added H$_2$O$_2$

Previous work by Tengvall et al.$^{44,45}$ had shown that stable titanium superoxide radicals (Ti(IV)O$_2^–$) can reside in a Ti-peroxy gel formed by Ti-H$_2$O$_2$ interactions. A later study by Wu et al.$^{46}$ suggested that such superoxide groups could be responsible for an initially high PCA on H$_2$O$_2$-oxidized titanium, but as the superoxide radicals became consumed, the PCA diminished. These findings support the abovementioned theory, but do not fully explain the process by which superoxide or other radical species are activated by UV light. In order to tackle the issue of diminishing PCA, yet trying to make use of the radicals residing in the titania gel layer after H$_2$O$_2$-oxidation, **Paper II** focused on stability and potential synergy effects in the TiO$_2$/H$_2$O$_2$/UV system.

Having H$_2$O$_2$ present during a photocatalytic reaction, which can either be residual from the oxidation process, or actively added prior to the reaction, may be beneficial as H$_2$O$_2$ can scavenge conduction band electrons ($e^–$) to yield $•$OH and OH$^–$, as shown in Equation 6. H$_2$O$_2$ can also absorb UV light directly to yield two $•$OH, as shown in Equation 7. The reaction in Equation 6 requires generation of $e^–$ from a photocatalytic process, while the photolytic splitting of H$_2$O$_2$ in Equation 7 generally requires high energy UV light ($λ$ < 300 nm).$^{50}$ Having an excess of H$_2$O$_2$, however, may also inhibit PCA according to Equation 8, which produces the comparatively less reactive perhydroxyl radical (HO$_2$$•$).

$$\text{H}_2\text{O}_2 + e^– \rightarrow •\text{OH} + \text{OH}^– \quad (6)$$

$$\text{H}_2\text{O}_2 + \text{hv} \rightarrow 2•\text{OH} \quad (7)$$

$$\text{H}_2\text{O}_2 + •\text{OH} \rightarrow \text{H}_2\text{O} + \text{HO}_2$$

For improved stability and PCA of the surfaces, crystallization heat treatments (HT) at 400 – 600°C were conducted on H$_2$O$_2$-oxidized and H$_2$O$_2$-aged cpTi and Ti64 samples in **Paper II**. At or above 500°C, HT demonstrated to induce anatase formation on cpTi, while both anatase and rutile polymorphs of TiO$_2$ was formed on Ti64. The PCA of the surfaces, quantified via degradation of rhodamine B and the rate constant $k$ in Figure 9, was shown to increase with increasing HT temperature, and could be correlated with degree of anatase formation on the samples. The highest noted value, for cpTi H$_2$O$_2$-oxidixed and H$_2$O$_2$-aged for 24 h and 72 h, respectively, then HT at 600°C, came close to matching the initial value of the non-heat treated samples in Figure 8. The PCA of the heat treated samples were also shown to remain stable over several test cycles. It was further noted that the surface morphology of the samples remained essentially unchanged after HT, although a densification of similar coatings have been reported in the litera-
A full treatment, i.e. H$_2$O$_2$-oxidation, H$_2$O-aging and HT at 600 °C, was also found to yield a higher PCA than when skipping the H$_2$O-aging, which indicates a significance of the surface events taking place during this step.

Figure 9. PCA of heat treated samples as a function of heat treatment (HT) temperature. The legend indicates substrate material and the duration (hours) of H$_2$O$_2$-oxidation and H$_2$O-aging.

The H$_2$O-aging step has previously been shown to generate an abundance of hydroxyl groups (Ti-OH) on the surface, which improves bioactivity. Here, the concept of bioactivity implies that bone-like apatite forms spontaneously on the surface when it is immersed in a simulated body fluid (SBF). It has been shown that Ti-OH enhances the apatite deposition process by attracting Ca$^{2+}$ ions present in the SBF, which in turn attracts HPO$_4^{2-}$ ions to form an amorphous calcium phosphate layer, later transforming into crystalline apatite. This surface property is an advantage for any material meant to integrate with bone. Moreover, having Ti-OH present can benefit the photocatalytic reaction by increasing oxygen adsorption, which results in an increased production of O$_2^-$ according to Equation 2. Having more electron scavengers (O$_2$, H$_2$O$_2$ etc.) present at the surface reduces the electron-hole recombination process, which then increases the amount of h$^+$ available to produce •OH, e.g. via Equation 3.

The consequence of adding H$_2$O$_2$ during the photocatalytic reaction is shown in Figure 10. Compared to the previously reported PCA (UV/TiO$_2$: $k \approx 5 \times 10^{-3}$ min$^{-1}$), the degradation rate was approximately five times faster with 3 wt% H$_2$O$_2$ present (UV/H$_2$O$_2$/TiO$_2$: $k \approx 25 \times 10^{-3}$ min$^{-1}$). This clearly demonstrated the capacity and potential synergy effects in the TiO$_2$/H$_2$O$_2$/UV system. Removing the TiO$_2$ samples to investigate photolytic
degradation (UV/H$_2$O$_2$) also revealed a substantial effect, indicating that splitting of H$_2$O$_2$ according to Equation 7 occurred at least to a certain degree, even though the incoming wavelength was > 300 nm. One should however consider that the photolytic reaction occurs throughout the entire volume of the rhodamine B solution, whereas the photocatalytic reaction is confined to the surface of the TiO$_2$ samples.

The considerably lower degradation rates noted for TiO$_2$, H$_2$O$_2$, and H$_2$O$_2$/TiO$_2$ when removing the UV illumination, also in Figure 10, demonstrated that the UV light activation contributes significantly to the capacity of the system. Nevertheless, a notable increase in the degradation rate was seen for the H$_2$O$_2$/TiO$_2$ system compared to H$_2$O$_2$ or TiO$_2$ alone, indicating an autocatalytic reaction at the surface.

![Figure 10](image.png)

*Figure 10.* Normalized degradation curves of rhodamine B under a variety of conditions, as indicated by the legend. Inset shows the quantified PCA of each condition/system. According to increasing value, the PCA ranked as follows: TiO$_2$ < H$_2$O$_2$ < H$_2$O$_2$/TiO$_2$ < UV < UV/TiO$_2$ < UV/H$_2$O$_2$ < UV/H$_2$O$_2$/TiO$_2$. Non-heat treated TiO$_2$ surfaces that were H$_2$O$_2$-oxidized and H$_2$O$_2$-aged for 24 h and 72 h, respectively, were used for the measurements.
Stability of the system

To investigate the stability of both heat treated and non-heat treated samples in the TiO$_2$/H$_2$O$_2$/UV system, cyclic tests, like the ones presented in Figure 8, were conducted. The results are shown in Figure 11, where the solid lines represent repeated tests with Ti64 samples H$_2$O$_2$-oxidized and H$_2$O-aged for 24 h and 72 h, respectively. The dashed lines represent identically H$_2$O$_2$-oxidized and H$_2$O-aged samples, but which were also heat treated for 1 h in air at 600°C.

![Figure 11. Repetitive tests of H$_2$O$_2$-assisted photocatalysis on heat treated (600 °C) and non-heat treated TiO$_2$ samples. The PCA, as quantified by the rhodamine B degradation rate constant $k$ ($\times 10^{-3}$min$^{-1}$), is indicated for each run.](image)

Both heat treated and non-heat treated samples demonstrated high PCA in the TiO$_2$/H$_2$O$_2$/UV system, and the activity was essentially maintained through four repeated runs. However, the non-heat treated samples still outperformed their heat treated counterparts.

