TiO₂ dark catalysis in biomedical applications

A doctoral thesis by
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It is difficult to find a black cat in a dark room, especially if there is no cat.

*An old Chinese saying*
ACKNOWLEDGEMENTS

This work was conducted as an industrial PhD project between Corticalis AS and the Department of Biomaterials, Faculty of Dentistry, University of Oslo during the years 2014-2017. The financial support was provided by Corticalis AS and the Research Council of Norway (Grant 257569).

I am indebted to Håvard J. Haugen, Janne Elin Reseland and Ståle Petter Lyngstadaas. I would like to thank you for the inspirational discussions, progress meetings and the encouragement to present my research outside the department. But most importantly, you backed me up when I needed it the most. You have my respect.

I never thought that microbiology is fun. That was before I met Jessica Lönn-Stensrud. Thank you for showing me bakteriernes forunderlige verden and for being an excellent supervisor.

Thanks, Hanna Tiainen, for absolutely everything. You have been a mentor for me and I enjoyed every bit of this three-year-long roller coaster ride. Our intense discussions have been a highlight of my PhD and I would like to thank you for passing on your passion for research.

Along the way, I had the privilege to work with several outstanding researchers in their field. For their infinite patience, and their willingness to share their knowledge with me, I owe particular thanks to Manuel Gomez, Alessandra Rinna, Catherine Heyward, Ken Welch, Fernanda C. Petersen and Einar Sagstuen. You enriched this thesis.

During my three years at the Department of Biomaterials, I shared countless great moments with my colleagues. Many of these colleagues became my friends during that time. Thank you Jonas Wengenroth, Sonny Margaret Langseth and Natalia Andronova for fixing everything around my PhD. Special thanks go to my former colleague Benjamin Müller. Representative for the best PhD office at the Faculty, my thanks go to Aman Chahal. You are the soul of this office and I know so much more about table tennis now.

When experiments did not go as planned, I could always have a coffee with Anne Klemm. Thanks for being a heart of gold and keep in mind that you are the future of TiO2 scaffolds.

Thank you Manuel Schweikle. We travelled along this PhD side by side and I could not have wished for a better friend to share this time with.

My sincerest thanks go to my mother and sister who supported me during the entire PhD and have been an anchor throughout this time. Gratitude alone cannot express my feelings for Miri. You were always there for me even if I could not be with you.

This ends here.

David Wiedmer
Oslo, June 2017
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1 INTRODUCTION

Biomaterials have become an integral part of modern health care. A missing tooth can be exchanged by a dental implant, hips regain strength by a total joint replacement and an unreliable heart is supported by the insertion of a pace maker. All these interventions can partly compensate the age-related loss of tissue functionality, and thereby, contribute to a high quality of life into old age. However, there has always been a percentage of implants which fail. One of the major causes for implant failure is the occurrence of biomaterial associated infections, which have been related to the colonisation of implant surfaces with bacteria. Current strategies to control and treat these persistent infections are insufficient and may become even less useful in the era of antibiotic resistant microbes.1-3

Therefore, novel strategies to combat implant-related infections are needed, and the development of new biomaterials can play an important role in this context. Biomaterials with antibacterial properties may be used to treat existing infections or prevent bacterial colonisation in the first place. The aim of this study was to assess the interaction of titanium dioxide and hydrogen peroxide as antibacterial strategy to encounter biomaterial associated infections. The introduction of this thesis provides the reader with the fundamentals of titanium dioxide as a biomaterial, implant infection and current strategies for infection control. On this basis, the role of radicals in biology and the state of the art in titanium dioxide dark catalysis is presented.

1.1 Titanium and titanium dioxide in biomedical applications

Titanium (Ti) and titanium alloys are among the most established and successful biomaterials in modern health care, particularly for load bearing and long term devices. Typical examples for Ti in medicine are dental implants, partial or full hip and knee replacements, and cardiovascular applications.4 The suitability of Ti in these applications is based on the triumvirate of good mechanical strength, high corrosion resistance in physiological environments and excellent biocompatibility. The corrosion resistance of metallic Ti is closely related to the formation of a thin oxide layer (3-7 nm) upon contact with air or aqueous fluid.5,6 Unlike other inert biomaterials, Ti implants show the ability to form direct anchorage with surrounding bone tissue (osseointegration).7 The recruitment and migration of osteogenic cells (osteogenesis) to the implant surface and formation of new bone, has been attributed to several physico-chemical properties of TiO2. The physi- and chemisorption of water results in the formation of titanium hydroxide (Ti-OH) at the outermost surface layer. The deprotonation of Ti-OH to Ti-O\(^+\) surface groups due to the lower isoelectric point (iep) of TiO\(_2\) compared to the pH of body fluids (iep\(_{\text{TiO}_2}\) = 5-6, physiological pH = 7.4) favours the electrostatic attraction of calcium ions.8,9 This has been shown to lead to the formation of metastable calcium phosphate which can be transformed to bone-like hydroxyapatite during bone remodelling.10,11 Further, Ti-OH surface groups play an important role in the interaction with biomolecules, particularly for the adsorption of serum proteins such as albumin and fibronectin.12 All these
factors contribute to the biocompatibility of Ti implants and have made Ti and TiO$_2$ the materials of choice in many applications where osseointegration is required.

1.1.1 Titanium implants and titanium dioxide scaffolds

Titanium-based materials have become a widely-used biomaterial for permanent devices in the oral and maxillofacial region.\textsuperscript{4} Figure 1 shows the two relevant applications in the context of this thesis: the Ti dental implant for the replacement of missing teeth and the TiO$_2$ scaffold for the guided repair of critical size bone defects.

![Schematic representation of a titanium dental implant for the replacement of a natural tooth and a titanium dioxide scaffold for the repair of a critical size bone defect.](image)

Screw-shaped endosseous implants have become the most commonly used implants to replace single or multiple teeth.\textsuperscript{13} The actual bone implant is complemented by the abutment for soft tissue attachment and a custom-made crown. The placement of a dental implant into bone tissue provides primary stability through mechanical friction. The primary stability typically declines within the first 2 - 3 weeks after placement due to bone remodelling processes, which eventually lead to a permanent, secondary stability by osseointegration. A high ratio of direct bone-to-implant contact has been shown to be essential for the long-term stability of the implant. In contrast to a natural tooth, a dental implant and the surrounding bone tissue are not separated by the periodontal ligament but are in direct contact with each other.\textsuperscript{14,15}

Commercially pure (cp) Ti is the most frequently used material for dental implants.\textsuperscript{16} The search for new bulk materials or mechanical designs has decreased in the last decades and has been replaced by the development of new implant surfaces.\textsuperscript{17} Various surface modifications have been applied to alter the original machine shaped surface, primarily its surface topography (e.g. by grit-blasting or acid etching) and surface chemistry (e.g. by calcium phosphate coatings).\textsuperscript{18-23} These modifications generally aim at a stronger implant-tissue interface and faster osseointegration.\textsuperscript{24,25} However, it has also been reported that infections are more persistent on modified surfaces compared to machined-shaped surfaces.\textsuperscript{26}
While Ti dental implants have been used clinically for decades, TiO$_2$ scaffolds for the repair of non-healing bone defects are not used in patients yet. Non-healing bone defects, e.g. from trauma or tumour resection, exceed the potential of bone tissue to self-repair and require surgical treatment for adequate healing. The gold standard in the therapy of such defects is the use of bone grafts (autogenous, allogeneous or xenogenous) to fill the defect volume, provide mechanical support and ultimately guide new bone formation to restore the initial functionality. However, scarcity of donor tissue, immunogenic reactions and pathogen transfer are some of the shortcomings for the conventional therapy with bone grafts. To overcome these shortcomings, synthetic scaffolds have been suggested as bone graft substitutes for guided bone repair.$^{27,28}$

Recently, it has been shown that synthetic scaffolds made from ceramic TiO$_2$ are a promising candidates for assisted repair of non-healing bone defects.$^{29,30}$ Ceramic TiO$_2$ scaffolds provide a 3D structure to fill the defect volume and promote the migration, proliferation and differentiation of osteoprogenitor cells to form new functional bone. The good performance of TiO$_2$ scaffolds \textit{in vitro}$^{31-33}$ and \textit{in vivo}$^{34-36}$ is related to a range of physical properties in combination with excellent biocompatibility of the material itself. TiO$_2$ scaffolds are characterised by the high porosity (80-90%) consisting of well-interconnected pores (~400 μm pore diameter) and provide an excellent microstructure for cell attachment, cell proliferation and ultimately ingrowth of new bone tissue.$^{33,34,37}$ In addition, TiO$_2$ scaffolds are simple to process and show superior mechanical properties compared to alternative materials for synthetic scaffolds such as calcium phosphates and bioglass.$^{37,38}$

\subsection*{1.1.2 Implant success and implant failure}

Implant success is defined as the absence of complications over the entire period of observation.$^9$ The fate of an implant is strongly dependent on a series of early biodynamic events during wound healing that lead to osseointegration. Peri-implant wound healing is triggered by tissue damage introduced during surgical placement of the implant. Within seconds after insertion, the implant surface is covered with ions, platelets and serum proteins, followed by the arrival of specialized inflammatory cells. An inflammatory response of the body is inevitable due to surgical trauma and the sole presence of a foreign body. The first inflammatory response, the acute inflammation, is characterised by sequential appearance and disappearance of phagocytes, which remove debris, apoptotic cells and pathogens. If wound healing progresses normally, acute inflammation ceases approximately one week after implantation, and proliferation begins. The proliferation phase is characterised by the formation of granulation tissue, angiogenesis, extracellular matrix (ECM) production and the subsequent arrival of osteoclasts and osteoblasts to form new bone tissue. The rapid formation of new, woven-like bone within 1-2 weeks undergoes further remodelling into lamellar bone directed by load adaption and may take several years to be completed.$^{40,41}$

The failure modes of dental implants have been divided in biologic, mechanic, iatrogenic and prosthetic failures.$^{42}$ Biologic failures are closely related to the inability of the implant to induce or maintain osseointegration. Several risk factors have been identified in this context. Systemic diseases such as osteoporosis and diabetes mellitus are suspected to reduce the rate
for implant success. Patients receiving radiation therapy for tumour treatment, or patients with periodontitis history, are further subjected to a higher risk of dental implant failure. However, the highest number of biologic implant failures has been related to the occurrence of biomaterial associated infections.

1.2 Biomaterial associated infections

Biomaterial associated infections (BAI) are a major cause of failure for orthopaedic and dental implants. With respect to the increase in total number of implanted devices and the difficulty in treating persistent infections, BAI has become a significant social and economic burden. An infection incidence of approximately 1-10% over lifetime has been reported for orthopaedic implants. In the case of dental implants, bacterial infections are believed to be among the main causes for peri-implant diseases, a condition characterised by inflammatory reactions around the implants. The inflammatory reactions in peri-implant diseases affect both soft tissue (peri-implant mucositis) and bone tissue (peri-implantitis) surrounding the dental implant. In peri-implantitis, the remodelling of healthy bone tissue is disturbed by the presence of chronic inflammation. As a consequence, supportive bone is resorbed which eventually leads to implant loosening, and ultimately, the loss of the dental implant.

Depending on the diagnosis criteria applied, the prevalence of peri-implantitis on a patient level ranges from 19% to 56% in studies of five years and longer. Similar numbers for the prevalence of peri-implantitis have been reported by Koldsland et al. The success rates for the treatment of peri-implantitis are equally concerning. Despite thorough debridement and decontamination combined with targeted use of antibiotics, Leonhardt et al. reported 42% re-infection of dental implants five years after peri-implantitis therapy. In a recent study by Carcuac et al., re-occurrence of peri-implantitis in patients could be prevented in 67% for unmodified and only 33% for modified surfaces one year after treatment.

1.2.1 Routes of infection

The origin of pathogenic invaders can be divided into perioperative and postoperative contamination. Perioperative contamination describes the route of infection before or during implantation. The partial destruction of the host epidermis during implantation destroys an important physical barrier against the invasion of pathogens. The vulnerable implant site can be readily contaminated with commensal bacteria or pathogens of the surrounding environment. In the last decades, the risk of perioperative contamination has been reduced successfully by minimising the bacterial load in operating theatres with advanced ventilation systems.

The occurrence of infections due to postoperative contamination is less controllable and less predictable. Pathogens in postoperative contamination are acquired during hospitalisation or can derive from infections elsewhere in the body (haematogeneous spreading). Therefore, postoperative infections can occur any time after the successful surgery and substantially contribute to a life-threatening re-infection spiral.

While the link between implant contamination and infection is far from understood, some of the key players have been identified. Staphylococcus epidermidis and Staphylococcus aureus
were found in 66% of the clinical isolates from infected orthopaedic devices. Both Staphylococci are commensal bacteria of the human skin and membrane mucosa and may enter the implantation site during the insertion of the implant. Staphylococci have also been reported as a main cause in the occurrence of peri-implantitis. However, the aetiology of dental implant infections is generally more complex. The microbiome of the oral cavity accommodates ~1000 different species, many of them living in a symbiotic relationship with the host. Yet, some pathogenic bacteria of the oral cavity, which are often associated with periodontal diseases and dental biofilm formation, have been reported to contribute to the development of peri-implantitis.

In a healthy human, the immune system provides an efficient line of defence against these pathogens. However, the host immune system in the tissue surrounding the implant is compromised due to trauma and the presence of a foreign body. In addition, many pathogens related with BAI form a biofilm on the implant surface as an efficient way to colonise new habitats. Furthermore, microorganisms in a biofilm are protected from the host defence, antimicrobial agents and environmental stresses.

1.2.2 Microbial colonisation of implant surfaces

In a clinical situation, the host tissue and microorganisms compete for the occupation of the newly available habitat. This competition between host tissue integration and microbial colonisation of a biomaterial surface has been visually described as the “race for the surface” by Anthony Gristina in 1987. If this race is won by the host, the implant surface will be covered by tissue and protected from bacteria. Unfortunately, the same surface properties of implants which promote tissue integration also provide excellent ground for microbial colonisation. In addition, the capability to colonise a broad spectrum of substrates as well as being able to form a protective biofilm, are two advantages why bacteria are often considered a step ahead in the race for the surface. The main stages of the development of a bacterial biofilm are illustrated in Figure 2.
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Figure 2: Biofilm development on implant surfaces. (1) Initial attachment of planktonic bacteria. (2) Aggregation of microorganisms and secretion of extracellular polymeric substances. (3) Accumulation in multi-layered cluster. (4) Maturation of biofilm. (5) Dispersal of planktonic bacteria from mature biofilm.