It is known that at temperatures above 300 °C, Ti-OH and radical species stabilized in the Ti-peroxy gel are effectively decomposed and crystallization of the layer starts to occur.$^{56}$ Hence, PCA of the heat treated samples was likely due to a heterogeneous photocatalytic reaction on crystalline anatase, enhanced by H$_2$O$_2$ which is a more efficient electron scavenger than O$_2$.$^{57,58}$ On the other hand, PCA of the non-heat treated samples relied less on crystalline anatase, and more on photo-activation of already present radical species. The abundance of Ti-OH would also aid any photocatalytic reaction at the surface. In both cases, photolysis of H$_2$O$_2$ occurred in the background and contributed to the total effect.
In summary, the experiments in Paper II revealed interesting synergies between TiO$_2$, H$_2$O$_2$, and UV light, which were not considered in Paper I. The fact that less crystalline samples, if equipped with Ti-OH and Ti-peroxy radicals, generated a greater effect in the TiO$_2$/H$_2$O$_2$/UV system than samples with anatase did, demonstrates an interesting phenomenon. Granted that the incoming photon energy is sufficient, similar systems have shown the photolytic contribution (UV/H$_2$O$_2$) to dominate the degradation process, while an excess of H$_2$O$_2$ can inhibit pathways of heterogeneous photocatalysis (UV/TiO$_2$) according to, e.g. Equation 8. As the heat treated samples were more dependent on heterogeneous photocatalysis, they were more susceptible to inhibitory effects of H$_2$O$_2$. The non-heat treated samples, on the other hand, could benefit from surface bound Ti-peroxy radicals and potentially have them restored when adding 3% H$_2$O$_2$ for the assisted reaction.

The studies in Papers I and II utilized an organic dye (rhodamine B) as a model substance to evaluate PCA, and although the same radical species that degrade the dye would be responsible for oxidation and mineralization of bacteria, the in vitro performance of the surfaces and systems could not be known for certain. Hence, the work in Paper III was directed against bacteria.

**Effects against bacteria**

After concluding in Paper II that the highest PCA on Ti64 substrates was obtained after 24 h H$_2$O$_2$-oxidation and 72 h H$_2$O-aging, and that this activity could be significantly enhanced and stabilized by adding a small amount of H$_2$O$_2$, these were the conditions tested further in Paper III.

As mentioned in the Bacterial colonization and infection section, one of the most common pathogens causing implant-associated infections is *S. epidermidis*. Another bacterial species, common in plaque and a leading cause for caries, is *S. mutans*. Both of these species are Gram-positive, meaning that they lack an outer membrane present in Gram-negative bacteria. Additionally, *S. mutans* is catalase negative, i.e. missing the enzyme responsible for decomposing hazardous H$_2$O$_2$, which makes it more sensitive to H$_2$O$_2$ exposure.

Both *S. epidermidis* and *S. mutans* are target species for the intended application of the system, i.e. debridement/cleaning of a dental or otherwise tissue penetrating implant surface, which could be at-risk or already afflicted with bacterial infection. Testing the TiO$_2$/H$_2$O$_2$/UV system against these clinically relevant strains would therefore give an interesting look into the different modes of bactericidal action of the system.

One of the challenges in evaluating a system where different modes of action are possible is designing a test that takes all these actions into account,
while making it possible to discern one bactericidal action from another. Such a test was developed for Paper III, and later adapted for bacteria tests in Papers IV and V. Termed a direct contact test (DCT), the idea was to optimize an adhesion-type test for short-range, UV-activated bactericidal surfaces. Briefly, a small amount of bacterial suspension (5-10 µl) is added to the sample surface, and the entity is incubated at 37 °C for a limited time to allow planktonic bacteria to settle on the surface while some of the suspension liquid is evaporated. Depending on the antibacterial function of the surface (contact killing or UV activated), the bactericidal action is initiated either immediately or when turning on the light. Quantification of adhered and viable bacteria can then be done after a certain time point, either by measuring fluorescence, as done in Papers III and V, or by counting colony-forming units (CFUs), as done in Paper IV. Further details on the DCT are given in the Analytical techniques section, under Bacterial viability assays.

For Paper III, the direct effect of H$_2$O$_2$ on S. epidermidis and S. mutans was first evaluated, and the result is shown in Figure 12. It was shown that both species are sensitive to H$_2$O$_2$ exposure but, at levels ≥ 0.1 wt%, S. mutans suffered greater losses. This was attributed to the fact that S. mutans lacks the catalase enzyme, which makes it unable to decompose H$_2$O$_2$. At 3 wt% H$_2$O$_2$, which is considered safe for human use and is routinely applied as an antiseptic in dentistry and on wounds, some 90% reduction in viability was seen for both species after 15 min exposure. In order to resolve synergy effects in the TiO$_2$/H$_2$O$_2$/UV system, however, the intermediate dose (0.1 wt%) was used for further tests.

Results with H$_2$O$_2$-oxidized and H$_2$O-aged Ti64 samples, 0.1 wt% H$_2$O$_2$, and UV light at 365 ± 10 nm in different combinations are presented in Figure 13. The control, denoted Ti in the figure, was a non-oxidized Ti64 surface. In these experiments, 5 µl of bacterial suspension, with or without a supplement of 0.1 wt% H$_2$O$_2$, was spread onto sample surfaces and immediately irradiated with UV light while being incubated at 37°C for 10 or 20 min. The UV light, intensity adjusted to 1.5 mW/cm$^2$, was applied right away since the action of H$_2$O$_2$ starts immediately upon mixing with bacteria.

Compared to the control surface, 20 min exposure to the TiO$_2$/H$_2$O$_2$/UV system caused 99.7% and 98.9% viability reductions of S. epidermidis and S. mutans, respectively. Significant reductions were also seen with photocatalysis (TiO$_2$/UV) and H$_2$O$_2$ exposure (TiO$_2$/H$_2$O$_2$) alone. Interestingly, the H$_2$O$_2$-oxidized and H$_2$O-treated surface (TiO$_2$) also showed antibacterial properties without UV activation, indicating that surface bound Ti-peroxy radicals remaining from the oxidation process had an effect on nearby bacteria.
Figure 12. Direct effect of different H₂O₂ concentrations on bacterial viability, analyzed after 15 min incubation at 37 °C. 100% viability corresponded to $7.8 \times 10^5$ CFU/ml $S. \text{epidermidis}$, and $7.3 \times 10^5$ CFU/ml $S. \text{mutans}$.

Figure 13. Viability of $S. \text{epidermidis}$ and $S. \text{mutans}$ bacteria after 10 and 20 min exposure to individual and synergetic effects of TiO₂ discs, UV light and 0.1 wt% H₂O₂. In the assay, 100% viability corresponded to $1.04 \times 10^6$ CFU/ml $S. \text{epidermidis}$, and $1.28 \times 10^6$ CFU/ml $S. \text{mutans}$. The asterisk (*) indicates a significant difference from Ti at $p < 0.05$. The double asterisk (**) indicates a significant difference from both Ti and TiO₂ at $p < 0.05$.