As illustrated in Figure 2, the initial attachment of planktonic bacteria is controlled by adhesive forces for the surface-cell and cell-cell interface. This reversible attachment is governed by electrostatic interactions between the cell wall and the surface. The adhesion of bacteria is complemented by binding to proteins which readily adsorb on the implant surface during implantation. Several serum proteins are recognised by bacterial adhesins for a firm connection between the microbe and the underlying substrate. The irreversible attachment phase is characterised by the aggregation of cells to form microcolonies, and by the beginning of the secretion of extracellular polymeric substances (EPSs). EPS is predominately composed of polysaccharides, but depending on the species, it might further contain teichoic acids and proteins. The secretion of EPS is essential for intercellular aggregation of microorganisms and the further maturation of the biofilm via multi-layered clusters into a complex 3D structure. The classical biofilm circle is completed by the dispersion of planktonic bacteria, possibly triggered by cell-density dependent cross-talk (quorum sensing), and thereby, their journey to conquer new surfaces continues.

The arrangement of bacteria in a sessile community, embedded in a protective polymeric matrix, is one of the key challenges in BAI treatment. Biofilms possess a series of strategies to evade the host immune defence, making the already compromised immune system even less efficient. Further, biofilms show a significantly lower susceptibility against antimicrobial agents compared to their planktonic counterpart. This has been related to the reduced diffusion of antimicrobial agents through the EPS, phenotypical changes and decreased metabolic activity. The dormant state of bacteria within a biofilm has further been linked to the occurrence of late infections, often several month after implantation.

1.3 Antimicrobial strategies

Biofilms on biomaterials play an essential role in the occurrence of BAI, and a tremendous amount of research has been done on how to prevent or disrupt biofilms. Figure 3 gives an overview of currently investigated antibacterial concepts by means of their mode of action.
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The vast majority of strategies under investigation aim at the inhibition of early microbial colonisation due to the low susceptibility of biofilms against antibacterial actions during later stages of their development. Only few biomaterials with clinical relevance show intrinsic antibacterial properties, and therefore, most biomaterials have to be modified to obtain antibacterial activity. In contrast to the large variety of antibacterial surface modifications for biofilm prevention, the use of biomaterials in the treatment of existing biofilms is a less noticed field of research. This is surprising in the context of low success rates of infection therapy and dramatic consequences of re-infection (Chapter 1.2).

1.3.1 Biomaterials for biofilm prevention

The integration of host tissue provides one of the most powerful anti-infective strategies for biomaterial surfaces. Once integrated, tissue cells are unlikely to be replaced by bacteria, thereby preventing microbial colonisation of the implant surface. However, the development of new implant surfaces designed for tissue integration in the last decades has not led to a decrease of BAI. Thus, the promotion of tissue integration alone may not be sufficient to prevent bacterial infections. Consensus has grown that implant surfaces have to provide antimicrobial functions in addition to tissue integrating properties to combat BAI efficiently.

Non-adhesive surfaces aim at the prevention of microbial colonisation by interfering with microbial adhesion mechanisms during the reversible attachment phase. The passive adhesion of bacteria is governed by material hydrophobicity and electrostatic interaction. Changes in surface topography or the application of polymer coatings have been shown to reduce bacterial adhesion significantly. Pre-adsorption of molecules, such as heparin, has further been shown to decrease bacterial colonisation by influencing protein specific adhesion. Several non-adhesive surfaces have made it into clinical practice, especially when biomaterials are used temporarily (e.g. vascular catheters) and are not in contact with protein-rich fluids (e.g. urinary catheters, contact lenses). However, non-adhesive surfaces are generally unsuitable or need to be further modified for applications which require tissue integration.
Contact killing and antimicrobial releasing surfaces may be more promising concepts in the search for anti-infective biomaterials in tissue integrating applications. Both rely on the activity of antibacterial compounds, either permanently immobilised on the surface (contact killing) or released from the surface over time (antimicrobial releasing). Thereby, biomaterials can be modified for bactericidal or bacteriostatic properties depending on the compound used. More recent approaches have also investigated the use of substances which interfere with biofilm development by targeting EPS components or distort cell signalling pathways.

Contact killing surfaces provide a long-lasting antibacterial activity by physically adsorbing or covalently binding active molecules onto the biomaterials surface directly, or aided by a polymeric sublayer. Examples of immobilized biocides are antimicrobial peptides, quaternary ammonium compounds and silver. All these biocides show strong bactericidal effects in vitro but only silver is applied clinically today. The stability of such coatings in vivo, as well as potentially cytotoxic effects of the used biocides, are some of the disadvantages of this approach. Further, the dependency on close contact with the bacterial wall generally lowers their efficiency in applications in which the surface is readily covered with a film of host proteins.

Compared to contact killing surfaces, antimicrobial releasing surfaces extend the range of action and are less compromised by the presence of a conditioning film. Most often this concept is realised by a biocide embedded in a carrier matrix and released passively by diffusion or actively e.g. by stimuli-dependent degradation of the matrix. Commonly used biocides are antibiotics, chlorhexidine and silver all of which are in clinical use as implant coatings or antibiotic-loaded bone cements. However, antimicrobial releasing biomaterials show weaknesses such as cytotoxic effects against host cells, low effect on specific strains or inadequate release kinetics. Especially the release of antibiotics below the minimal inhibitory concentration, decades after implantation, has raised concern regarding the development of antibiotic resistance.

All surfaces described above show distinct limitations, and multifunctional surfaces are required to compensate for their weaknesses or to create synergistic effects. Non-adhesive surface coatings have been successfully functionalised for cell specific attachment and several surface topographies, which favour cell adhesion and compromise bacterial adhesion simultaneously, have been developed. Despite these great possibilities, the development of new and simple monofunctional surfaces remains a vital part in the development of multifunctional surfaces.

1.3.2 Biomaterials for biofilm disruption

The development of anti-infective surfaces may solve the problem of BAI to some extent in the future, but the translation into clinical practice is difficult. Today, the efficiency of an anti-infective surface has to be validated in human clinical trials. These studies provide experimental proof that the risk of infection is significantly reduced for the tested surface compared to controls. With respect to the low infection prevalence of osseointegrated implants, this translates to long-term studies with 5000 and more patients, accompanied with enormous cost. Hence, clinical trials have been a major hurdle in the translation of new implant surfaces to
As a consequence, no antibacterial surface for tissue integrating implants is available today. With no quick solution for infection-resistant implants ahead, the therapy of infected implants has become an important task in implantology. The treatment of infected permanent implants aims at the establishment of a non-infectious environment for re-osseointegration of an implant. In revision surgery of orthopaedic devices, the infected implant is generally removed and replaced by a new implant. This differs from the therapy of infected dental implants, where the goal typically is to reintegrate the original implant. This exceptional situation requires the best decontamination of the implant surface possible to prevent re-infection.

Surgical treatment has become the gold standard in the therapy of peri-implantitis. In a first step, full access to the infected site is provided by incising the gingiva (open flap procedure). The subsequent debridement aims at the complete removal of inflamed tissue around the dental implant. The decontamination of the infected implant itself can be divided into the mechanical removal of adherent biomass and the chemical disinfection of the implant surface. Commonly used tools for mechanical debridement are curettes, lasers, air abrasion or brushes. More recently, implantoplasty, which describes the grinding and destruction of the exposed implant surface, has gained considerable attention as a technique to remove adherent biofilms. The mechanical debridement is generally supported by chemical decontamination with an antimicrobial agent such as H2O2, chlorhexidine (CHX) or citric acid. The choice of adjacent antibacterial agents is often based on experiences from periodontitis therapy. However, several of these agents have shown low efficiency in the therapy of peri-implantitis, and no gold standard for the decontamination of dental implants has been identified yet.

Biomaterials or biomaterial-based strategies for the treatment of existing infections are scarce. One approach is the design of implant surfaces which are easy to decontaminate in an infection scenario. Several groups have proposed surface modifications which would allow the destruction of biofilms a posteriori by thermal inactivation. A different approach is the development of biomaterials which are used to destroy or support the destruction of existing biofilms during infection therapy. A hydrogel enriched with TiO2 microparticles has been proposed to improve the mechanical decontamination in peri-implantitis treatment. Further, it has been suggested that the bactericidal activity of H2O2 can be enhanced by the addition of TiO2 particles due a catalytic reaction. However, the antibacterial effect due to the interaction of H2O2 and TiO2 has not been ascertained yet.

1.4 TiO2 dark catalysis

The dark catalytic effect is a yet undefined term for the formation of oxygen centred radicals by the catalytic decomposition of H2O2 on TiO2 surfaces. It differs from classical TiO2 photocatalysis by the independency from irradiation, and shows a different reaction pathway compared to similar Fenton-like reactions (Chapter 1.4.2). The generation of bactericidal free radicals is the foundation of TiO2 dark catalysis as potential antimicrobial strategy in biomedical applications, particular in scenarios where irradiation is not feasible.
1.4.1 Bactericidal free radicals

A free radical is defined as an atom, molecule or ion with one or more unpaired electrons. Radicals are formed by losing or gaining a single electron from a non-radical, or due to dissociation of chemical bonds of a molecule.\textsuperscript{116} The unpaired electron is the reason for the high reactivity of most radicals with a wide range of substances. Because of the high reactivity, the half-life of free radicals in physiological environment is very short. Radicals differ in their chemical structure, their reduction potential and the location of the unpaired electron. In biology, the most relevant radicals derive from diatomic oxygen (O\textsubscript{2}) and are summarised under the term oxygen centred radicals. Together with some non-radical species (H\textsubscript{2}O\textsubscript{2}, O\textsubscript{2}\textsuperscript{1\Delta g}), oxygen centred radicals form the important group of reactive oxygen species (ROS) which are shown in Figure 4.\textsuperscript{117,118}

ROS play an important role in many biological processes such as phagocytosis, energy production or oxidative stress mediated cell death. The molecular mechanisms of these processes are to some extent understood. Many of them can be referred to the initial formation of the superoxide radical anion (O\textsubscript{2}•\textsuperscript{-}) by the reduction of O\textsubscript{2} during the respiratory burst.\textsuperscript{117} Some of the most relevant interactions of ROS with biomolecules in the context of cytotoxicity and phagocytosis are illustrated in Figure 4.

Phagocytosis is an essential part of the immune system in the defence against pathogens, and it relies on the intra- and extracellular formation of ROS and ROS-derivatives.\textsuperscript{119,120} Bacterial targets for ROS-mediated damage are DNA, lipids and proteins. A direct damage by O\textsubscript{2}•\textsuperscript{-} is generally negligible in biological systems. The low reactivity of O\textsubscript{2}•\textsuperscript{-} with most biomolecules and powerful enzymatic defence mechanisms of many pathogens reduce its antibacterial effect.\textsuperscript{119} Further, due to its negative charge, O\textsubscript{2}•\textsuperscript{-} is unable to pass the bacterial membrane and cause intracellular damage. However, the bactericidal effect of O\textsubscript{2}•\textsuperscript{-} has been suggested to increase by protonation and the formation of the uncharged hydroperoxyl radical (HO\textsubscript{2}).\textsuperscript{121,122}

The dominant bactericidal action of ROS has been related to the intracellular formation of highly reactive hydroxyl radicals (•OH). •OH can be formed by the decomposition of H\textsubscript{2}O\textsubscript{2} in the presence of a Fenton catalyst such as iron (Fe) or copper (Cu). Both catalysts are essential for the protein synthesis of bacteria and are stored in the cytoplasm. When reacting with H\textsubscript{2}O\textsubscript{2}, Fe and Cu catalyse the formation of •OH, which causes severe protein and DNA damage.\textsuperscript{119,123} Alternatively, H\textsubscript{2}O\textsubscript{2} can react with halide ions (Cl\textsuperscript{-} and Br\textsuperscript{-}) to form highly bactericidal hypohalous acids (HOCl and HOBr) with multiple targets within microorganisms.\textsuperscript{124,125}

In addition to intracellular damage, several extracellular targets for ROS attack have been identified. A major damaging mechanism of extracellular ROS is the oxidation of polyunsaturated fatty acids (PUFAs) to cause lipid peroxidation. The oxidation of PUFAs is the initial step in a chain reaction that propagates the formation of oxidation products such as lipid hydroperoxides. This causes severe damage to the lipid bilayer of cells which eventually results in cell death due to the loss of membrane integrity.\textsuperscript{126}

Bacteria have developed a range of defence and repair mechanisms to cope with ROS-mediated killing. Many of those strategies are species and strain specific and include the evasion of regions with high ROS concentrations, the physical protection in capsules and biofilms, strict
regulation of intracellular iron and the enzymatic regulation of ROS levels by antioxidants.\textsuperscript{127-129} In addition, bacteria possess a sophisticated system to repair oxidative damage on DNA, cell envelope proteins or intracellular enzymes.\textsuperscript{127} Despite these countermeasures, ROS remain a highly toxic substance for microorganisms. Therefore, the formation of ROS on TiO\textsubscript{2} provides an interesting approach in the development of a biomaterial with antibacterial properties.

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{reactive_oxygen_species.png}
\caption{Reactive oxygen species}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{damage_to_biomolecules.png}
\caption{Damage to biomolecules}
\end{figure}
1.4.2 Catalytic activity of titanium dioxide

The formation of free radicals on TiO₂ and other transition metal oxides has been primarily investigated from the perspective of photocatalysis. The role of TiO₂ in the catalytic decomposition of H₂O₂ is still elusive and has been the topic of only a limited number of publications. Figure 5 illustrates the differences in the underlying molecular mechanisms between irradiation dependent and independent formation of free radicals.

![Figure 5: Molecular mechanisms in the formation of free radicals on TiO₂ surfaces under irradiation (photocatalysis) and in the absence of light by the interaction with H₂O₂ (dark catalysis). Only reactions with reference to this thesis are shown in the illustration.](image)

In TiO₂ photocatalysis, the irradiation of TiO₂ induces the formation of an electron hole pair by promoting an electron from the valence into the conduction band. The energy required to form an electron-hole pair is determined by the material specific band gap energy (Eₙ) to convey the material in a conductive state. Environmental oxygen can function as an electron acceptor to form O₂⁻, while H₂O can be oxidized and form •OH. Both the electron hole itself, and the formed free radicals, are highly reactive and can undergo secondary reactions to oxidize a wide range of organic materials. The energy required to overcome the band gap energy of TiO₂ (Eₙ, anatase = 3.2 eV, Eₙ, rutile = 3.0 eV) requires irradiation in the UV range and often limits the use of TiO₂ photocatalysis in industrial applications. Therefore, recent developments have focused on modifications of the catalyst to extend the photocatalytic effect to visible light.

The antibacterial effect of TiO₂ photocatalysis has been predominantly related to loss of membrane functionality by lipid peroxidation due to the extracellular formation of ROS. TiO₂ photocatalysis has been shown to be effective against different pathogens in vitro with certain degree of range activity. So far, the photocatalytic effect of TiO₂ has been primarily utilised in environmental applications for water and air purification or self-cleaning surfaces. Several biomedical applications such as photodynamic therapy for cancer treatment or self-sterilisation of medical instruments are under development. However, the necessity of irradiation often excludes the use of photocatalysis for devices within the human body.
The dark catalytic effect of TiO$_2$ describes the catalytic decomposition of aqueous H$_2$O$_2$ into water and oxygen in the absence of light. The overall reaction (0) and the main subreactions (1-3) have been proposed to follow the following scheme:\textsuperscript{131}

\begin{align*}
\text{H}_2\text{O}_2 &\rightarrow \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \\
\text{H}_2\text{O}_2 + \text{M} &\rightarrow 2 \cdot \text{OH} + \text{M} \\
\cdot \text{OH} + \text{H}_2\text{O}_2 &\rightarrow \text{HO}_2 \cdot + \text{H}_2\text{O} \\
2 \text{HO}_2 \cdot &\rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\end{align*}

where M is an undefined site at the liquid-metal oxide interface. This reaction pathway has been used to describe the catalytic decomposition of H$_2$O$_2$ in systems where the metal cation cannot undergo further oxidation. For these systems, the classical redox path by the Haber-Weiss reaction is invalid.\textsuperscript{145} However, it should be noted that a redox pathway has also been proposed for TiO$_2$ in which Ti$^{IV}$ is reduced to Ti$^{III}$ by the reaction with H$_2$O$_2$.\textsuperscript{146} For the presented reaction pathway (Reaction 0-3), the activation energy (E$_a$) required for the cleavage of the O-O bond and the formation \textsuperscript{\textbullet} OH (Reaction 1) has been shown to be reduced in aqueous H$_2$O$_2$ suspension in the presence of a number of metal oxides including TiO$_2$.\textsuperscript{130,131} The formation of \textsuperscript{\textbullet} OH as the primary product of the reaction has been experimentally verified.\textsuperscript{147} The reaction kinetics were shown to depend strongly on the solid-surface-area-to-solution-volume-ratio (S$_a$/V), pH and temperature.\textsuperscript{147,148} Some of the \textsuperscript{\textbullet} OH radicals formed contribute to the formation of HO$_2 \cdot$ by the reaction with H$_2$O$_2$ (Reaction 2). While the dissociation of H$_2$O$_2$ and \textsuperscript{\textbullet} OH is negligible in physiological conditions ($pK_{a, H_2O_2} = 11.8, pK_{a, \cdot OH} = 11.9$), the deprotonation of HO$_2 \cdot$ ($pK_{a, HO_2 \cdot} = 4.88$) needs to be considered.\textsuperscript{149}

\begin{equation}
\text{HO}_2 \cdot \leftrightarrow \text{O}_2 \cdot^- + \text{H}^+
\end{equation}

The superoxide anion radical readily adsorbs on several metal oxide surfaces.\textsuperscript{150} In the case of TiO$_2$, O$_2 \cdot^-$ coordinates to the Ti(IV) metal cation and becomes a long-lived radical species.\textsuperscript{136} Several superoxo- and peroxy-metal complexes have been suggested as possible configurations of adsorbed O$_2 \cdot^-$ species on TiO$_2$.\textsuperscript{136}

Surprisingly, only a few studies have investigated the potential of TiO$_2$ dark catalysis in biomedical applications. Several groups have investigated the dark catalytic effect of TiO$_2$ from a toxicological point of view, particularly regarding the toxicity of TiO$_2$ nanoparticles. Based on their results, the cytotoxicity due to the interaction of TiO$_2$ with H$_2$O$_2$ is still controversial.\textsuperscript{133,151-153} On the other hand, the dark catalytic effect as an antibacterial strategy for TiO$_2$ has gained little attention. Tengvall \textit{et al.} have reported the formation of a Ti-peroxy gel for the interaction of Ti with H$_2$O$_2$.\textsuperscript{154,155} These gels have shown high bactericidal activity against \textit{Escheria coli} in the presence of myeloperoxidase (MPO) and halogens.\textsuperscript{124} The antibacterial effect has been related to the presence of stabilised O$_2 \cdot^-$ in the hydrated TiOOH-matrix and the release of H$_2$O$_2$ during gel degradation.\textsuperscript{156} Further, Henderson \textit{et al.} have recently shown a strong bactericidal effect of H$_2$O$_2$-TiO$_2$ suspension against \textit{S. epidermidis} biofilms.\textsuperscript{114} In this study, the bactericidal activity has been associated with the formation \textsuperscript{\textbullet} OH
at the H$_2$O$_2$-TiO$_2$ interface. Therefore, the dark catalytic effect of TiO$_2$ shows great potential as an antibacterial strategy in both, the prevention and the treatment of BAI.
2 RESEARCH CONCEPT

So far, the dark catalytic effect of TiO₂ has not been explored as a strategy to reduce the risk of biomaterial associated infections. This thesis investigated the interaction of TiO₂ and H₂O₂ to form radicals with antibacterial activity in the absence of light. This phenomenon was examined for H₂O₂-TiO₂ particles suspensions and for surface modified TiO₂ scaffolds.

The general hypothesis of this thesis was that TiO₂ dark catalysis can be used as an antibacterial strategy in biomedical applications. With respect to this hypothesis, two specific applications were investigated:

1. The decontamination of implant surfaces by H₂O₂-TiO₂ suspensions in a peri-implantitis scenario
2. The prevention of biofilm formation on TiO₂ scaffolds for bone repair without adverse effects on host tissue cells

2.1 Aims of research

The overall aim of this study was to develop catalytic active H₂O₂-TiO₂ systems with appropriate biological response regarding the applications stated above. The overall aim was further divided into more specific objectives which were addressed in the corresponding papers.

Paper I

- Evaluation of the catalytic activity of five TiO₂ powders in aqueous H₂O₂ suspensions and identification of material properties responsible for high oxidative power
- Verification and identification of reactive species formed during the interaction of TiO₂ powders and H₂O₂

Paper II

- Verification of the antibacterial activity of H₂O₂-TiO₂ suspensions in vitro
- Comparison between H₂O₂-TiO₂ suspensions and conventional chemical agents in the treatment of contaminated implant surfaces in vitro

Paper III

- Development and characterisation of a surface modification for open porous TiO₂ scaffolds with high catalytic activity
- Assessment of the antibacterial effect of modified TiO₂ scaffolds in vitro
- Assessment of the cytotoxic and genotoxic effect of modified TiO₂ scaffolds in vitro
2.2 Experimental design

An experimental design was developed to investigate the specific aims stated above. A schematic flow chart of the experimental strategy in this thesis is shown in Figure 6.

![Experimental design flow chart](image)

Figure 6: Experimental design followed in this thesis to evaluate the potential of H$_2$O$_2$-TiO$_2$ suspensions as an antibacterial agent and the translation to TiO$_2$ bone scaffolds with antibacterial properties.
3 METHODOLOGICAL CONSIDERATIONS

The methods used in this study aimed at the fabrication and characterisation of TiO₂-based biomaterials and the subsequent assessment of their biological performance in vitro. While many of the selected methods are well-established in the field of biomedical research, some others, such as EPR spin trapping, may be considered more exotic techniques. Therefore, this chapter should introduce the reader to the applied techniques, critically review their strengths and weaknesses and justify why these methods have been used to investigate the aim of research defined in the previous chapter.

3.1 Particle characterisation

Five commercially available TiO₂ powders were characterised in Paper I. The conducted experiments aimed at identifying physico-chemical properties related to a high catalytic activity in the decomposition of H₂O₂. This was important to be able to select the most suitable TiO₂ powder for the assessment of the antibacterial activity in vitro in Paper II. Further, the identification of relevant material properties guided the development of a thin film coating for TiO₂ scaffolds in Paper III.

3.1.1 BET surface area and hydrodynamic diameter

The specific surface area (SSA) of five different TiO₂ powders was determined by the Brunauer-Emmet-Teller (BET) method. The BET theory describes the multilayer adsorption of chemically inert gas molecules on a solid surface. In Paper I, TiO₂ powders were degassed and the physical adsorption of nitrogen (N₂) over the relative pressure of the gas (p_rel) was measured. During the increase of p_rel, a monolayer of N₂ molecules adsorbs onto the surface. The formation of a monolayer is represented by the Langmuir isotherm in the BET plot. The SSA of the solid can then be estimated by the number and the cross-sectional area of adsorbed N₂ molecules.

BET measurements are a well-established, simple and fast method to determine and compare the surface area of different solids. However, the assumption of a homogeneous and non-porous solid in BET-theory needs to be considered in the interpretation of SSA values derived from BET measurements. Further, the physical adsorption of inert gas molecules on TiO₂ particle is of limited use to describe their behaviour in suspensions. Hence, light scattering experiments were performed to determine the size of particles in a liquid and examine the stability of the suspension.

The particle size of different TiO₂ powders in suspension was determined by dynamic light scattering (DLS) in Paper I. In DLS, the particle size is calculated from the intensity fluctuations of a laser beam scattered by the surface of a particle. The principle of this technique is illustrated in Figure 7. Small particles in suspension move randomly due to the collision with atoms and molecules of the fluid (Brownian motion). The speed of the particles is linked to their size and described by the Stokes-Einstein equation. The intensity of a laser beam which is scattered by
a particle fluctuates due to the particle motion in suspension. The fluctuation is high for a fast moving, small particle and low for a slow moving, large particle. This relation is used in DLS to determine the size of a particle in suspension. The calculated size refers to a hypothetical, spherical particle with the same behaviour in a fluid than the measured sample (Figure 7). Therefore, the hydrodynamic diameter (D\textsubscript{hyd}) measured may vary strongly from the primary particle size especially in the presence of agglomerates or aggregates.

DLS measurements are a simple, fast and straightforward technique to characterise the particle size and size distribution in suspensions. One challenge in the sample preparation of diluted and monodisperse TiO\textsubscript{2} suspensions in Paper I was the presence of TiO\textsubscript{2} aggregates. The presence of these microstructures resulted in a polydisperse sample and high values for the average particle size were measured. Further, gravitational forces dictated the motion of large aggregates which sedimented at the bottom of the cell during the experiment. Therefore, all samples were bath sonicated immediately before use. In addition to the sonication step, all samples were rested for 20 min to allow remaining TiO\textsubscript{2} aggregates to sediment. Only the supernatant was used for further particle size analysis. Multiple scattering was not detected for the low particle concentration of the supernatant, and no further dilution steps were required for high quality measurements.

The results from DLS measurements complemented the BET results and gave insight about the available surface area of TiO\textsubscript{2} powders in colloidal suspensions. During the sample preparation for DLS measurements, differences in the stability between each TiO\textsubscript{2} suspension were observed. Thus, the stability of TiO\textsubscript{2} suspensions at the different solution pH was further examined by electrokinetic measurements.

### 3.1.2 Electrokinetic potential

The zeta potential (ζ-potential) was examined by electrophoretic light scattering (ELS) as a measure for the stability of aqueous TiO\textsubscript{2} suspensions. The ζ-potential is the electrokinetic potential at a defined distance from the particle surface in a liquid phase, and can be used to describe the net surface charge of a particle in suspension. When a solid is immersed in a fluid,
it forms an electric double layer (EDL) by the adsorption of ions and counterions on its surface. This double layer consists of an inner layer with strongly bound ions (Stern layer), and a diffuse outer layer with weakly bound ions. When exposed to an electro-magnetic field, the particle moves to the electrode of opposite charge (electrophoresis) and drags the EDL with it. The slipping plane describes the boundary in the diffuse layer beyond which ions are unaffected by the motion of the particle. The electrokinetic potential at this boundary is defined as the $\zeta$-potential.\textsuperscript{160,161} Figure 8 illustrates the formation of an EDL at a TiO$_2$ particle and the measurement principle to determine the $\zeta$-potential.

![Figure 8](image)

**Figure 8:** (Left) The formation of an electric double layer on a particle surface when exposed to a fluid. The zeta potential is defined as the electrokinetic potential at the slipping plane, where ions remain in place when the particle moves. (Right) Schematic illustration of the measurement principle of electrophoretic light scattering (ELS). The intensity of an incident laser beam fluctuates due to the movement of particles along an electro-magnetic field. The zeta potential is correlated to the electrophoretic mobility of a particle and can therefore be determined by ELS.

The electrophoretic mobility and the $\zeta$-potential of a particle in suspension are linked by the Henry equation.\textsuperscript{162} Hence, the $\zeta$-potential can be measured by applying an external electric field to a measurement cell and analyse the speed of the particles in the liquid phase by ELS. The working principle of ELS is analogous to DLS with minor changes in the instrumental setup.\textsuperscript{163} In general, any relative motion between a solid and a liquid phase induces an electrokinetic potential and ELS provides a method to determine it. One alternative for ELS is the determination of the $\zeta$-potential by electroacoustic attenuation (EAA),\textsuperscript{164} which could have been used to confirm the results of Paper I.

The same sample properties that were required for an accurate DLS measurement were essential for ELS measurements (monodisperse suspensions with low particle concentration). Therefore, the same sample was used for both measurements in Paper I. Additionally, several aspects regarding sample preparation specific for ELS had to be considered. Electrophoretic measurements need to be performed in an inert electrolyte. Commonly used inert electrolytes for TiO$_2$ suspension are low concentrated NaCl and KCl solutions, which show no specific adsorption on TiO$_2$.\textsuperscript{165} Specific ion adsorption interferes with the naturally occurring EDL in
aqueous suspensions and influences its electrophoretic mobility. This can result in changes for the absolute values of the $\zeta$-potential or an overall shift of the $\zeta$-potential curve along solution pH. Specific adsorption is depending on both, the type of electrolyte and its ionic strength. In Paper I, 1 mM NaCl was used as an inert electrolyte in ELS measurements. Specific adsorption and a strong influence on ELS measurements of TiO$_2$ suspensions have been reported for the monovalent salt NaCl at high ionic strength. However, electrolytes with C$_{NaCl}$ < 0.1 M show inert behaviour and have been used frequently to examine the electrokinetic potential of TiO$_2$.

A pH titration study was planned in addition to the $\zeta$-potential measurements at pH 3, 6.7 and 9 published in Paper I. The $\zeta$-potential of TiO$_2$ is a function of solution pH. At low pH, Ti-OH$_2^+$ surface groups are predominant, while at high pH Ti-O$^-$ surface groups dictate the net charge of the metal oxide. The solution pH of neutral charge in an inert electrolyte is defined as the isoelectric point (iep) of TiO$_2$ and can be determined in a pH titration experiment. However, TiO$_2$ suspensions showed an intrinsic buffer capacity and > 2 h of stirring were required for the pH stabilization in aqueous suspensions. Further, TiO$_2$ agglomerates sedimented in the measurement cell at pH > iep, which distorted the measurements during titration. Hence, the determination of the iep was discarded and the $\zeta$-potential of the five different TiO$_2$ powders was measured at the three specific pH levels.

3.2 Scaffold fabrication

TiO$_2$ scaffolds were used to investigate the antibacterial activity of TiO$_2$ surfaces due to the interaction with H$_2$O$_2$. Infection around permanently implanted scaffolds is believed to cause major complications, and therefore, scaffolds to guide bone repair were considered a relevant study subject for antibacterial surfaces.