The results obtained from the bacteria tests in Paper III, shown in Figure 13, correlate well with the rhodamine B degradation data recorded in Paper II. It is obvious, however, that bacteria are more sensitive than rhodamine B to H₂O₂ exposure. Nevertheless, using an organic dye rather than bacteria suspensions to optimize the TiO₂ surface and devise a system for adequate ROS production was shown successful.

The challenge that followed, i.e. developing a rather simple bacterial viability assay that accounts for multiple short-range bactericidal actions, was also quite successful. Through the assay, it was demonstrated that a TiO₂ surface, at-risk of becoming colonized by biofilm-forming bacteria, could be effectively disinfected by a low concentration H₂O₂ and low intensity UV light treatment within an acceptable timeframe. Increasing the H₂O₂ concentration (up to 3 %), as well as the UV light intensity, especially with a device designed to shield sensitive host tissue, could also significantly increase the efficiency of the treatment in a clinical setting.
Silver nitrate (AgNO₃) has been applied to treat wounds, burns and infections for centuries, but its use diminished significantly with the introduction of penicillin in the 1940s. Twenty years later, it returned in the form of silver sulfadiazine, a combined silver/antibiotic cream, to manage burns. More recently, however, silver wound dressings have returned yet again to counter the emergence of antibiotic resistance. Along the way, some more questionable medicinal uses of nanoparticulate or colloidal silver have also been exploited.

The primary use of silver has thus far been as a topical agent, but incorporation of silver nanoparticles (AgNPs) in implant coatings or on medical devices is emerging as a viable antibacterial strategy. Silver-hydrogel coatings on urinary catheters are already in clinical use and have significantly reduced the occurrence of urinary tract infections. Orthopedic and dental implant coatings containing silver or AgNPs are yet to be introduced in a wide clinical setting, but early trials and intense research efforts suggest that it is only a matter of time.

One of the major hurdles to be overcome is the question of dose. For both implant coatings and wound dressings, there has been a lack of standard procedures for determining efficient release rates and minimum inhibitory concentrations (MIC), largely due to the broad scope of technologies applied. It is also worth noting that the ideal Ag⁺ release rate differs widely between different applications. For wound dressings, Ag⁺ concentrations up to 100 ppm are applicable, whereas a suitable Ag⁺ release from an implant surface lies in the ppb range, i.e. three to four orders of magnitude lower.

Mechanism of action

In its ground state, silver is a rather stable noble metal. Nevertheless, small amounts of ions are constantly released in aqueous solutions, and it is the Ag⁺ ion that is responsible for the bactericidal effect.

In a study by Feng et al., it was shown that Ag⁺ interferes with bacterial DNA molecules of Escherichia coli and S. aureus, causing DNA condensation that result in lost replication abilities. The same study also suggested that Ag⁺ interacts with essential thiol groups, inducing the inactivation of
proteins and critical enzymes for the respiratory chain. Similar results have been shown for a wide range of bacterial species and strains, including MRSA, making silver a broad-spectrum antibacterial agent. These bactericidal effects are enhanced by the use of AgNPs, which, by means of their small size can release more Ag\(^+\) due to an increased surface to volume ratio, and can also disturb or penetrate the bacterial cell membrane to induce further damage.

Aside from the bactericidal effects of Ag\(^+\), nanoparticles (NPs) in general have been reported toxic to bacteria. Electrostatic interactions enable NPs to attach to, and disrupt the integrity of the bacterial cell membrane, and similarly to photocatalytic disinfection, NPs can induce oxidative stress on bacteria by triggering the production of ROS.

In suitable doses, silver presents little or no cytotoxic effects. It can also quite easily be deposited on titanium or other biomaterials via a range of established techniques, such as sol-gel, plasma immersion ion implantation, or various physical vapor deposition (PVD) techniques such as magnetron sputtering. As an antibacterial agent with low propensity to develop resistance in bacteria, silver has therefore gained momentum against antibiotics when developing implant coatings to reduce infection. Questions still remain, however, on what is a suitable dose and how silver is best incorporated to provide an adequate Ag\(^+\) release for antibacterial effect, while still maintaining good osseointegration properties and avoiding potential cytotoxicity.

In the work leading up to this thesis, silver and titanium was co-deposited in a reactive environment by means of a combinatorial synthesis approach. The concept of combinatorial materials science is further explored in the next section. The resulting Ag-Ti oxide gradients were characterized and evaluated for antibacterial properties in Papers IV and V.
Combinatorial materials science

Conventional materials research and development is typically conducted by synthesizing and testing a series of samples within a predetermined design space. Further optimization to meet specific property demands is then done by iterative steps where the input parameters are altered individually. This “trial-and-error” approach is a scientifically sound model, but the process can be tedious and the knowledge gained does not necessarily extend beyond our predictive abilities.

An alternative approach to discovery and optimization of new compounds, structures, alloys or molecules is combinatorial materials science. The concept revolves around rapid synthesis and high-throughput screening of different material combinations, which helps to build materials libraries and to find qualitative trends in composition-structure-property relationships. Further exploration or optimization can then be done in areas of interest.

Considering the complexity of just binary or ternary inorganic systems, which can be complemented with oxides and nitrides, it becomes clear that our understanding of possible material combinations is far from complete. The same holds true for organic molecules. Hence, if the goal is to discover new materials or efficiently optimize the properties of known ones, the methods of doing so have to cover more parameters and a larger design space than any conventional method.

This approach can be applied by a number of means, such as automated fabrication and high-throughput screening of polymeric microarrays for cell studies, stem cell growth, or comprehensive surface characterization. Various sputtering or deposition techniques can also be used to create compositional or structural gradients between inorganic materials, which have been applied to produce materials libraries for a wide range of applications.
An illustrative example of a binary concept system, synthesized and evaluated by traditional and combinatorial means, is given in Figure 14. The obvious advantage of the combinatorial approach is that only a single sample is used, saving both time and resources. Another feature is that, with a continuous gradient any number of points can be evaluated, and potential unintended variations between multiple samples due to process parameters can be avoided. Creating combinatorial arrays of materials or molecules also significantly enhances the probability of making new discoveries, which has been shown for both organic and inorganic materials, as well as for pharmaceutical drugs.95,97,98

![Figure 14. Illustration of two different approaches to materials research and development in a binary system: traditional and combinatorial. The combinatorial approach strives to produce a “library” sample that contains the compositional or structural material varieties of interest, rather than producing multiple samples with discrete compositions or structures.](image)

A binary gradient between silver and titanium oxide, like the system illustrated in Figure 14, was synthesized by PVD, characterized and evaluated for antibacterial properties in Paper IV. Further development and evaluation of how incorporation of silver could potentially affect the photocatalytic properties of TiO<sub>2</sub> led to the results presented in Paper V, which demonstrated some intriguing secondary effects of UV illumination on multifunctional coatings.
Physical vapor deposition

PVD involves the physical removal of atoms from a source material, called a target, and the deposition of said atoms as a coating on a designated substrate. A common PVD technique is magnetron sputtering, in which a plasma is generated from ionization of inert gas atoms, usually argon (Ar). The Ar⁺ ions in the plasma are then accelerated towards the target by a magnetic field, and physically eject atoms from the target material. The ejected (sputtered) atoms then journey through the plasma to condense on the substrate, as well as on any other surface in the sputtering chamber, to form a coating. Upon depositing on a surface, there is very little energy remaining to form a thermodynamic equilibrium, meaning that atomic migration to form a crystalline structure is limited. Crystallization of the coating can however be done by heating the substrate, either during or after the deposition.