TiO$_2$ scaffolds used in Paper III were fabricated by the polymer sponge method (PSM). The main stages of the process are illustrated in Figure 9. The method has been developed by Schwartzwalder and Somers for the fabrication of macroporous ceramics which can be used in various industrial applications. PSM offers some advantages compared to alternative fabrication methods, such as direct laser sintering or foaming techniques. It is a cost-efficient method to produce porous ceramic foams with high reproducibility. The chemical and physical properties of the TiO$_2$ scaffold can be tuned by the composition of the ceramic slurry and the applied heat treatment during sintering. Polyurethane foams, which are used as sacrificial templates, are easy to machine, and therefore, PSM provides a simple way to produce complex ceramic 3D structures. Further, the complete burnout of the polymer and the absence of slurry additives result in a toxin-free ceramic with optimized architecture for bone tissue engineering applications. The compressive strength of TiO$_2$ scaffolds lays within the range of cancellous bone and can be controlled by the applied heat treatment. High sintering temperatures lead to the stabilization of the rutile phase and an increase in compressive strength due strut folding. Unfortunately, the findings of Paper I indicated that anatase is more active in the decomposition of H$_2$O$_2$ than rutile. Thus, TiO$_2$ scaffolds needed to be coated for high catalytic activity and potentially high antibacterial activity.
3.3 Surface modification

A thin-film coating was applied to improve the catalytic activity of TiO$_2$ scaffolds for the decomposition of H$_2$O$_2$. Further, a pre-treatment of coated scaffolds with high concentrated H$_2$O$_2$ for prolonged oxidative behaviour was examined.

3.3.1 Sol-gel dip coating

Sol-gel dip coating was used to deposit a thin TiO$_2$ film on TiO$_2$ scaffolds to alter the crystal structure of the substrate (Paper III). The sol-gel process provides a controlled and simple method to produce homogeneous, thin ceramic films of high quality. Several alternative methods, such as chemical or physical vapour deposition, are commonly used to deposit thin films on various substrates. The main reason for choosing sol-gel dip coating was the simplicity of the method which can easily be realised in standard lab. The main steps of the procedure are illustrated in Figure 10.
METHODOLOGICAL CONSIDERATIONS

Figure 10: Schematic illustration of the sol-gel coating process. A titanium alkoxide is used as a precursor and hydrolysed by the addition of water. Hydrolysed precursor molecules simultaneously undergo polymerization to form a dispersed TiO$_2$ sol. Substrates are coated prior to the development of a gel by the arrangement of particles in a polymeric network. The xerogel deposited on a substrate can be calcified by heat treatment to form a dense, crystalline ceramic coating.

A sol is a colloidal suspension in which gravitational forces of the solid phase are negligible due to the small size of the solid. In the sol-gel process, the sol gradually evolves into a gel by forming a polymeric network which encloses the liquid phase. The first step in this reaction is the hydrolysis of a precursor molecule (1), typically a metal alkoxide, and the subsequent formation of small colloidal particles by polycondensation (2 or 3):

\[
\begin{align*}
M(OR)_4 + H_2O &\rightarrow HO-M-(OR)_3 + ROH \\
(OR)_3M-OH + HO-M(OR)_3 &\rightarrow (OR)_3M-O-M(OR)_3 + H_2O \\
(OR)_3M-OR + HO-M(OR)_3 &\rightarrow (OR)_3M-O-M(OR)_3 + ROH
\end{align*}
\]

In Paper III, titanium isopropoxide (TTIP) was dissolved in isopropanol (iPrOH) and used as a precursor. In addition, hydrochloric acid (HCl) was used as catalyst to accelerate the reactions above. Polycondensation of the hydrolysed precursor molecules can either occur by oxolation (2) or alcoxolation (3). The crosslinking of precursor molecules by M-O-M bonds leads to the formation of dispersed macromolecules and ultimately to the formation of a polymeric network by continuous polycondensation. The ideal sol for the deposition on a substrate consists of small TiO$_2$ particles that are homogeneously dispersed in the liquid phase. One challenge in sol-gel dip coating is the prevention of microparticle precipitation in the sol, which results in inhomogeneous coatings. In Paper III, precipitation was prevented by determining an adequate hydrolysis rate ($n_{H_2O}/n_{TTIP} = 2$) and avoiding precursor hydrolysis in moist environments. The acquired sol was clear and stable for several days at 4 °C before gradually turning into a gel. Therefore, it was not necessary to further stabilise the process by the addition of complexing agents.

Several techniques exist to deposit the precursor sol on a substrate. The most common ones are spin coating and dip coating. Dip coating was chosen for the surface modification of TiO$_2$
scaffolds because this method is easy to implement at a laboratory scale and has been reported more adequate for the coating of complex 3D structures.\textsuperscript{171,173} The different stages of the coating procedure are illustrated in Figure 11. A thin TiO\textsubscript{2} film was required to maintain the open porous architecture of the scaffolds. The thickness of films applied by dip coating can be controlled by the viscosity of the sol and the withdrawal speed of the substrate.\textsuperscript{174} A custom-made machine was used to withdraw the substrate from the sol at low constant speed (Figure 11). A thin layer of TiO\textsubscript{2} sol remains on the surface of the substrate after gravitational drainage of excess sol-gel and the evaporation of iPrOH. During the subsequent drying step, this layer further shrinks in volume due to solvent evaporation and polymerization of unreacted precursor molecules.

In Paper III, dried films were calcified in a two-step heating cycle. First, samples were heated to 120 °C for complete evaporation of the solvent. Second, the remaining amorphous TiO\textsubscript{2} layer was calcified at 500 °C for which several other studies have reported the formation of anatase coatings.\textsuperscript{175,176}

Typical failure modes after the heat treatment of the film were coating residues at the pore windows of the scaffolds and thick flakes on the TiO\textsubscript{2} struts (Figure 12). In addition, cracks along the grain boundaries were frequently observed. These failure modes are a result of internal stresses in the sol-gel matrix induced by volume shrinkage during the drying and heating stage. Shrinkage dependent failure has been described to occur for coatings which exceed a critical thickness.\textsuperscript{177} In Paper III, the thickness was controlled by the withdrawal speed of the substrate and high quality coatings were achieved for 10 cm/min (Figure 12). It should be noted that alternative approaches such as variation of the hydrolysis rate, addition of chelating agents or modified heating cycle have also been shown to reduce the occurrence coating defects.\textsuperscript{177,178}
Figure 12: Exemplary SEM images of open-porous TiO\textsubscript{2} scaffolds before and after the deposition of a thin film by sol-gel dip coating. Major coatings defects (white arrows) were observed for a withdrawal speed of 30 cm/min. A thin, homogeneous film was achieved for a lower withdrawal speed of the substrate (10 cm/min) with only minor delamination effects present (black arrow).

3.3.2 \textit{H\textsubscript{2}O\textsubscript{2} pre-treatment}

Coated and uncoated samples were further modified by a H\textsubscript{2}O\textsubscript{2} pre-treatment for a prolonged oxidative effect due to the adsorption and stabilisation of reactive species on the TiO\textsubscript{2} surface. The pre-treatment procedure is illustrated in Figure 13. The oxidative behaviour of modified surfaces was quantified by the degradation of methylene blue. For the assessment of the biological response \textit{in vitro}, autoclaved TiO\textsubscript{2} discs were pre-treated with H\textsubscript{2}O\textsubscript{2} as described and handled antiseptically afterwards. A sterilization step after the pre-treatment was avoided because adsorbed radicals may destabilise at high temperatures. The absence of contamination \textit{in vitro} was verified by using appropriate control groups.

Figure 13: Coated and uncoated TiO\textsubscript{2} scaffolds were pre-treated with H\textsubscript{2}O\textsubscript{2} for prolonged oxidative behaviour. Samples were exposed to 30 % H\textsubscript{2}O\textsubscript{2} for 1 h and dried at 37 °C for 24 h.

3.4 \textit{Surface characterisation}

The surface modification described in the previous chapter was applied to change the crystal structure of TiO\textsubscript{2} scaffolds for high catalytic activity. At the same time, the open-porous microstructure had to be maintained. Therefore, several methods were applied to characterise the structural and chemical changes by sol-gel dip-coating.

3.4.1 \textit{Scanning electron microscopy}

The morphology of modified and unmodified samples was analysed by scanning electron microscopy (SEM) as shown in Figure 12. SEM is a fast and simple method for high-resolution imaging based on the interaction of the sample with colliding electrons. This interaction induces the formation of secondary electrons (SE), backscattered electrons (BSE) and emitting X-ray
The tabletop SEM used in Paper III detects the signal from BSE to create a sharp image of TiO₂ scaffolds. The resolution achieved by the detection of BSE was adequate to qualitatively assess the microstructure of the scaffold surface. The limitation of SEM as a method to assess the morphology of the scaffolds is the restricted view, and thus, the inability to quantify changes in porosity, pore size and interconnectivity. Micro-computed tomography (micro-CT) has been used to quantify these important structural properties of the 3D scaffold. However, the goal in Paper III was to characterise the thin film rather than the scaffolds as a whole. Hence, qualitative SEM images were found a good enough estimate to assess the microstructure of coated TiO₂ scaffolds.

### 3.4.2 Ellipsometry

The film thickness of TiO₂ sol-gel coatings was determined by ellipsometry, a fast and non-invasive method which uses the shift in polarization of an incident light beam upon reflection with a surface. Sol-gel coated Si wafers were used as samples to distinguish the coating from the underlying substrate. Further, mirror polished Si wafers reduced the effect of surface roughness in the measurements compared to TiO₂ discs. The incident, polarized beam is changed depending on the film thickness by means of its amplitude ratio and phase difference of the reflected beam. The experimental data is fitted to an ellipsometric function to receive information about the thickness and the refractive index of the coating. Spectroscopic ellipsometry is a very sensitive technique for the characterisation of thin films with sub-nanometer resolution. One drawback of this technique is that fitting the experimental data to the optical model can be a complex and time consuming process. Atomic force microscopy (AFM) may be used as an alternative method to determine the thickness of thin films but requires the presence of uncoated areas on the sample as a reference. Recently, optical profiling has been used as an alternative optical method to determine coating thicknesses. In preliminary experiments, thickness measurements with profilometry were found not sensitive enough for the accurate determination of the thickness of sol-gel films. Nevertheless, profilometry was found very useful to determine the surface topography of TiO₂ discs.

### 3.4.3 Optical profilometry

The change in the microscale topography of TiO₂ surfaces by the deposition of a thin film was characterised by optical image profilometry. This technique requires relatively plane surfaces and thus TiO₂ discs instead of scaffolds were used as samples. The surface roughness was determined by scanning a defined area of the disc with a confocal microscope. Multiple focal planes in z-direction were recorded and compiled for a three-dimensional surface profile. The average deviation of the surface roughness ($S_a$) was calculated from the topographical analysis. Profilometry is a fast and non-invasive optical method to analyse the surface of a sample but its applicability is limited by the physical properties of light. The lateral resolution is determined by the wavelength of the incident beam and thereby unable to detect features below ~200 nm. Further, several topological structures such as undercuts or step edges cannot be analysed by
profilometry due to insufficient reflection of the incident beam.\textsuperscript{181} This limitation was relevant in the analysis of TiO$_2$ discs in Paper III and is shown in Figure 14.

![Figure 14: The topographical analysis of TiO$_2$ surfaces by optical profilometry. (Left) Measurements at grain boundaries were error-prone (white arrows). (Right) Digital filters were applied to identify and remove artefacts for the determination of the surface roughness.](image)

The surface of unpolished discs consisted of several smooth grains which were separated by distinct grain boundaries. Grain boundaries appeared as steep and deep valleys which were challenging to image by optical profilometry. However, over 95% of the surface could be analysed for all tested samples and only 5% incorrectly measured areas needed to be extrapolated. Atomic force microscopy (AFM) may be used in future studies to assess potential changes by thin film coatings in the nanoscale topography of TiO$_2$ surfaces.

### 3.4.4 X-ray diffraction

In Paper III, the crystal structure of the applied thin film coating was examined by X-ray diffraction (XRD). XRD is a commonly used, fast and non-destructive method to determine the crystal phases of thin film coatings.\textsuperscript{179} The measurement principle of XRD is illustrated in Figure 15. In XRD measurements, the incident angle $\theta$ of x-rays with a wavelength $\lambda$ is varied in the range of 0 - 20. X-rays are reflected at the atomic planes of the lattice and a diffraction pattern is recorded. Constructive interference occurs if $\theta$ and the distance between two atomic planes fulfil Bragg’s law ($n\lambda = 2d \sin \theta$). This interference depends on the arrangement of atoms in a crystal lattice and recorded XRD spectra can therefore be correlated to a specific crystalline phase of a material.\textsuperscript{179}
Si wafers were used as a substrate for the identification of the crystal structure of sol-gel coatings. The porous 3D structure of TiO2 scaffolds was not suitable for XRD analysis. The same TiO2 discs used for in vitro studies in Paper III were tested in a preliminary experiment. However, the obtained XRD-spectra were prone to misinterpretation due to the strong background signal of the underlying rutile structure for those samples. Si wafers provide a plane surface of known crystallography and no overlapping XRD peaks with TiO2, and were therefore found suitable for XRD analysis of TiO2 coatings. Multiple coating layers were necessary for a sufficient signal intensity of the TiO2 film. This may be related to the small grain size or the low TiO2 content of a single layer as has been described earlier.182 It should also be noted that the chemistry of the substratum may influence the calcification of thin films. The diffusion of ions from the substrate has been reported to change the phase transition temperature for TiO2.183 However, similar temperatures have previously been reported to result in the complete amorphous-anatase transition for TiO2 films derived from sol-gel coating.176

3.5 Catalytic activity

The catalytic activity of TiO2 powders and TiO2 scaffolds due to the interaction with H2O2 was assessed by the degradation of a model dye (Paper I & III) and EPR spin trapping (Paper I). The degradation of a model dye is commonly used for the early assessment of the oxidative power in catalytically reactive systems.184-186 However, the decomposition of a dye molecule is generally not specific to one reactive species. Thus, dye degradation is valuable to assess and optimize the overall catalytic activity of a system but unable to verify the presence of free radicals. EPR spin trapping measures the paramagnetic behaviour specific to individual radicals and can be used to get information about the underlying molecular mechanisms. Therefore, the advantages of both techniques were used in this thesis for a comprehensive study on the dark catalytic effect of TiO2.
3.5.1 Methylene blue degradation

Methylene blue (MB) is commonly used as a model dye or pollutant to quantify the photocatalytic activity (PCA) of TiO₂ particles and surfaces. It has also been used to study the oxidative behaviour of H₂O₂-TiO₂ suspensions in a previous study. MB is a cationic thiazine dye with a distinct double peak absorbance in the visible light region (λ₁,₂ = 664 and 620 nm) and one peak absorbance in the UV-light region (λ₃ = 290 nm). The chemical structure of the molecule and the UV-vis adsorption spectrum is given in Figure 16. The intensity of peak absorbances can be determined by UV-vis spectroscopy and are linearly correlated to the concentration of the dye (Cₘₜₚ) in the range defined by the Beer-Lambert law. This correlation provides a simple way to determine Cₘₜₚ and its decrease due to the decomposition of the dye.

![Chemical structure of methylene blue](image1)

**Figure 16:** (Left) The chemical structure of methylene blue. (Right) The absorbance spectrum of methylene blue in the wavelength range of visible light with the characteristic double peak at 664 and 620 nm.

The decomposition of MB by H₂O₂-TiO₂ suspensions was quantified in Paper I. The experimental setup is shown in Figure 17. Samples were taken from the stock suspension and centrifuged prior to UVvis measurements to avoid interference with remaining TiO₂ particles. The supernatant appeared slightly turbid after centrifugation for some experimental conditions, particularly for Degussa nanoparticles at low pH. Filtration as a complementary method to separate solid particles from liquid was tested in preliminary experiments. Filtration could only slightly reduce the turbidity of the sample due to a too large mesh size for the separation of nanoparticles, and was therefore not used in the published study.

Prior to the introduction of H₂O₂, MB-TiO₂ suspension were stirred for 1 h to allow dye adsorption on the TiO₂ particles. The adsorption of the positively charged dye is favoured at high solution pH in which the TiO₂ surface is negatively charged. However, the introduction of high concentration H₂O₂ resulted in a change of the suspension pH. This led to the rapid desorption processes of MB at pH 9 and the increase in MB concentration for early measurement points. Therefore, it should be noted that changes in Cₘₜₚ during the degradation phase were accompanied by adsorption/desorption processes and cannot be related exclusively to the decomposition of the dye molecule. The contribution of adsorption/desorption processes to the overall change in Cₘₜₚ was strongly depending on the pH of the suspension and the type
of TiO$_2$ powder used. Hence, the analysis of the reaction kinetics and the determination of an apparent rate constant as a quantitative measure to compare different catalysts were found invalid for the described experimental setup.

Figure 17: (Left) Test setup for the degradation of MB by aqueous H$_2$O$_2$-TiO$_2$ particles suspensions in Paper I. Dye adsorption on TiO$_2$ particles was determined prior to the introduction of H$_2$O$_2$. (Right) Test setup for the degradation of MB by TiO$_2$ scaffolds in the presence of H$_2$O$_2$ or by H$_2$O$_2$ pre-treated scaffolds. All experiments were performed under minimal light exposure.

Preliminary experiments showed insignificant adsorption of MB on TiO$_2$ scaffolds most likely due to the lower available surface area compared to particle suspensions. Hence, a separated adsorption phase was excluded in the test design for Paper III. In addition to the decomposition of MB in the presence of H$_2$O$_2$, scaffolds pre-treated with H$_2$O$_2$ were tested. The observed decrease in C$_{MB}$ was unlikely due to increased dye adsorption on pre-treated surfaces. No significant desorption of MB was observed when the solution pH was lowered by the introduction of HCl. However, analysis of the ζ-potential of unmodified and modified TiO$_2$ surfaces could have been helpful to further assess the adsorption capacity of TiO$_2$ substrates.

One major limitation of both studies is the use of MB as an indicator molecule for the dark catalytic effect of TiO$_2$. Even though MB is extensively used to assess the PCA of different photocatalysts, several shortcomings of MB as an indicator catalytic activity have been reported. MB is a photosensitive molecule which can be reduced to its colourless leuco-form or act as a photosensitizer in TiO$_2$ photocatalysis. Therefore, all experiments in Paper I & III were conducted under minimal light exposure. Further, leuco methylene blue (LMB) may be formed by the reaction with reactive species such as O$_2^*$ which can act as both, an oxidizing and reducing agent. The reversible formation of LMB may therefore be misinterpreted as complete decomposition of the dye, and thereby, lead to an overestimation of the catalytic activity of the tested system. The complementary assessment with a different dye (e.g. malachite green) could have been used to reduce the probability of misinterpretation. Further, several indicator molecules are available which additionally provide information about the underlying reaction mechanism. Among others, coumarin-3-carboxylic acid (CCA) and luminol have been used as fluorescent probes for the detection of *OH and O$_2^*$ respectively. The disadvantage of this method is that fluorescent products of these molecules may not necessarily derive from the reaction with only one specific radical. In general, EPR spin trapping is considered the only technique to verify the presence of a specific radical and was therefore used in Paper III.
The investigation of the dark catalytic effect of TiO$_2$ in this thesis focused on the verification of free radicals (Paper I) and the oxidative behaviour of the latter (Paper I-III). However, the overall decomposition of H$_2$O$_2$ into oxygen and water (see Chapter 1.4.2) was not investigated. This is a limitation of the presented study and additional experimental data are necessary to support the proposed dark catalytic effect of TiO$_2$. One approach may be to spectrophotometrically measure the decline of CH$_2$O$_2$ due to the interaction with TiO$_2$ by the oxidation of I$^-$ to I$_3^-$ (Ghormley triiodide method).$^{191}$

3.5.2 EPR spin trapping

Electron paramagnetic resonance (EPR) spectroscopy combined with radical spin trapping was used to verify and identify the free radicals responsible for MB degradation in Paper I. EPR is the only technique to directly and specifically measure radicals since it measures the unique behaviour of a molecule with an unpaired electron in a magnetic field.$^{190}$ When exposed to a strong magnetic field, the spin of an electron (+$\frac{1}{2}$ or -$\frac{1}{2}$) aligns parallel or antiparallel to the applied field to create two spin states (m$_s$ = $\frac{1}{2}$ or m$_s$ = -$\frac{1}{2}$). These spin states have two distinct energy levels and the difference between these energy levels can be described by:$^{192}$

$$\Delta E = E_{1/2} - E_{-1/2} = g_e \mu_B B_0$$

where $g_e$ is the g-factor of an electron, $\mu_B$ is the Bohr magneton, and $B_0$ is the applied magnetic field. In EPR, the transition between the two energy levels can be measured by applying an external microwave field to create a resonance condition:

$$hf = g_e \mu_B B_0$$

where $h$ is the Planck constant and $f$ is the microwave frequency. This resonance condition is the fundamental relation of EPR and is illustrated in Figure 18. Most EPR instruments use a fixed microwave output and a sweeping magnetic field to fulfil the equation above. The energy adsorption due the resonance condition is typically represented as the first derivative of the adsorption spectrum. The energy levels of an unpaired electron are not only influenced by the applied magnetic field but also by the associated nuclei of the molecules. Therefore, energy levels are further refined (hyperfine coupling) which results in characteristic EPR spectra that are unique for different radical species Figure 19.
The half-life of radicals is generally too short to achieve concentrations high enough for the detection with EPR. Therefore, EPR is often combined with spin trapping. Spin traps are molecules which react with unstable free radicals to form stable radical adducts that can be observed with EPR. Nitrones and nitroso compounds are commonly used for spin trapping of oxygen centred radicals. A free radical can covalently bind to the α-carbon of the spin trap to form a nitroxide radical. The formation of larger radical species leads to a more detailed EPR spectrum based on the presence of a larger number of interfering nuclei. The corresponding hyperfine coupling constants and g-factors for the most common spin adducts have been summarised and were used to relate experimental spectra from Paper I to the associated free radical.

The molecular structure and the characteristic EPR spectrum of the OH-adduct of the well-established spin traps 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and N-tert-Butyl-α-phenylnitrone (PBN) are shown in Figure 19. Both spin traps have been tested in a preliminary study regarding their suitability to investigate TiO₂-H₂O₂ suspension. TiO₂ powders were mixed in aqueous H₂O₂ and the spin trap was added immediately before the sample was transferred to a glass capillary. EPR measurements were started immediately after the introduction of the glass capillary in the sample cavity and several spectra were recorded to assess the time-dependent formation and depletion of spin adducts. All experiments were conducted under minimum light exposure to avoid the formation of free radicals by the photocatalytic effect of TiO₂. Further, all experiments were performed in unbuffered, aqueous H₂O₂ suspension which is an important aspect to consider when comparing the results of Paper I with other similar studies. Buffered systems are commonly used for EPR measurements in biological systems and can also be used to dictate the pH-dependent half-life of spin adducts. However, the degradation of MB was performed in unbuffered systems and comparison between the two studies was desired. Moreover, several buffers have shown to interfere with EPR measurements of H₂O₂ containing samples either by acting as a competitive radical scavenger or by undesired catalytic reactions with metal ion impurities of the buffer.
METHODOLOGICAL CONSIDERATIONS

Figure 19: The chemical structure of the two common spin traps DMPO and PBN. The reaction of DMPO with *OH forms the EPR-sensitive DMPO-OH spin adduct. The EPR line spectrum of DMPO-OH is a quartet with the intensity ratios 1:2:2:1. The characteristic spectrum PBN-OH consists of a triplet of double peaks with the intensity ratio 1:1:1. Both spectra were recorded for H2O2 at the presence of a Fenton reagent (FeCl3).

In Paper I, DMPO was used as a spin trap to detect oxygen centred free radicals. DMPO was found more suitable than PBN, even though both spin traps could successfully trap *OH and O2*/HO2* in H2O2-TiO2 suspensions. DMPO was selected because of the higher half-life of its spin adducts and the higher rate constant for superoxide trapping. However, DMPO has several disadvantages over PBN, particularly the presence of impurities in commercially available spin traps which can result in artificial signals during EPR measurements. Among these impurities, iron (Fe) has been recognised as the most problematic. Fe is a strong Fenton catalyst which decomposes H2O2 via radical intermediates. These radicals can be trapped and falsely taken as a reaction product of the studied system. This problem has been addressed by the use of purified DMPO in Paper I. An alternative approach may be the use of an iron chelator such as diethylenetriaminepentaacetic acid (DETAPAC).

EPR spectra are sensitive to misinterpretation due to the potential formation of free radicals from other sources than TiO2 dark catalysis, such as TiO2 photocatalysis or catalytically active impurities. In addition, several false-positive artefacts have been reported when DMPO is used as a spin trap. DMPO spin adducts may be formed without the actual reaction with the corresponding radical. A typical example is the decomposition of the less stable DMPO-OOH spin adduct into DMPO-OH as illustrated in Figure 20. DMPO-OH has also been reported to form DMPO-OH when exposed to UV irradiation or H2O2 in the absence of free radicals. It was therefore important to verify that DMPO-OH was due to the presence of *OH. The verification was performed by an additional experiment in which ethanol was used as a competitive *OH radical scavenger. The corresponding α-hydroxyethyl radical can further react with DMPO to give rise to a characteristic EPR signal and thus confirm the presence of *OH radicals (Figure 20).
3.6 Antibacterial activity in vitro

The first step in the assessment of the antibacterial activity of a biomaterial is to test its effect in vitro. The evaluation in model culture systems provide a fast and cost-efficient method in the screening of new antibacterial surfaces or disinfectants.\textsuperscript{200} The simplicity and high reproducibility of in vitro studies makes them particularly suitable to study cell-material interactions such as the antibacterial mode of action. Depending on the underlying mechanism, several assays have been established to quantify the antibacterial activity (Table 1). In addition, several in vitro assays have been developed assessing the prevention or destruction of EPS in a biofilm scenario.\textsuperscript{201,202}

Despite these advantages, in vitro studies are unable to represent the complex biological processes happening in living organisms. In the context of antibacterial biomaterials, this includes neglecting the interaction of the surface with host proteins, tissue cells and the immune system among others. With respect to these shortcomings, co-cultures have recently been developed to study the biological response of host cells and bacteria to biomaterials simultaneously.\textsuperscript{203} This may give a more accurate representation of “the race for the surface” and may help to further reduce the number of animal studies.
### METHODOLOGICAL CONSIDERATIONS

#### Table 1: In vitro assays for the evaluation of the antibacterial effect of biomaterial surfaces and disinfectants.
Modified from Zaborowska et al.\textsuperscript{200}

<table>
<thead>
<tr>
<th>Method</th>
<th>Surfaces</th>
<th>Disinfectants</th>
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<tbody>
<tr>
<td></td>
<td>Non-adhesive</td>
<td>Contact</td>
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<tr>
<td></td>
<td></td>
<td>killing</td>
</tr>
<tr>
<td>CFU counting</td>
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<td>X</td>
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<tr>
<td>Disk diffusion</td>
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<td>Broth dilution</td>
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<td>Fluorescence</td>
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<tr>
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<td>X</td>
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<tr>
<td>Confocal laser scanning</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Scanning electron</td>
<td></td>
<td></td>
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<tr>
<td>microscopy</td>
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#### 3.6.1 Bacteria cultures

The antibacterial effect of H\textsubscript{2}O\textsubscript{2}-TiO\textsubscript{2} suspensions (Paper II) and modified TiO\textsubscript{2} surfaces (Paper III) was assessed against \textit{Staphylococcus epidermidis} Xen 43. \textit{S. epidermidis}, along with its relative \textit{Staphylococcus aureus}, are Gram-positive bacteria and common colonisers of the human skin and mucosa membranes.\textsuperscript{204} Their habitat make staphylococci the most likely microorganisms to contaminate indwelling medical devices.\textsuperscript{205} As a result, staphylococcal infections have been recognised as the number one cause for BAI of orthopaedic devices.\textsuperscript{3,58,206}

The pathogenesis of peri-implant diseases is generally more complex compared to the infection of orthopaedic devices. Peri-implantitis has been associated with strains related to dental biofilm formation and periodontitis.\textsuperscript{49} Therefore, the use of a single-strain \textit{S. epidermidis} biofilm model in Paper II is a strong simplification of the clinical situation. However, staphylococci have been isolated from peri-implantitis sites and have been addressed an important role as an early coloniser in the development of pathogenic biofilms on dental implants.\textsuperscript{207} Further, the formation of a biofilm is characteristic of the infection of dental and orthopaedic implants, and \textit{S. epidermidis} has been extensively used to study this behaviour \textit{in vitro}.\textsuperscript{114,115,203,208} It readily forms biofilms on various substrates in a monoculture and is less virulent compared to \textit{S. aureus}.\textsuperscript{58} In addition, \textit{S. epidermidis} grows under aerobic and anaerobic conditions with high tolerance against exogenous oxidative stress.\textsuperscript{128} Thus, it represents a robust strain to assess the antibacterial effect of modified TiO\textsubscript{2} surfaces which were hypothesised to kill via a ROS-mediated pathway (see Chapter 1.4.1). With respect to the above mentioned reasons, \textit{S. epidermidis} was considered a safe, well-established and suitable model in the assessment of antibacterial surfaces and agents.
3.6.2 Experimental setup

Both in vitro studies (Paper II & Paper III) were performed in a monoculture under static conditions at 37 °C in aerobic atmosphere with tryptic soy broth (TSB) as culture medium. TSB is a complex culture medium for bacterial growth with a relatively high iron content. Iron is needed for bacterial growth but excessive iron may act as a competitive Fenton catalyst in the decomposition of H2O2. An iron-depleted culture medium or the addition of a chelator may be used to clarify this subject.