Aside from the sputtering gas (Ar), the process is performed under vacuum. If non-metallic compounds such as carbides, nitrides or oxides are to be deposited, an additional gas (CH₄, N₂ or O₂) is added. The process is then termed reactive magnetron sputtering, and the reactive gas is ionized along with Ar in the plasma and can react with metal atoms sputtered from the target to form, e.g. an oxide coating on the substrate.

In Papers IV and V, a custom-built combinatorial magnetron sputtering system was used to co-deposit silver and titanium in a reactive (O₂) environment. By means of the geometrical set-up illustrated in Figure 15, binary, combinatorial samples containing a compositional and structural gradient between silver and titanium oxides were produced. To facilitate uniform depositions, and handy characterization and analysis, the coatings were deposited on silicon wafers.

![Figure 15](image)

*Figure 15.* Illustration of the combinatorial magnetron sputtering system used to deposit binary gradients between silver and titanium. The geometrical set-up, with opposing targets facing the substrate at a 45° angle, allows for deposition of a continuous gradient with enrichment in Ag and Ti close to each respective target.
Composition-structure-property relationships

As previously established, titanium is a biocompatible material suitable for hard tissue replacement, whereas silver is intrinsically antibacterial due to leaching of Ag$^+$ ions. These characteristics are also enhanced for their respective oxides. Hence, in an attempt to map and characterize the composition-structure-property relationships that govern the bioactive features of these materials, while demonstrating the benefits of a combinatorial approach, a single Ag-Ti oxide gradient sample was synthesized and evaluated for Paper IV. By co-sputtering silver and titanium in a reactive environment, rather than, e.g. depositing AgNPs on a prefabricated TiO$_2$ surface, the potential for discovering new structures or features would also be improved.

Gradient thin film characteristics

The crystalline structures at extreme positions of the Ag-Ti gradient sample produced in Paper IV are given in Figure 16. Spectrums intermediate of these were found along the gradient.

![Grazing incidence X-ray diffraction patterns](image)

*Figure 16. Grazing incidence X-ray diffraction patterns of the gradient Ag-Ti oxide thin film, showing presence of metallic Ag and traces of rutile TiO$_2$.*

The majority of crystalline content on both ends of the gradient was metallic silver, while signs of rutile TiO$_2$ was found towards the titanium end. Ag-doping of TiO$_2$ has previously been shown to inhibit the growth of anatase in
favor of rutile.99 The significant difference between metallic content of silver and titanium is explained by the oxygen affinity of titanium, causing it to deposit as amorphous or poorly crystalline oxide. The noble silver, however, is less prone to oxidation and could deposit in its metallic form. By refraining from a crystallization heat treatment, the as-deposited metastable structure could be preserved to reveal any potentially new materials with beneficial properties.

An example of a structure unlikely to have remained after heat treatment is shown in Figure 17, which depicts the morphology and cross-sectional view of the gradient coating towards the Ag-side. Here, a rough and porous, yet rather homogeneous surface appeared. Enrichment in Ag could only be seen as slightly more electron dense regions (appearing brighter) in the cross-sectional scanning electron microscopy (SEM) images, which also indicated a potential presence of intermetallic AgTi phases or corresponding oxides. A gradual phase separation, coupled with nucleation and growth of metallic Ag particles, was seen when moving towards the center and Ti-rich end of the gradient, shown in Figure 18. At the Ti-side, silver was present as AgNPs with diameter ranging from 20 to 200 nm, scattered across a considerably denser coating. Smaller AgNPs, having been lodged in the TiO₂ structure during deposition, were seen when examining the cross-section.

![Figure 17. SEM images showing surface morphology at the Ag-side of the gradient coating in (a) and (b). The cross-sectional view in (c) and (d) was cut using a focused ion beam (FIB), and show (from the bottom): Si wafer, Ag-Ti oxide coating (approx. 600 nm thick), and two layers of protective Pt film deposited at different currents.](image-url)
Composition along the gradient was dominated by silver, largely due to its softer nature that gives it a considerably higher sputtering rate. As shown in Figure 19, the compositional spread ranged from 62 wt% Ag, 21 wt% Ti, and 17 wt% O on the Ag-side, to 35 wt% Ag, 35 wt% Ti and 30 wt% O on the Ti-side. In atomic terms, the corresponding content was 27-11 at% Ag, 20-27 at% Ti, and 38-53 at% O, counting from the Ag-side to the Ti-side.

Considering the Ti:O ratio, which is thermodynamically most stable at 1:2, forming compounds of TiO₂, it ranged from 1:2.65 on the Ag-side to 1:2.3 on the Ti-side. This implies that more oxygen was bound to silver at the Ag-side than at the Ti-side, further supporting the claim that the silver content shifted from oxide to metallic form along the gradient.

On the right hand side in Figure 19, contact angle measurements along the gradient are shown, which demonstrated hydrophilic properties on the Ag-side, but an increase in contact angle towards the Ti-side. Illuminating the sample with UV-light to probe for photocatalytic or superhydrophilic properties resulted in a general decrease of the contact angle, indicating a slight degree of photoactivity. The lower contact angles on the Ag-side were in line with its more porous and rough appearance, contributing to a larger specific surface area and higher surface energy.¹⁰⁰
Resulting properties

The antibacterial properties of the Ag-Ti oxide gradient were tested against *S. aureus* in a bacterial adhesion test, quantified by CFU counting. Briefly, 10 µl of *S. aureus* suspension was spread onto 1 cm² samples cut from three parts along the gradient (Ag-side, center, and Ti-side), and incubated at 37°C for 2 h. Adhered bacteria were then removed by sonication, plated on agar, and incubated overnight before counting viable colonies. The test was performed twice with duplicates for each position on the gradient, resulting in n = 4.

The result, showed in Figure 20, demonstrated a clear antibacterial effect compared to the control, with statistically significant reductions in survival rate for all positions tested along the gradient (*p* < 0.05). The differences, however, were great along the gradient with 99.6% reduction at the Ag-side compared to 17% reduction at the Ti-side. This was correlated with the Ag⁺ release curves, obtained via inductively coupled plasma-atomic emission spectroscopy (ICP-AES), shown to the right in Figure 20.

The Ag⁺ ion release study demonstrated a significantly higher dissolution rate of the coating at the Ag-side than at the Ti-side. This difference was much greater than the reported difference in composition (see Figure 19), illustrating that the structural variety along the gradient had a major impact on the resulting properties.
Figure 20. On the left: calculated survival rate (%) and number of colonies remaining after 2 h contact with different parts of the Ag-Ti oxide gradient coating and plating on agar. On the right: cumulative Ag⁺ ion release from the Ag-side and Ti-side of the gradient.

The Ag⁺ ion release study was performed in Dulbecco’s phosphate buffered saline (D-PBS), containing MgCl₂ and CaCl₂. Since the D-PBS has an ion concentration somewhat similar to that of blood plasma, it can be likened with an SBF and the release study doubled as an assay for in vitro apatite formation on the Ag- and Ti-sides of the sample. In short, if HA deposits on the surface when submerged in D-PBS, it can be considered an indication of bioactivity.¹⁰¹

Both the Ag-side and Ti-side of the gradient sample demonstrated bioactive properties, but with varying degree. Figure 21 shows the precipitated HA layer on the Ag-side in (a), and the scattered HA particles found on the Ti-side in (b). The GI-XRD spectra in (c) verified the precipitated species as HA, but also demonstrated that they contained traces of silver.