Static culture conditions are unable to represent the flow situation in vivo which have been shown to influence the development of bacterial biofilms. Fluid flow alone has been reported to act as an environmental signal for the formation of EPS in S. epidermidis biofilms. Further, the assessment of the antibacterial effect against one specific strain needs to be considered. In vivo, multiple strains compete with tissue cells for the colonisation of the implant surface. Microbial findings from failing implants often reveal the presence of multispecies biofilms on the implant surface. Multi-species biofilms are highly complex microstructures which may respond differently to the applied treatment (Paper II) or modified surfaces (Paper III). The monoculture is also unable to represent the interaction with host tissue cells. Co-culture systems with S. epidermidis and U2OS cells have pointed out the importance of simultaneous culturing in the assessment of new biomaterial surfaces. Despite these limitations, static monocultures remain a valuable tool in the early assessment of promising antibacterial strategies. They are simple, fast, and a large number of samples can be processed simultaneously. Further, monocultures are particularly useful to investigate the biochemical processes of the antibacterial effect. In order to confirm the antibacterial effect of dark catalytic TiO2 in the future, it could be tested against Gram-negative bacteria and in co-culture systems as a representation of the race for the surface in vitro.

The experimental setup for the treatment of contaminated Ti surfaces with different antibacterial agents (Paper II) is illustrated in Figure 21. The antibacterial activity of the H2O2-TiO2 suspension was compared to H2O2 alone or to the commonly used antiseptic chlorhexidine (CHX). Control groups included the treatment with H2O-TiO2 to verify the absence of an antibacterial effect due to the sole presence of TiO2 nanoparticles.

Grit-blasted, acid etched Ti coins were used to resemble the commercially available OsseoSpeed implant surface. Ti coins were inoculated with S. epidermidis and incubated for 8 h for biofilm formation. The incubation time was determined in a preliminary study, which showed that a thin biofilm of few bacterial layers covered most of the substrate after approximately 6 h incubation. An incubation time of 8 h was chosen in Paper II to guarantee complete coverage of the substrate with bacteria.
Biofilms were treated by exposing them to different antibacterial agents for 1 minute. This time period was chosen since it represents the time for cleansing in a clinical situation. The treatment was stopped by removing the antibacterial agent and consecutive rinsing with phosphate buffered saline (PBS). However, it has to be considered that CHX has been reported to adsorb on Ti surfaces. Therefore, surfaces treated with CHX may show prolonged antibacterial activity after the rinsing steps in the described test setup.

The decontamination outcome was assessed by luminescence readings during re-incubation, SEM and confocal laser microscopy after live/dead staining. One limitation of this study is the absence of a quantifiable viability assay such as CFU counting. This is partly compensated by the quantification of luminescence recordings and the qualitative assessment of fluorescent images. Alternatively, biofilm mass can be measured spectrophotometrically by the optical density (OD) of detached biomass or by staining with e.g. safranin red or crystal violet. However, these methods have been used in similar studies and were not found sensitive enough to measure the enhanced antibacterial effect of H$_2$O$_2$ by the addition of TiO$_2$ particles. Therefore, the remaining biofilm mass after treatment was not quantified in Paper I. Contrary, luminescence readings provided a sensitive method to assess decontamination outcome after the treatment with different chemical agents.

The experimental setup for the assessment of pre-treated TiO$_2$ surfaces (Paper III) is illustrated in Figure 21. TiO$_2$ discs were used as substrates for biofilm formation to resemble the surface of TiO$_2$ scaffolds. Samples were inoculated with a small amount of bacterial suspension in the centre of the disc for initial adherence. Biofilm formation by immersion in a bacterial suspension was avoided in this setup, since the modified surface is believed to act primarily on adherent bacteria. Therefore, only a fraction of bacteria would be affected by the surface
modification if an experimental setup similar to Paper II was used. After the initial adherence, TSB was added and samples were re-incubated for 12 h. The regrowth phase of bacteria on the TiO$_2$ coin and in planktonic state was recorded continuously by luminescence measurements. In addition to luminescence recordings, the effect of TiO$_2$ surfaces on _S. epidermidis_ biofilm formation was assessed by SEM, live/dead staining and CFU counting after 4 and 12 h. The time points were chosen to assess the antibacterial effect of the surface at the beginning of the exponential growth curve (4 h), and after termination of the experiment (4 h). The assessment of the biofilm after 4 h was found interesting since it was expected that the oxidative behaviour of H$_2$O$_2$ pre-treated surfaces quickly ceases when in contact with bacterial suspension. With a doubling time of approximately 30 min for _S. epidermidis_, the consequences of such a burst are likely to be overlooked for later time points.

### 3.6.3 Luminescence

A luminescent strain was used to continuously monitor biofilm regrowth after treatment with antibacterial agents (Paper II) and the biofilm growth on modified TiO$_2$ surfaces (Paper III). The bioluminescent strain _S. epidermidis_ Xen 43 has been constructed from the parental strain _S. epidermidis_ ATCC 1457. Both strains have been reported to be phenotypically equal and bacterial suspensions in Paper III were prepared directly from a frozen stock to avoid phenotypical changes due to passaging. The bacterial luciferase (luxABCDE) gene cassette has been inserted to _S. epidermidis_ Xen 43 by plasmid transformation analogous to the engineering strategy for _S. aureus_ Xen 23. In contrast to firefly luciferase, the insert encodes both the enzyme (luciferase) and the substrates (reduced flavin mononucleotide = FMNH$_2$, O$_2$, fatty aldehyde = RCHO) required for light emission. It can therefore function autonomously. The net reaction catalysed by bacterial luciferase is given by:

\[
\text{FMNH}_2 + \text{O}_2 + \text{RCHO} \rightarrow \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light}
\]

The antibacterial strategy in Paper II and Paper III is based on the formation of free radicals which may interfere with this reaction. One possible reaction is the oxidation of RCHO leading to a decrease in light emission. On the other side, the dependency of O$_2$ and the formation of O$_2$ during dark catalysis (see Chapter 1.4.2) may enhance the catalytic reaction and lead to an increase in luminescence. It is unlikely that extracellularly formed radicals directly react with bioluminescence educts and products, and therefore, an acceleration of the light production due to excessive oxygen is more plausible. This is supported by findings in _Vibrio fischeri_ which exploit the high oxygen affinity of luciferase to lower oxidative stress related damage. The influence of free radicals on bioluminescence was not further investigated, but considered in the development of the experimental setup for Paper II and Paper III.

The use of a bioluminescent strain enables the non-destructive, real-time monitoring of the metabolic activity during the growth of a bacteria population. Since the emission of light is an energy demanding process, bioluminescence has widely been used as an indicator for the metabolic activity of cells. Further, a linear correlation between luminescence and
viability is valid until a peak luminescence ($RLU_{\text{max}}$) is reached. This relation and a characteristic luminescence curve are shown in Figure 22.

![Figure 22](image-url)

**Figure 22**: (Left) Schematic representation of a luminescence curve during bacterial growth: (1) Lag phase, (2) exponential increase, (3) peak, (4) decrease and (5) stagnation of luminescence at a higher level as (1). (Right) Calibration curve for the linear correlation between CFU and luminescence.

The characteristic five segments of the luminescence curve were found among all measurements and test setups. After reaching a peak ($RLU_{\text{max}}$), the luminescence intensity dropped, but to a higher value than the initial lag phase. This was different to the bacterial growth curve determined by CFU in which a stationary phase was reached after exponential growth. Hence, the linear correlation between luminescence and CFU was only valid for $0 < t < t_{RLU_{\text{max}}}$. The drop in luminescence may be related to a decrease in metabolic activity due to a shortage of nutrients or oxygen. This phenomenon appeared independent of whether the well-plates were sealed or not. Moderate shaking before each measurement should further provide the population with sufficient oxygen, thus oxygen shortage was unlikely to cause the reduction in luminescence. Nutrients provided by the culture medium are considered sufficient for 12-24 h. One explanation for the drop in luminescence is that the metabolic activity of bacteria was slowed down by reaching a critical cell number and switching into a dormant state.

Absolute values for $RLU_{\text{max}}$ were not used as a quantitative measure due to the strong variability of $RLU_{\text{max}}$ between each measurement (every 15 min). Instead, $t_{RLU_{\text{max}}}/2$ was used as a reproducible measure for the bacterial regrowth within the linear region of CFU-luminescence. In addition, an overall shift of the characteristic luminescence curve to later time points was considered an indicator for antibacterial activity.

Despite the advantage of monitoring bacterial growth continuously, the use of bioluminescence as a reporter for antibacterial activity has several shortcomings. It remains unclear to which degree the formation of ROS interacts with the $\text{lux}$-cascade. This circumstance may be examined by a separate experiment in which the interference of ROS with either luciferase or the substrates for light production is investigated. Further, both planktonic bacteria and bacteria in a biofilm can be present in a single well and contribute to luminescence. Hence, the origin of luminescence could not be allocated specifically to one or the other, and therefore, complementary methods (CFU, live/dead staining) were required for a conclusive assessment of the antibacterial efficiency. One benefit of the conducted luminescence assay was that luminescence recordings gave a first impression of bacterial growth over the entire observation
time. This was useful for choosing time points of special interests, such as the beginning of exponential growth, for CFU and imaging analysis.

3.6.4 Colony forming units

CFU counts were used to determine the CFU-luminescence calibration curve in Paper II, and to complement the luminescence results in Paper III. Bath sonication at low power output was found the most efficient method to detach bacteria from the surface without damaging them. Phosphate buffered saline (PBS) was used as a dilution medium to avoid further bacterial growth prior to plating on tryptic soy agar (TSA). Direct plate counting complemented the results from luminescence readings by separating planktonic from adherent bacteria. In comparison to all other applied assays, CFU counting was the only method to directly measure the viability of microorganisms, and therefore, the ability to grow and divide under defined conditions. A common problem with CFU counting is that one colony is not necessarily formed by a single bacterium. The number of CFU can be severely underestimated in the presence of microbial aggregates, which consist of several bacteria but only form one CFU. This has been reported to be particularly problematic in biofilm studies, even though CFU counting is a well-established technique to evaluate the efficiency of antibacterial surfaces (Table 1). Bacteria in biofilms are incorporated in EPS, and may therefore be detached from substrates in clusters rather than separated microorganisms. A sonication step, as used in Paper III, has been shown to separate bacteria from surrounding EPS, but may also damage suspended bacteria. Yet, this was found acceptable in Paper III since the same sonication step was performed for all test groups. In addition to microbial aggregates due to the presence of EPS, aggregates have also been shown to form aggregates in the presence of particles. Several agglomerates of TiO$_2$ nanoparticles were observed on Ti surfaces after the treatment with H$_2$O$_2$-TiO$_2$ suspensions as shown in Figure 23. Hence, CFU counting was excluded to determine the antibacterial efficiency of chemical agents in Paper II, and imaging techniques were used to partly compensate the absence of a viability assay in this study.

3.6.5 SEM and confocal microscopy

SEM was used as a fast and simple method to visualise decontaminated surfaces (Paper II), as well as the development of biofilms on TiO$_2$ discs (Paper III). In contrast to SEM analysis of TiO$_2$ samples in Chapter 3.4.1, biological samples were imaged using a field emission (FE) SEM. FE-SEM was run at low operating voltage (5 kV) and high resolution images were obtained from the detection of secondary electrons. Contaminated samples were fixed, dehydrated and sputter coated prior to imaging. SEM images have been used to show alterations in cell morphology and relate them to the underlying killing mechanism. However, such analyses are error-prone due to the occurrence of artefacts by sample preparation (collapse of bacterial wall during dehydration) or the work under vacuum. It is generally dubious to make assumptions on the viability or damage of bacteria based on SEM images and atomic force microscopy (AFM) has shown to be superior in this context. AFM measurements can be
performed in air or liquid, and therefore, avoid cell wall deformation due to vacuum. Further, AFM allows quantitative analysis of the cell wall at nanometre scale. On the other hand, SEM was found a suitable technique for observing a large area of decontaminated Ti surfaces in a relatively short time. Further, SEM revealed the presence of TiO$_2$ agglomerates after the treatment with H$_2$O$_2$-TiO$_2$ suspensions and fibrous microstructures on all decontaminated surfaces (Figure 23). Those features could not be detected by scanning confocal microscopy or any other method applied in Paper II.

![Figure 23](image)

Figure 23: SEM and confocal images of contaminated Ti coins after the treatment with H$_2$O$_2$-TiO$_2$ suspensions. Fibrous structures remained on the surface, independent of the antibacterial agent used for decontamination. Large TiO$_2$ agglomerates were present on decontaminated surfaces after the treatment with H$_2$O$_2$-TiO$_2$ and H$_2$O-TiO$_2$. Only TiO$_2$ agglomerates on surfaces treated with H$_2$O$_2$-TiO$_2$ appeared green fluorescent in scanning confocal microscopy after live/dead staining.

In addition to SEM, samples were stained with propidium iodide and SYTO9, and then imaged by confocal laser microscopy in Paper II and III. SYTO9 is a nucleic acid stain which can penetrate intact cell membranes and stain both live and dead bacteria. Propidium iodide is a nucleic acid stain with a higher binding affinity than SYTO9. It thereby reduces the SYTO9 fluorescence of bacteria with a damaged membrane. Both dyes have specific excitation/emission wavelengths and allow the differentiation of viable (fluorescent green) and dead (fluorescent red) microorganisms. The stained samples were imaged by confocal scanning microscopy in z-stack mode and focal planes were compiled for a full projection 2D image. One limitation of this method is the necessity to remove excess dye by several rinsing steps which can mechanically remove parts of the biofilm from the surface. This may explain the qualitative differences in biomass between SEM and confocal microscopy images seen in Paper II and III. Generally, quantitative analysis of live/dead images can be performed if the corresponding calibration curves are determined and background fluorescence is absent. However, TiO$_2$ agglomerates which remained on the surface after the treatment with H$_2$O$_2$-TiO$_2$ suspensions were strongly green fluorescent as shown in Figure 23. Interestingly, no green fluorescent TiO$_2$ agglomerates were observed for surfaces treated with H$_2$O-TiO$_2$, even though agglomerates were also found on the H$_2$O-TiO$_2$ sample as confirmed by SEM.

3.7 Biocompatibility in vitro

The biocompatibility assessment in vitro is essential for all biomaterials, and particularly for surfaces with antibacterial properties. The ideal antibacterial surface shows high activity against pathogens and no adverse effects on tissue cells. Once the antibacterial activity has been
confirmed \textit{in vitro}, the next step is generally a toxicity screening on tissue cells \textit{in vitro}. The reaction of ROS with biomolecules is unspecific and ROS-mediated damage on host cells has been demonstrated numerously.\textsuperscript{225,226} Analogous to the bactericidal action of ROS, DNA damage has been reported as a major damaging mechanism of free radicals in host cells.\textsuperscript{226} Therefore, a genotoxicity assay complemented the analysis of membrane related damage in Paper III. \textit{In vitro} toxicity screenings give a first indication of potential toxic effects of the modified TiO\textsubscript{2} surfaces but provide no information about the influence of the functionalization on the osteoconductive properties of the tested bone scaffolds. The complexity of an \textit{in vivo} situation is simplified drastically \textit{in vitro} and several toxic molecules which require ROS as a precursor (see Chapter 1.4.1) cannot be formed under these conditions. Therefore, the absence of toxic effects in the conducted cell study may not exclude ROS-mediated damage \textit{in vivo}.