The greater coverage of HA on the Ag-side than on the Ti-side goes hand in hand with the contact angle measurements shown in Figure 19, which indicated a higher surface energy towards the Ag-side. Increased surface hydration and HA deposition also indicates a greater surface hydroxylation, as was discussed in relation to the H₂O₂-oxidized and H₂O-aged titanium in Paper II.
Figure 21. SEM images showing precipitated HA on the Ag-side in (a) and on the Ti-side in (b) after being submerged in D-PBS for 7 days. GI-XRD spectrum in (c) verifying the layers as HA with traces of silver.

The deposited HA layer was poorly adhered and easily removed. In Figure 22, the structural appearance and composition of the underlying Ag- and Ti-side surfaces are shown. Summarizing the above-mentioned findings and looking at these surfaces, one can appreciate the difference in corrosion and consequent release of Ag\(^+\) ions, governed by structural features such as crystallinity and oxidation state. On the Ti-side, previously adhered AgNPs were no longer present, but the underlying structure and composition was nearly intact. This indicated poor adhesion of the formed AgNPs, and that the released Ag\(^+\) ions primarily came from this source. The Ag-side, on the other hand, demonstrated a visibly corroded surface with considerably less silver than before, indicating more structural dissolution of the coating. To draw any conclusions regarding biocompatibility, further \textit{in vitro} cell studies would have to be done. It is likely that the high dose of Ag\(^+\) released from the Ag-side would have caused adverse effects also in mammalian cells, but a more acceptable release is also plausible within the confines of the gradient. What is more important, however, is that the study showed clearly how structure trumps composition in terms of Ag\(^+\) release and resulting antibacterial effect, which is a key aspect to consider.
Figure 22. SEM images of the Ag-side (a) and Ti-side (b) surfaces after performing the Ag\(^+\) ion release study in D-PBS. Surface composition quantified by EDS in (c). The precipitated HA layer was removed prior to analysis.

As a tool to explore potentially new compounds and structures, or efficiently screen materials for new applications, the combinatorial approach has several advantages over more traditional methods. As demonstrated in Paper IV, a single gradient sample can be produced to have very distinct properties from one end to another. Producing the same results by traditional means would require a series of coatings and extremely tight control over process parameters. A true combinatorial (or high-throughput) process, however, should preferably be complemented with automated or otherwise highly efficient characterization and property screening. To this end, the current approach is still in need of some development. Nevertheless, the composition-structure-property relationships displayed in Paper IV can very well shape the development of future silver-based antibacterial implant coatings, where structure can be tailored to provide a therapeutic but non-toxic release of Ag\(^+\).
UV-induced SOS response

Similarly to the study in Paper IV, a combinatorial approach was used to deposit Ag-Ti oxide gradients in Paper V. The aim was to further evaluate antibacterial properties along the gradient, this time against *S. epidermidis*, and investigate potential photocatalytic properties of Ag-doped TiO₂.

The photocatalytic disinfection process on TiO₂, as well as the antibacterial function of Ag⁺, has already been laid out. What remains to explore, however, is how these two strategies can be combined to further improve the antibacterial effect in multifunctional coatings.

Photocatalysis on Ag-doped TiO₂

Noble metal doping, or deposition of noble metals on TiO₂ coatings have been shown to enhance the photocatalytic antibacterial effect in two ways; by providing electron traps that reduce electron-hole recombination, and by interfering with the band-gap to increase visible light absorption. The proposed process is illustrated in Figure 23. The trapping of electrons, excited across the band-gap, occurs according to scheme (a) and can increase the incidence of charge transfer to adsorbed O₂ and H₂O. Sensibility to visible light is enabled by the surface plasmon resonance of adhered AgNPs, which can render an injection of e⁻ to the TiO₂ conduction band, as illustrated in (b).

Again, a number of technologies are available to Ag-dope or deposit Ag particles on TiO₂, and the reactive magnetron sputtering used in Paper IV is a good and versatile alternative. However, the coating was deposited at room temperature to enable characterization and property evaluation of metastable phases, resulting in poor TiO₂ crystallinity. Heat treating the amorphous TiO₂ layer, e.g. like was done for the H₂O₂-oxidized samples in Paper II, would likely induce crystallization for improved PCA, but would most certainly also induce phase separation and a loss of some beneficial properties discovered in Paper IV. Hence, the samples produced for Paper V were still sputtered at room temperature and tested without any crystallization heat treatment. Additionally, similar structures of Ag-doped TiO₂, deposited via magnetron sputtering at low temperature, have previously shown an enablement or enhancement of photocatalytic reactions.
Figure 23. Illustration of how Ag-doping can aid or enhance photocatalytic reactions on TiO$_2$. In (a), Ag deposits can act as electron traps; reducing electron-hole recombination and thereby enhancing the charge transfer to adsorbed O$_2$ and H$_2$O. In (b), surface plasmon resonance effects in adhered AgNPs, responsive to visible light, can render an injection of electrons to the conduction band of TiO$_2$.

New gradient characteristics

Using the same set-up and similar deposition parameters as in Paper IV, a series of identical Ag-Ti oxide gradient coatings, along with Ag and Ti oxide reference coatings, were produced for Paper V. Three areas along the gradient (A, B, and C as depicted on top in Figure 24) were characterized and tested. The elemental composition and crystalline structure of these areas, compared to Ag and Ti reference samples that were also produced via magnetron sputtering, are also presented in Figure 24. A thin Ti binding layer was first deposited on all substrates, explaining the presence of Ti in the Ag reference sample. This presence, however, should be considered an artifact on part of the EDS quantification, which is related to the excitation volume created by the incoming electron beam, in this instance set to 7 kV. As seen in the cross-section images at the bottom of Figure 24, the Ag reference coating was thin (70 nm) compared to the Ag-Ti gradient (310–360 nm) and the Ti reference (200 nm). Hence, no Ti was present at the very top of the Ag reference, and the Ti content in the other samples might be slightly over-estimated.

Compared to the gradient produced in Paper IV, where the Ag content varied from 11 to 27 at%, the gradient in Paper V had an Ag content between 9 and 34 at%. The structural variety, however, was not as distinct as before. Area “A”, close to the Ti-side on samples produced in Paper V, bore more resemblance to the center of the gradient produced in Paper IV.
Moreover, no columnar TiO$_2$ structure laden with AgNPs could be seen along the gradient in Figure 24, indicating a general shift towards Ag oxide in samples produced for Paper V. The GI-XRD data also indicated that crystalline Ag$_2$O was present on the Ag reference, and that the Ti reference was essentially amorphous.

![Figure 24. Summary of sample characteristics from Paper V: composition, crystalline structure and cross-sectional appearance of Ag-Ti oxide gradient and reference coatings. Illustration on top shows geometry of the Ag-Ti gradient and the position of areas A (Ti-rich), B (center), and C (Ag-rich).](image)

**Secondary effects of UV illumination**

The bacterial viability assay in Paper V was developed from the methods used in Papers III and IV, and the target bacterium was *S. epidermidis*. In brief, 10 µl of bacteria suspension was spread onto 1 cm$^2$ samples cut from each area of interest, then incubated for 30 min at 37°C and 100% relative humidity. One set of samples was then irradiated with UV light (365 nm, 3.0 mW/cm$^2$) for 10 min, while another set was kept in darkness. All compositions and conditions were tested in triplicates to allow for statistical evaluation. The remaining viable bacteria were then quantified by fluorescence
measurements, and compared to two negative controls representing 0 % and 100 % viability.

The results, displayed in Figure 25, demonstrated a significant antibacterial effect along the entire gradient, and near complete reduction (97%) for area C. However, the results also revealed another intricate effect: for the Ti oxide reference (denoted Ti), area C, and the Ag oxide reference (denoted Ag), the bacterial viability was greater after UV irradiation. For area C and the Ag oxide reference, these differences were significant at $p < 0.05$.

Since there were no appreciable amounts of crystalline anatase in the coatings, the photocatalytic properties were poor even with the Ag dopant. Hence, little or no ROS were produced by electron-hole generation, and the reduction in bacterial viability could be attributed entirely to Ag$^+$ release. With a non-crystalline TiO$_2$ contribution, the effect of UV irradiation was thus opposite from what was hypothesized.

To investigate the matter further, S. epidermidis was again seeded on Ti and Ag oxide reference samples, which showed the most diverse properties, and irradiated with UV light or kept in darkness as previously described. Adhering bacteria were then fixated for inspection in the SEM, and the results are shown in Figure 26 and Figure 27.

The visual inspection corroborated the results shown in Figure 25, displaying healthy bacteria and some early biofilm formation on the Ti oxide
reference, while remnants of lysed cells were scattered across the Ag oxide reference. Adding UV light caused a debatable reduction in biofilm formation on the Ti oxide reference, which otherwise hosted a similar amount of bacteria. UV irradiation of bacteria on the Ag oxide reference, however, yielded a greater difference since all bacteria appeared intact. These findings suggested a defensive reaction within the bacteria in response to UV irradiation, especially visible in those also subjected to Ag$^+$ ions.

**Figure 26.** Visual inspection of *S. epidermidis* on the Ti oxide reference; kept in darkness (Ti) in (**a**) and (**b**), and irradiated with UV light for 10 min (Ti-UV) in (**c**) and (**d**). The SEM images were color enhanced after acquisition, highlighting biofilm in yellow and bacteria in pink.

**Figure 27.** Visual inspection of *S. epidermidis* on the Ag oxide reference; kept in darkness (Ag) in (**a**), (**b**), and (**c**), and irradiated with UV light for 10 min (Ag-UV) in (**d**) and (**e**). The SEM images were color enhanced after acquisition to highlight traces of biofilm (yellow), intact bacteria (pink), and remnants of lysed bacteria (blue).
High energy UV-C is known to cause irreparable DNA damage and is used as a disinfection method.\textsuperscript{106,107} Sublethal doses of UV, however, can trigger an SOS response in \textit{E. coli} and other bacteria,\textsuperscript{108,109} which pauses the DNA replication process while the damage is being assessed and potentially repaired.

In \textbf{Paper V}, it was thus stipulated that Ag\textsuperscript{+} induced damage to bacteria could be limited by a UV-induced SOS response. Since both UV irradiation and Ag\textsuperscript{+} ions affect the bacterial DNA, a pause in replication and hindering of premature cell division could, if only momentarily, help survival.

By surveying the viability of bacteria 2 and 4 h after treatment, it was noted that it increased with time for Ti and Ti-UV samples, suggesting that bacterial communities thrived after contact with these surfaces. However, for the Ag and Ag-UV samples, as well as for samples along the gradient, the viability, declined steadily with time, which supports the claim of reduced replication abilities after Ag\textsuperscript{+} exposure. Moreover, since all bacterial communities exposed to Ag\textsuperscript{+} demonstrated a gradual decline in viability with time, the relatively higher levels seen after UV irradiation should be interpreted as a slightly slower reduction rate, rather than a recovery in viability. Hence, the suggested UV-induced SOS response could not stop the damage caused by Ag\textsuperscript{+}, only slow the process down.

The qualitative reduction in biofilm after UV irradiation observed with the SEM inspection, most notable on the Ti oxide reference, could partly explain the increase in viability, as more bacteria would still be in a metabolically active planktonic state. For the Ag oxide reference, a UV-induced pause in replication, or rendering of an active but nonculturable state by Ag\textsuperscript{+} exposure, as suggested by Jung et al.,\textsuperscript{110} could explain the numerous bacteria seen in \textit{Figure 27d-e}, compared to the poor viability seen for Ag-UV samples in \textit{Figure 25}.

In summary, the Ag-Ti oxide gradient thin film produced in \textbf{Paper V} was clearly antibacterial due to Ag\textsuperscript{+} ion release, but it lacked photocatalytic properties since the deposited TiO\textsubscript{2} was amorphous. These characteristics led to some interesting findings when testing antibacterial properties under UV illumination. Contrary to what was hypothesized, UV light did not generate electron-hole pairs to yield ROS, but rather provoked an SOS response in the bacteria that limited its reduction rate. For the development of multifunctional coatings with antibacterial properties that wholly or partially depend on UV activation, this is a secondary effect to keep in mind.
Concluding remarks

Developing and evaluating antibacterial surfaces is not a trivial matter, certainly not when potential adverse effects on friendly cells have to be mitigated. The extent of the work presented in this thesis was limited to the manufacturing, characterization, photocatalytic property and in vitro antibacterial property evaluation of two principal surfaces; H$_2$O$_2$-oxidized and H$_2$O-aged Ti, and co-deposited Ag-Ti oxide. As for the antibacterial properties of these surfaces, they were both demonstrated effective against planktonic cells of clinically relevant species, encouraging further development and evaluation to ensure safety aspects and maintained efficiency in an in vivo situation.

Concerning the H$_2$O$_2$-oxidized and H$_2$O-aged Ti and Ti-6Al-4V in Papers I, II, and III, it was concluded that an extended treatment time (24 h + 72 h) was necessary to obtain satisfactory results. It was further revealed that the ROS generation on these surfaces was not necessarily reliant on photocatalysis, but rather on an activation of Ti-peroxy radical species. Both the degradation of rhodamine B, which served as an indicator of PCA, and the actual antibacterial activity was enhanced by adding a small amount of H$_2$O$_2$ to the reaction, demonstrating synergy effects in the TiO$_2$/H$_2$O$_2$/UV system.

For the Ag-Ti oxide gradients in Papers IV and V, a combinatorial approach was applied by means of concurrent magnetron sputtering from two opposing targets in a reactive environment. This enabled deposition of continuous, binary gradients containing a range of compositions and structures that would have been impossible or very cumbersome to produce by traditional means. The advantages of the combinatorial approach were highlighted in Paper IV, where the entire study was based on a single Ag-Ti oxide gradient sample. In Paper IV, the amount of Ag$^+$ released from parts of the coating likely exceeded acceptable levels in a normal case scenario, but the compositional and structural properties affecting the release were clarified. This led to the conclusion that future implant coatings could be better tailored for clinical needs by altering structure of incorporated silver species rather than amount.