3.7.1 Cell cultures

The toxicity of modified TiO\textsubscript{2} surfaces in Paper III has been tested on the murine osteogenic cell line MC3T3-E1. This cell line has been used extensively for the early assessment of cellular interactions with surfaces designed for bone tissue applications.\textsuperscript{227} MC3T3-E1 is an immortalized primary cell line of pre-osteoblasts, which can differentiate into osteoblasts and form calcified nodules \textit{in vitro}.\textsuperscript{228} In comparison to \textit{in vitro} testing with primary human osteoblasts, tests with MC3T3s are often cheaper, faster and show a lower degree of variability. However, the \textit{in vivo} situation is generally represented more accurately with primary cells which are directly isolated from donor tissue.\textsuperscript{229} Continuous cell lines are immortalized by genetic modification and often lose a series of cell-specific functions due to high passaging numbers. This loss of function may include the cell response to extracellular ROS formation and MC3T3s may therefore respond differently to oxidative stress compared to primary cells. Further, it has been shown that the \textit{in vitro} behaviour of human cells varies strongly compared to cells derived from mice and other mammals.\textsuperscript{229} Therefore, MC3T3s cultures cannot be used to predict the actual cell-material interaction in the human body but are valuable tool for a first toxicity screening.

3.7.2 Experimental design

The biocompatibility assessment was performed under static conditions at 37 °C in humidified atmosphere and 5\% CO\textsubscript{2}. Cells were cultured on TiO\textsubscript{2} discs resembling the surface of a porous 3D scaffold. The use of a static 2D model was found adequate for a toxicity screening but neglects the influence mechanical and microfluidic stimuli of dynamic 3D cultures.\textsuperscript{230} The experimental setup used in Paper III is illustrated in \textbf{Figure 24}. Cells were seeded on TiO\textsubscript{2} surfaces for 15 min for the initial attachment of a monolayer. A high cell density was chosen for adequate imaging of comets in the comet assay. After the incubation for 24 h, cell culture media was collected for LDH analysis and the cell morphology was qualitatively assessed by confocal microscopy after fluorescent staining. In addition, cells were detached from the surface for genotoxic analysis by means of a comet assay.
3.7.3 Confocal microscopy

The cell morphology and cell spreading was analysed by confocal laser microscopy after 24 h culture on TiO₂ surfaces. Green fluorescent phalloidin Alexa Fluor 488 was used to stain the actin cytoskeleton, and blue fluorescent DAPI was used to stain the cell nuclei. Imaging was performed to confirm cell spreading on the TiO₂ discs and visualise potential adverse effects due to the high cell density which was required for the comet assay. Alterations in cell morphology were assessed qualitatively with focus on spherical cell shapes as an indicator for cytotoxic effects. Fluorescent staining and imaging with confocal laser microscopy provides a simple and well-established technique to visualise cell morphology and can therefore complement quantitative toxicity assays.

3.7.4 LDH activity

The cytotoxic effects of modified TiO₂ surfaces was determined by the lactate dehydrogenase (LDH) activity in collected cell culture medium after 24 h. LDH is cytoplasmic enzyme which is released in the surrounding cell culture medium if the integrity of the cell membrane is lost. Therefore, it has been used extensively as a simple and fast method to determine the cytotoxicity of biomaterial surfaces in vitro.²³¹ By adding a reaction mix, the LDH activity can be measured indirectly by the formation of formazan which can be quantified spectrophotometrically. The cytotoxicity was calculated relative to a low control (tissue culture plastic) and a high control (1% Triton X-100):

\[
\text{Cytotoxicity} = \frac{\text{experimental value} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100
\]

Cytotoxicity relative to control groups allows the comparison between test groups and avoids misinterpretation of cell death due to naturally occurring cell death. The loss of membrane integrity is a clear indicator for cell death, but several damaging mechanisms prior to membrane disruption may occur. For example, cytotoxic biomaterials can alter the metabolic activity of host cells which can be analysed by MTT and related assays.²³² Moreover, irreparable DNA damage should be considered if genotoxic effects are expected, as in the case of a ROS-
mediated bactericidal biomaterial. Hence, a comet assay was performed to assess potential genotoxic effects of the modified TiO$_2$ surfaces.

3.7.5 Comet assay

ROS can damage DNA in several ways such as strand breakages, deoxyribose damage, DNA-DNA crosslinks and many more.\textsuperscript{226} Depending on the mechanism, several \textit{in vitro} assays exist to quantify the damage. Hence, the potential reaction pathway has to be assumed in order to choose an adequate genotoxicity assay. The single-cell gel electrophoresis or comet assay in Paper III was selected with respect to the formation of OH radicals and their role in DNA strand breakages.\textsuperscript{226} The main process steps of the comet assay which was used to determine the genotoxic effects of modified TiO$_2$ surfaces on MC3T3 cells is illustrated in Figure 25.

The comet assay is a simple, fast and sensitive technique which is used extensively in ecogenotoxicology and for genotoxicity screening of chemicals.\textsuperscript{233} It is used less often in biomaterials research with only a few studies that investigated the genotoxic effect of surfaces.\textsuperscript{234,235} One limitation of the presented experimental setup is that cells which are killed by the oxidative surface may detach from the sample and will therefore not be represented in the analysis of the comets. Therefore, a trypan blue cell count was performed in a preliminary experiment to confirm a similar number of collected cells compared to a positive control (tissue culture plastic).

The attack by ROS can cause single and double strand breakages of DNA and fragment the double helix.\textsuperscript{226} Therefore, a high number of small fragments imply a high number of strand breakages, possibly due to the reaction with ROS. The comet assay utilises the negative charge of DNA to separate smaller and larger fragments during electrophoresis and gives a quantitative measure for DNA damage \textit{in vitro}. In Paper III, the quantification was performed automatically with a plugin for ImageJ to circumvent manual scoring which has been shown to be more error-prone.\textsuperscript{233}

Ideally, comets derived from this assay resemble the damage on MC3T3 cells due to the exposure to the tested surface. However, several experimental parameters can influence the appearance of comets and have to be considered carefully to avoid misinterpretation of the results obtained. For example, a large comet may be a result of increased damage, decreased...
repair or both. This misinterpretation concerns both the under- and overestimation of genotoxic effects caused by the tested surface.

Underestimation of DNA damage can occur due to repair processes during culturing. This was relevant in the presented study since MC3T3 cells were incubated for 24 h after seeding. With respect to the expected burst effect of the oxidative layer, it could not be excluded that DNA repair took place during incubation. The time frame for incubation is similar to the doubling time of MC3T3 cells, which may further lead to underestimation of genotoxic effects. The re-joining of DNA fragments has also been reported to take place during sample preparation after the collection of cells. This problem was addressed by constantly working at 4 °C after the trypsinization of cells, and thereby, minimising re-joining events during sample preparation.

Overestimation of DNA damage in the conducted assay could occur in at least two different ways. Several sources of DNA damage were identified in the experimental setup which could add up to the potential genotoxic effects form the modified surface. These sources include naturally occurring DNA damage but also external damage by irradiation or exposure to high levels of O₂. Both, irradiation and O₂ exposure were carefully avoided during sample preparation and a TCP control group was used to assess natural occurring DNA damage. Yet, high baseline damage was observed for all tested groups and control groups (Appendix A). Apart from unwanted DNA damage during sample preparation this may be a result of the poor automatic shape detection for small comets. Hence, the applied protocol could be used determine relative genotoxicity between groups but was unable to provide information about the overall genotoxicity of the test samples.
4 KEY FINDINGS

4.1 Oxidative behaviour of TiO$_2$ particles and surfaces by the dark catalytic effect

In Paper I, the model dye methylene blue (MB) was degraded by aqueous H$_2$O$_2$-TiO$_2$ suspensions in the absence of light. The highest degradation was found for TiO$_2$ nanoparticles (Degussa P25) at pH 6.7. The degradation study and particle analysis implied that the oxidative power of TiO$_2$ was higher for anatase than for rutile. Further, the degradation process was shown to take place at the liquid-solid interface or in its proximity. EPR spin trapping verified the catalytic activity of TiO$_2$ in the decomposition of H$_2$O$_2$. Hydroxyl radicals (•OH) were present in all samples, while superoxide radicals (HO$_2^\cdot$/O$_2^\cdot$) were found in TiO$_2$-H$_2$O$_2$ suspensions with high degradation rates for MB. The important role of HO$_2^\cdot$/O$_2^\cdot$ in the oxidative power of TiO$_2$-H$_2$O$_2$ suspension was attributed to its ability to stabilise on the TiO$_2$ surface. It was suggested that adsorbed O$_2^\cdot$ species favour the adsorption of dye molecules, or form an oxidative layer at the TiO$_2$-liquid interface.

Sol-gel dip coating was used to deposit a homogeneous, thin anatase coating on highly porous rutile scaffolds for guided bone repair in Paper III. The interconnected pore network of TiO$_2$ scaffolds remained unaltered by the surface modification. Coated scaffolds showed significantly higher degradation of MB in the presence of H$_2$O$_2$ compared to uncoated scaffolds. The result supported the higher activity of anatase compared to rutile in TiO$_2$ dark catalysis. The pre-treatment with high concentration H$_2$O$_2$ resulted in prolonged oxidative behaviour on coated, but not on uncoated samples. The oxidative behaviour of pre-treated scaffolds in the absence of light and H$_2$O$_2$ was related to the presence of radicals stabilised on the surface. The oxidative layer on coated TiO$_2$ scaffolds was stable for at least 24 h in air at room temperature.

4.2 TiO$_2$ dark catalysis as an antibacterial strategy

The addition of TiO$_2$ nanoparticles enhanced the antibacterial effect of H$_2$O$_2$ in an S. epidermidis biofilm model in vitro. Control experiments confirmed the absence of intrinsic antibacterial properties of TiO$_2$ nanoparticles. The interaction of TiO$_2$ and H$_2$O$_2$ was therefore suggested to be responsible for the enhanced antibacterial activity of H$_2$O$_2$. In Paper II, the treatment of contaminated Ti surfaces with H$_2$O$_2$-TiO$_2$ suspensions delayed bacterial regrowth after decontamination, but could not prevent it. Strong eradication of the bacterial biofilm was observed for the decontamination with H$_2$O$_2$ and H$_2$O$_2$-TiO$_2$ suspensions. The treatment with chlorhexidine (CHX) could prevent bacterial regrowth on Ti surfaces, but not eradicate the existing biofilm. Based on the results of the in vitro study, a synergistic approach for efficient implant decontamination was proposed.

Sol-gel coated TiO$_2$ discs showed antibacterial activity against S. epidermidis in vitro after the pre-treatment with H$_2$O$_2$. Pre-treated, coated samples showed a bactericidal effect at early stages of biofilm formation in a modified direct contact test. No antibacterial effect was
observed for uncoated discs before or after the treatment with high concentration H₂O₂. It was suggested, that the antibacterial effect of pre-treated coated discs was due to O₂•⁻ adsorbed on the TiO₂ surfaces. The bactericidal activity was correlated to the formation of a reactive surface layer. The same surface layer showed no cytotoxic effect in MC3T3s pre-osteoblasts assessed by LDH activity after 24 h of culture. Further, a comet assay showed no increase in DNA strand breakages for pre-treated, coated surfaces compared to controls groups.
5 DISCUSSION

The development of new antibacterial strategies has become a driving force in implant research with good reason.\textsuperscript{76} The prevention and management of biomaterial associated infections (BAIs) is one of the key challenges for a future in which an increasing number of people receive implants for the restoration of body functions.\textsuperscript{2,76} The occurrence of BAI is closely related to the colonisation of implant surfaces with bacteria, most often in the form of strongly adherent biofilms. Biofilms provide a successful mode of life for bacteria in order to conquer new habitats and make them their own, as well as protect themselves from environmental attack. The formation of a biofilm can also cause a strong inflammatory response by the human body which may endanger tissue integration of the implant.\textsuperscript{52} The establishment and maintenance of a direct anchorage between the implant and the surrounding tissue is essential for long-term stability of the device.\textsuperscript{4} Eventually, the non-integrated implant has to be removed, leaving behind a functionally impaired part of the body with increased risk of re-infection.\textsuperscript{2}

A vast number of approaches for infection-resistant biomaterials are currently under development. The main strategy followed, is the functionalisation of implant surfaces with antibacterial properties to prevent biofilm formation. By inhibiting bacterial adhesion, tissue cells may have an advantage in occupying the available surface, and thereby, hinder bacterial colonisation during later stages of implant healing. The interference at early stages of biofilm development is undoubtable a promising approach to combat BAI, but this should not distract from the fact that there is also plenty of room for improvement in the treatment of existing implant infections.\textsuperscript{76,100}

In this thesis, the dark catalytic effect of TiO\textsubscript{2} was studied as a strategy to combat BAI. In contrast to photocatalysis, the dark catalytic effect of TiO\textsubscript{2} describes the formation of free radicals in the absence of light. Hence, TiO\textsubscript{2} dark catalysis extends the field of application to areas where irradiation is not feasible, such as permanently implanted devices. Thus, the interaction of TiO\textsubscript{2} with H\textsubscript{2}O\textsubscript{2} offers a novel and simple approach to functionalise TiO\textsubscript{2} in biomedical applications for antibacterial activity.

5.1 On the interaction of TiO\textsubscript{2} with H\textsubscript{2}O\textsubscript{2}

The catalytic decomposition of H\textsubscript{2}O\textsubscript{2} by TiO\textsubscript{2} under the formation of intermediate radicals is still elusive, and this thesis offers a glimpse into the interplay between the two substances. The experimental work focused on the verification of free radicals during the H\textsubscript{2}O\textsubscript{2}-TiO\textsubscript{2} interaction, and as a result, the oxidative behaviour of such systems (Paper I & III). Secondly, the adsorption of reactive species on the TiO\textsubscript{2} surface and the formation of a stable oxidative layer was investigated (Paper III). These two associated reactions, the formation and the adsorption of reactive species, are considered individually in the following discussion.

5.1.1 Catalytic decomposition of H\textsubscript{2}O\textsubscript{2} on TiO\textsubscript{2}

H\textsubscript{2}O\textsubscript{2} is a strong oxidant which decomposes in aqueous solutions to form O\textsubscript{2} and H\textsubscript{2}O via several reactions pathways. The auto-decomposition of aqueous H\textsubscript{2}O\textsubscript{2} is a relatively slow process at
room temperature, but increases strongly when heated. Apart from temperature, the decomposition rate is further depending on the concentration of H$_2$O$_2$. Hence, H$_2$O$_2$ is commonly stored at concentrations above 30 wt.% at 4 °C, and often blended with stabilizing agents to minimise auto-decomposition.