While trying to combine and improve on the two antibacterial strategies explored in this thesis, i.e. photocatalysis and Ag$^+$ release, it was found in Paper V that on amorphous TiO$_2$, UV illumination caused an SOS response in bacteria that limited the reduction rate caused by Ag$^+$ exposure. Hence, multifunctional implant coatings that rely on UV activation should be developed with such secondary effects in mind.
Future challenges

Translation of scientific findings from the lab bench to a successful clinical application, which would be the end goal of the work presented in this thesis, is a long endeavor. The natural next step for the surfaces presented here would be mammalian cell studies and thorough evaluation of potential cytotoxicity, followed by in vivo animal trials given the biocompatibility is not compromised. In short, all surfaces and systems presented would require further development before being considered clinically.

In its present state, the H$_2$O$_2$-oxidized and H$_2$O-aged Ti surface could likely find success on abutments, a component of the dental implant construct that is susceptible to infection, but also easily accessible by UV light. It is also a component with fewer requirements for direct tissue integration, making potential adverse effects of the surface less impactful. For application as an implant coating, in vivo trials to confirm osseointegration and/or form basis for a risk-benefit analysis would be required. For controlled UV-activation of the surface, a hand-held device delivering the right dose of light would also have to be developed.

Concerning the Ag$^+$ releasing surfaces, much research and development remains. The presented studies form more of a basis for understanding composition-structure-property relationships than demonstrating a prototype surface for antibacterial applications. Continued development of the combinatorial deposition and analysis approach, including more streamlined property evaluation is needed. Once the right balance between biocompatibility and antibacterial properties have been struck by further tweaking the composition and structure, other fabrication methods than PVD might also benefit.

For the combined Ag$^+$ releasing and photoactivated surfaces, further development is obviously needed to optimize both Ag$^+$ release and photocatalytic properties, all while keeping potential SOS responses in mind.
Analytical techniques

Rhodamine B degradation

In Papers I and II, degradation of the organic dye rhodamine B was used to evaluate PCA of \( \text{H}_2\text{O}_2 \)-oxidized and \( \text{H}_2\text{O} \)-aged titanium samples. ROS generated from the photocatalytic process will degrade the dye molecules, and the resulting loss of color is easily monitored by measuring UV/Vis absorption.

For each test, four identical samples were placed in a flat-bottomed quartz cuvette with 1 cm optical path length, containing 2.5 ml of 5 µM rhodamine B solution, as shown in Figure 28. A UV transparent glass lid covered the cuvette to avoid evaporation, and a small well drilled in the bottom of the cuvette allowed magnetic stirring for homogenization and oxygenation during the tests. The cuvette was placed in a UV/Vis spectrophotometer, fitted with a UV light source (\( \lambda = 365 \pm 10 \) nm) irradiating the samples and solution with pulsed light (100 Hz) at 6.7 mW/cm\(^2\) intensity. The UV light was momentarily blacked out for automated stepwise absorbance measurements of the solution, taken at 554 nm every 5 min for up to 8 h.

Converting the absorbance values to rhodamine B concentration then allowed quantification of PCA via the function \( \ln(C/C_0) = -kt \), where \( C/C_0 \) expresses the normalized concentration, \( k \) is the reaction rate constant reflecting the PCA, and \( t \) is the reaction time.

Figure 28. Illustration of the method used to quantify PCA. Degradation of rhodamine B was monitored over time in presence of TiO\(_2\) samples and UV light.
Scanning electron microscopy (SEM)

SEM is a widely used technique for high resolution imaging of surfaces. It also features a large depth of field, giving images a three-dimensional appearance. Rather than using visible light photons, like in an optical microscope, the SEM uses a focused beam of electrons to scan the surface, which have significantly shorter wavelengths than visible light.

The incident beam of electron interacts with the surface in various ways, giving rise to a number of detectable signals. Secondary electrons are produced during inelastic scattering of the incident beam, i.e. when incident electrons knock out K-shell electrons from specimen atoms. The secondary electrons generated close to the surface have a greater chance of escape to reach the detector, resulting in topographical information about the surface. Backscattered electrons are electrons that originate from the electron beam, but have been elastically scattered by coming close to atomic nuclei in the specimen. Heavier elements generate more backscattered electrons, making the signal an indicator of composition.

SEM was used to image surfaces in Papers I, II, IV, and V, primarily using the InLens detector (secondary electrons) in a Zeiss LEO 1550 operated a 3-7 kV. For Paper IV, a Zeiss crossbeam Neon 40 operated at 5 kV was also used. For non-conducting surfaces, e.g. the HA deposits in Paper IV and the bacteria imaged in Paper V, a thin layer of Au/Pd was sputter deposited on top of the samples to avoid charge build-up.

Energy dispersive X-ray spectroscopy (EDS)

In the SEM, inelastic scattering or excitation of electrons creates atomic inner shell vacancies. This state of ionization forces outer shell electrons to drop down in energy to fill the vacancy, resulting in release of excess energy in the form of an X-ray photon or an Auger electron. The energy differences between electron shells in different elements are well defined, making each X-ray photon constitute a characteristic X-ray of its chemical element. Hence, by detecting the energy and amount of emitted X-ray photons, the chemical composition of a specimen can be determined.

The surface sensitivity and accuracy of EDS is related to the acceleration voltage used in the SEM. A higher acceleration voltage creates a larger (deeper) excitation volume, generating more X-ray photons from bulk material that may suppress the surface signal, but a lower acceleration voltage may not be sufficient to excite electrons with distinguishable energies in all elements present.

EDS was used to determine the elemental compositions in gradient Ag-Ti oxide thin films in Papers IV and V, using detectors fitted to above-mentioned SEMs and the INCA AZtec software.
Focused ion beam (FIB)

The FIB, which can be incorporated into the SEM, uses an ion beam source (usually Ga⁺) to sputter or mill the surface of a specimen. It is commonly used to prepare exquisitely thin samples for transmission electron microscopy (TEM), which have to be electron transparent. It can also be used for micromachining or milling of surfaces, e.g. to analyze a cross-section of a coating.

In relation to this thesis, a FIB (fitted in a Zeiss crossbeam Neon 40) was used to mill a cross-section for analysis at the Ag-side of the gradient coating produced in Paper IV.

Grazing incidence X-ray diffraction (GI-XRD)

XRD uses an incident beam of monochromatic X-rays, usually Cu-Kα (λ = 0.154 nm), to determine the phase composition of a crystalline material. The analysis is done by enumeration of the amount and position of diffracted X-rays, and result in a spectrum displaying intensity vs. angular position.

Crystalline materials have a long-range periodical arrangement of atoms, built on specific crystal lattices that constitute a number of crystallographic planes. In XRD, constructive interference between X-rays diffracted from different parallel planes, according to Bragg’s law (nλ = 2dhkl sinθ), will generate a signal, as described in Figure 29. Scanning a range of angles will thus create an XRD pattern, which can be matched with reference patterns to determine the crystalline composition of the sample.

GI-XRD is based on the same principle, but the incident beam is fixed at a low angle, typically 1°. This allows for analysis of crystalline structures in thin films, since the X-ray penetration depth at low angles is shallow (order of nanometers). GI-XRD was used on thin films in Papers I, II, IV, and V.

![Figure 29. The wavelength of incident X-rays (λ), along with the distance between crystallographic planes (dhkl) and the angle θ constitute Bragg’s law of diffraction, which is the basis for XRD. Scanning θ, a spectrum peak occurs when the diffracted beams are “in phase”.](image-url)
Surface roughness (R$_a$, R$_q$)

Surface roughness is, as previously discussed, an important parameter for dental and orthopedic implants. For this thesis, the average roughness (R$_a$) and root mean square roughness (R$_q$) of sample surfaces in Papers I and IV were determined by white light interferometry.

This optical, non-contact method uses light that is separated into two beams; one directed at the sample and one directed at a reference mirror. When the reflected light is recombined, interference patterns according to sample topography appear. By scanning a surface, the vertical position of each point can thus be determined at the nanometer level by measuring the level of interference with the reference beam. A WYKO NT1100 optical profiler was used for the measurements.

Contact angle (CA) measurements

Water CA measurements are often used to determine the wettability of a surface. The CA is defined as the angle between the solid surface and the tangent line of the liquid phase at the solid-liquid-gas interphases, as shown in Figure 30. Surfaces with a CA above 90° are considered hydrophobic, while a CA below 90° signifies a hydrophilic surface. If the CA is close to 0° or above 150°, the surface is considered superhydrophilic or superhydrophobic, respectively.

In Paper IV, the sessile drop method was used to determine the CA along the Ag-Ti oxide gradient.

![Figure 30. Contact angle (θ) measured by the sessile drop method.](image)

Bacterial viability assays

There are a number of techniques available to evaluate the antibacterial or bacteriostatic properties of surfaces and materials. However, depending on the antibacterial function, or the materials mode of action against bacteria, all techniques are not suitable, and careful planning and sterile execution is required to obtain reliable results.
A quite common and simple method is to evaluate bacterial adhesion, in which the sample of interest is generally submerged in a single strain suspension of bacteria. After some time of incubation, the sample is removed and washed from non-adhering bacteria. The remaining, adhering bacteria are then suspended in solution by sonication or vigorous vortex shaking. Quantification of the adhered bacteria can then be done by, e.g. optical density measurements (rough estimate), serial plating on agar followed by CFU counting (good estimate), mixing with a fluorescence marker sensitive to bacterial viability (good estimate, but requires calibration), or visual inspection by light microscopy or SEM (variable estimate).

For the surfaces evaluated in this thesis, which were photoactivated and/or deposited on Si-wafers, submerging the entire sample in bacterial solution would constitute a significant disadvantage, primarily since only bacteria adhered to the active (UV-illuminated or Ag\(^+\) releasing) surface would be affected. Hence, a DCT method was devised where only the active surface is evaluated. In effect by allowing a small volume (5-10 µl) of bacteria in suspension to adhere to the surface of interest, then testing while losses due to other factors such as desiccation can be controlled. The approach, as applied with UV light and TiO\(_2\) in Paper III, is illustrated in Figure 31.

In Papers III and V, bacterial viability was quantified by mixing suspended bacteria with culture medium and the metabolic activity indicator resazurin. Resazurin is a blue non-fluorescent dye, which in presence of metabolic intermediates is reduced to pink, fluorescent resorufin. As such, the fluorescence becomes a sensitive indicator of viable bacteria. To correlate the fluorescence readings with known amounts of bacteria, however, a standard curve has to be prepared in parallel with every experiment. With the aid of standard curves and negative controls, the viability of bacteria after exposure to various surfaces and conditions could be expressed in percent compared to a control surface.

In Paper IV, surviving bacteria detached from the sample surfaces were quantified by CFU counting. From the suspensions, ten-fold serial dilutions were plated on brain heart infusion (BHI) agar, and colonies were counted the following day.

*Figure 31. Illustration of the DCT method used to evaluate photocatalytic samples.*
Inductively coupled plasma–atomic emission spectroscopy (ICP-AES)

ICP-AES is an analytical technique used to detect trace amounts of metals in solution. In the equipment, a radio frequency coil ionizes Ar gas to generate a high temperature plasma. When the sample to be analyzed is introduced, it is evaporated and broken down into atoms, which collide with electrons and Ar$^+$ ions in the plasma to repeatedly lose and gain electrons, giving off characteristic radiation in the process. The wavelength and intensity of each emission is then used to identify and quantify the elements present in the sample.

In Paper IV, ICP-AES was used to quantify the amount of Ag$^+$ that was released in D-PBS from different parts of the Ag-Ti oxide gradient over time.
Svensk sammanfattning


Denna avhandling syftar till att utveckla tekniker för att förhindra eller begränsa spridningen av infektioner på titanimplantat, och två huvudspår har följts upp: fotokatalys på titandioxidytor (TiO$_2$), samt inkorporering och frisättning av silverjoner (Ag$^+$).

Fotokatalys på kristallin TiO$_2$ sker under belysning av UV ljus, vilket skapar fria radikaler på materialets yta som kan oxidera organiska föreningar. Om dessa organiska föreningar utgör byggnesterna i en bakterie kan denna komma att utplånas.

I avhandlingen utvecklades och användes en enkel och kostnadseffektiv metod för att oxidera titanytor till TiO$_2$. Den baserades på oxidering i väteperoxid (H$_2$O$_2$) följt av en åldringsprocess i vatten, och visade sig generera ytor som var aktiva mot organiska föreningar under UV ljus. Däremot visade
sig dessa ytor vara amorfa (icke-kristallina) och mindre stabila vid uppre-
pade försök, vilket föranledde misstankar om att aktiviteten berodde på nå-
got annat än fotokatalys. Vidare undersökningar visade att aktiviteten till
stor del berodde på UV-aktivering och förbrukning av Ti-peroxy föreningar
som skapats vid H₂O₂-oxideringen. För att gå vidare och på ett smart sätt
utnyttja denna kunskap så utvecklades ett system där TiO₂ ytor, UV ljus
och en svag H₂O₂ lösning kombinerades och testades mot bakterier. Det
visade sig vara ett effektivt system, där cirka 99 % av två kliniskt relevanta
bakteriearter kunde elimineras på 20 min.

Gällande inkorporering och frisättning av silverjoner så utnyttjades en
kombinatorisk materialutvecklingsmetod, där silver och titan deponerades
tillsammans på ett substrat i syreatmosfär, men kommandes olika från källor
placerade mittemot varandra. Detta möjliggjorde deponering av en kompo-
sitions- och strukturmässig gradient, innehållandes en silverdominerad och
en titandominerad ände. De antibakteriella egenskaperna hos denna gradient
följde tydligt dess benägenhet att frisätta silverjoner. Den silverdominerade
änden visade starkast antibakteriella egenskaper, men mer på grund av att
ytan var mer porös och troligen innehöll med silveroxid än vad som fanns på
den titandominerade ytan, där silvret var representerat i form av metalliska
nanopartiklar.

De två spåren för att utveckla antibakteriella ytor kombinerades även i
den sista studien, där fotokatalys på silverdopad TiO₂ utvärderades. För att
behålla de intressanta ytegenskaper som hade påträffats tidigare, med tydlig
skillnad i frisättning av silverjoner beroende på skillnader i ytstruktur, gjor-
des ingen värmebehandling för att kristallisera ytan. Detta ledde, återigen,
till en amorf TiO₂ utan tydlig fotokatalytisk aktivitet. I test med bakterier
visades en distinkt effekt på grund av frisättning av Ag⁺, men mot förmodan
så begränsades effekten då ytor och bakterier belystes med UV ljus. Detta
ledde till slutsatsen att UV ljuset, i avsaknad av kristallin TiO₂ som kunde
generera fria radikaler, istället framkallade ett försvar hos bakterierna som
till viss mån bromsade den skadliga effekten av Ag⁺.
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References


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)