In addition to homogeneous reactions, decomposition of H$_2$O$_2$ by heterogeneous reactions has been studied extensively. H$_2$O$_2$ is decomposed in the presence of several catalysts, such as biological enzymes (peroxidases) and some metals, such as Fe and Cu. The catalytic reactions via radical intermediates for these metals follow the redox cycle described by the Haber-Weiss mechanism. A similar mechanism has also been used to explain the catalytic activity of some metal oxides, such as FeO, in which the metal ion is not in its highest oxidation state.

A redox pathway is typically not valid to explain the decomposition of H$_2$O$_2$ on ceramic oxides where the metal ion cannot undergo further oxidation. This applies to transition metal oxides such as SiO$_2$, CeO$_2$, Al$_2$O$_3$, ZrO$_2$, and most importantly for this thesis, TiO$_2$. Hence, several authors have proposed an alternative reaction pathway for the catalytic decomposition of H$_2$O$_2$. In this reaction, H$_2$O$_2$ decomposes on a yet unspecified site of the ceramic oxide via the formation of intermediate free radicals (Figure 5). In Paper I, the formation of *OH and HO$_2$•/O$_2$• for TiO$_2$ suspensions was verified in H$_2$O$_2$-TiO$_2$ suspensions by EPR spin trapping. The reaction was accompanied by gas evolution which has been attributed to abundant oxygen. Hence, the results from Paper I support the formation of radical intermediates for the catalytic decomposition of H$_2$O$_2$ into O$_2$ and H$_2$O.

Several groups have studied the interaction of TiO$_2$ and H$_2$O$_2$ by EPR spin trapping with different outcome. Sanchez et al. have also observed *OH and HO$_2$•/O$_2$• for TiO$_2$ suspensions with 10-fold higher H$_2$O$_2$ concentrations. Such concentrations are interesting from a basic science perspective but are too high for the use in most biomedical applications. Fenoglio et al. have studied the catalytic decomposition of H$_2$O$_2$ for concentrations similar to Paper I. They have shown the irradiation independent formation of *OH, while sunlight was required for the formation of HO$_2$•/O$_2$•. The dependency on irradiation to generate HO$_2$•/O$_2$•, but not *OH, has also been shown by Lee et al. for similar test conditions. However, there are also several groups which did not observe radical species for the interaction of TiO$_2$ and H$_2$O$_2$ in the absence of irradiation. These conflicting results may derive from the experimental conditions of the EPR spin trapping studies. Among others, solution pH, measurements in buffered or aqueous samples, or the used spin trap, have all been shown to influence the detection of radicals in similar systems. The experimental conditions chosen in Paper I differed from the above mentioned studies to a varying degree. Hence, direct comparison with these studies remains cumbersome and may be invalid in some cases.

In their pioneering work, Tengvall et al. have thoroughly investigated the interaction of metallic Ti and TiO$_2$ with H$_2$O$_2$ to illuminate the biocompatibility of titanium implants. The focus on their work has been based on the formation of intermediate radicals by the metal-ion-catalysed decomposition of H$_2$O$_2$. Several transition metal ions have been shown to catalyse the decomposition of H$_2$O$_2$ via a chain reaction. In the case of Ti, this chain reactions includes the reduction from Ti$^{IV}$ to Ti$^{III}$ by O$_2$• to complete the redox cycle.
However, no free radicals were observed for Ti- and TiO$_2$-H$_2$O$_2$ suspensions in EPR spin trapping experiments in these studies. The absence of free radicals has been related to the formation of a Ti-OOH adduct with stabilised O$_2^*$ radicals and subsequent quenching of the redox cycle.\textsuperscript{154}

The studies by Tengvall \textit{et al.} have demonstrated that the mechanism of H$_2$O$_2$ decomposition on metallic Ti does not follow the classical Fenton reaction.\textsuperscript{154} On this basis, they have proposed a reaction pathway via Ti-H$_2$O$_2$ complexes, which explained the absence of free radicals during H$_2$O$_2$ decomposition.\textsuperscript{154} Since intermediate radicals were present in our studies, a different reaction pathway was used to explain the formation of radicals for TiO$_2$-H$_2$O$_2$ systems studied in this thesis. The reaction through a redox path is generally not assumed for transition metal oxides in which the metal ion is in its highest oxidation state. While the redox cycle described by Haber and Weiss is triggered by an electron transfer from the metal ion to H$_2$O$_2$, the catalytic decomposition on metal oxides is based on the breakage of the O-O bond of H$_2$O$_2$.\textsuperscript{131,145} The O-O breakage results in the formation of two *OH radicals, which can undergo secondary reactions to form HO$_2^*/$O$_2^-$. Hence, the presence both species (*OH and HO$_2^*/$O$_2^*$) in Paper I is in line with the reaction pathway described in Chapter 1.4.2.

Interestingly, the results from Paper I showed that HO$_2^*/$O$_2^*$ are present for some, but not all, tested TiO$_2$ powders. The three powders which formed HO$_2^*/$O$_2^*$ also showed significantly higher degradation of MB, compared to powders where only *OH was present. This result was surprising, since HO$_2^*/$O$_2^*$ is a fairly weak oxidizer compared to highly reactive *OH radicals.\textsuperscript{121} This observation raises two questions: why are samples with strong *OH signals less oxidising than samples with strong HO$_2^*/$O$_2^*$ signals? And secondly, what material properties of TiO$_2$ favour the formation of one or the other reactive species?

One explanation why samples with strong *OH signals had low oxidative power may be found in the control sample which contained H$_2$O$_2$, but not TiO$_2$. This control group also showed strong *OH signals but was unable to decompose MB. In a separate experiment, it was confirmed that the *OH signal originates from *OH radicals and not from a spin trap artefact (Appendix B). If *OH radicals in this control sample derived from the auto-decomposition of H$_2$O$_2$, it can be assumed that high levels of *OH were also present in MB degradation experiments with H$_2$O$_2$ alone. This would imply that free *OH radicals in solution were unable to decompose MB. However, this hypothesis is in conflict with the strong degradation rates of MB observed in UV-photolysis of H$_2$O$_2$, in which *OH radicals are formed in solution.\textsuperscript{244,245}

An alternative explanation is that *OH radicals were exclusively formed during EPR spin trapping experiments, but not during MB degradation experiments. The formation of *OH in EPR spin trapping may be based on the catalytic decomposition of H$_2$O$_2$ by impurities of the spin trap. The susceptibility of DMPO for catalytically active impurities is well-known,\textsuperscript{198} and this was the reason why purified DMPO was used in Paper I. Nevertheless, the origin and the role of *OH could not be completely clarified. Thus, the DMPO-OH signal in EPR spin trapping experiments alone should not be taken as an indicator for the dark catalytic effect of TiO$_2$ in the presented study.
In contrast to \( {\cdot}OH \) radicals, \( \text{HO}_2^{\cdot}/\text{O}_2^{\cdot} \) may be a more valuable indicator for the catalytic decomposition of \( \text{H}_2\text{O}_2 \) on TiO\(_2\). \( \text{HO}_2^{\cdot}/\text{O}_2^{\cdot} \) was only present in \( \text{H}_2\text{O}_2\)-TiO\(_2\) suspensions with high decomposition rates for MB. It was absent in control samples without TiO\(_2\) particles, and has been proposed to be an important intermediate reactive species in the dark catalytic effect of \( \text{H}_2\text{O}_2 \) (Chapter 1.4.2). Thus, \( \text{HO}_2^{\cdot}/\text{O}_2^{\cdot} \) may represent the clearest experimental evidence in the presented study that \( \text{H}_2\text{O}_2 \) is decomposed at the TiO\(_2\)-liquid interfaces under the formation of oxygen centred radicals.

While a correlation between high degradation of MB and the presence of \( \text{HO}_2^{\cdot}/\text{O}_2^{\cdot} \) is obvious, it remains unclear what the exact role of \( \text{HO}_2^{\cdot}/\text{O}_2^{\cdot} \) in the decomposition of MB is. One possibility is the adsorption of \( \text{O}_2^{\cdot} \) on the TiO\(_2\) surface causing a more negative surface. This would favour the electrostatic attraction of the positively charged MB molecules. The results from Paper I indicated that the decomposition of MB occurs at the liquid-solid interface or in its proximity. Hence, the role of \( \text{O}_2^{\cdot} \) may be the attraction of MB molecules to this region of high oxidative power. In a recent study, Zhao et al. have reported a strong decrease in MB concentration in the presence of Ti-peroxide. \(^{246}\) They related the decrease in MB concentration to strong dye adsorption to peroxy surface groups but not to active decomposition of the MB molecules. In the study by Zhao et al., MB adsorption was clearly visible by the change in the colour of Ti-peroxide from pale yellow to dark blue. In Paper I, dried powders after dye degradation experiments showed no signs of adsorbed MB molecules and remained pale yellow after the experiment. This indicates that the adsorption of MB in \( \text{H}_2\text{O}_2\)-TiO\(_2\) suspensions may be followed by the decomposition of the dye. It is therefore suggested that the continuous MB decomposition observed in Paper I is a result of the constant consumption and renewal of reactive species at the TiO\(_2\)-liquid interface.

This leads to the question which material properties of the catalyst favour the decomposition of \( \text{H}_2\text{O}_2 \). In their comprehensive work, Hiroki et al. \(^{131}\) and Loussa et al. \(^{130,147,148}\) have investigated the influence of different metal oxides on the decomposition rates of \( \text{H}_2\text{O}_2 \). They found that the decomposition rate depends on the type of metal oxide, and increases with increasing surface area. Interestingly, similar activation energies (\( E_a \)) for the decomposition of \( \text{H}_2\text{O}_2 \) have been observed for all tested catalysts. \(^{131}\) However, the decomposition rate increased strongly in the order \( \text{SiO}_2 < \text{Al}_2\text{O}_3 < \text{TiO}_2 < \text{CeO}_2 < \text{ZrO}_2 \). \(^{131}\) These differences in the reaction rate were related to differences in the number and/or efficiency of catalytically active sites at the oxide-liquid interface. \(^{131}\) Unfortunately, no similar studies exist, comparing the catalytic activity of different TiO\(_2\) powders. However, a large number of excellent studies has been published which compared the photocatalytic activity (PCA) of different TiO\(_2\) powders. \(^{247-250}\) Degussa P25, an anatase-rutile nanoparticle mixture, which also showed the highest dark catalytic activity in Paper I, has been widely used as a reference catalyst with high PCA. \(^{250}\) The high PCA of P25 has been related to charge carrier mechanisms which are specific to UV-induced formation of free radicals. \(^{251}\) With respect to the differences in the underlying reaction pathway of photocatalysis and dark catalysis (Figure 5), different material properties may be decisive for high catalytic activity.

Much experimental effort is required to determine specific material properties responsible for high catalytic activity in the absence of irradiation. This was beyond the scope of this thesis,
and a more pragmatic approach was followed to select an adequate catalyst for the decontamination of implant surfaces (Paper II) and the development of antibacterial surfaces (Paper III). Degussa P25 showed the highest degradation of MB among all tested TiO₂ powders, and was therefore chosen as the most promising candidate in the decontamination of Ti surfaces. The decomposition of MB by TiO₂-suspensions was not normalised with respect to the different surface areas of the tested TiO₂ powders. Hence, the very high decomposition rates observed for P25 may be primarily due to the high surface area of this catalyst compared to other catalysts tested.

In contrast to TiO₂ suspensions, unmodified TiO₂ scaffolds showed low catalytic activity in Paper III. The results from Paper I indicated that a high surface area and an anatase crystal structure presumably favour the decomposition of H₂O₂. Fenoglio et al. have shown a correlation between the type of radicals formed, and the crystal phase of TiO₂.¹³³ In their study, anatase has been shown to favour the formation of O₂⁻, the same species which was found to play an important role in the decomposition of MB. At first glance, the findings in Paper III, in which anatase coated scaffolds showed high oxidative power, support the idea that anatase is required to form O₂⁻, which again is required to decompose MB. However, it is questionable whether the high degradation of MB by anatase scaffolds is only due to the crystal phase of the tested catalysts. This correlation is further challenged by preliminary experiments, in which scaffolds coated by atomic layer deposition (ALD) were tested regarding their catalytic activity. The ALD coating has previously been applied to protect scaffolds from grain boundary corrosion in acidic environments.²⁵² For this purpose, a thin TiO₂ film with crystallites of approximately 30 nm has been deposited on TiO₂ scaffolds. The crystal structure of these coatings has also been shown to be pure anatase.²⁵² Further, the ALD coating significantly increased the surface area of TiO₂ discs similar to the ones used in Paper III.²⁵² However, no MB degradation was observed when ALD coated anatase scaffolds were tested in the presence of H₂O₂ (Appendix C). This finding indicates that the high catalytic activity of sol-gel coated scaffolds is not solely due to the anatase phase, or the larger available surface area compared to uncoated scaffolds. Hence, it is presumed that other surface properties are responsible for the strong degradation observed for sol-gel coated scaffolds.

One possible explanation for the catalytic activity of sol-gel coated scaffolds may be the presence of a high number of oxygen vacancies in the deposited film. Point defects such as oxygen vacancies play an important role in heterogeneous catalysis, and are often the reason why metal oxides are catalytically active.²⁵³ The sequential film growth in the ALD coating procedure has been shown to result in a low number of coating defects.²⁵³ On the contrary, sol-gel coatings typically show a high number of defects due to the less controllable hydrolysis and polymerisation steps in the sol-gel route (Figure 10).²⁵⁴ The short sintering time in low oxygen environments applied in Paper III may have further contributed to the formation of oxygen vacancies. Hence, a high number of surface defects is presumed to be the predominant reason for the high catalytic activity of the sol-gel coated TiO₂ scaffolds. This is in good agreement with a recent study by Lousada et al., in which a theoretical model was used to investigate the catalytic decomposition of H₂O₂ on TiO₂.¹³⁰ A small cluster model with reduced coordination
of the metal ions has been shown to describe the decomposition of H$_2$O$_2$ on metal oxides accurately. These results have pointed out the importance of defects on metal oxide surfaces and may explain the high catalytic activity of the sol-gel coated scaffolds.

Even though the experimental work in Paper I and III leave many questions regarding the interaction of H$_2$O$_2$ and TiO$_2$ unanswered, the findings have indisputably set the foundation for the assessment of TiO$_2$ dark catalysis \textit{in vitro}. Based on these findings, Degussa P25 and sol-gel coated TiO$_2$ scaffolds were identified as catalysts with high oxidative power in the presence of H$_2$O$_2$. Interestingly, the oxidative power of the coated scaffolds could be conserved to some degree when these samples were pre-treated with H$_2$O$_2$. This effect was suggested to be a direct consequence of the formation of O$_2^\cdot$ during the decomposition of H$_2$O$_2$ and is discussed in the following.

5.1.2 Oxidative behaviour of modified TiO$_2$ scaffolds after H$_2$O$_2$ pre-treatment

In addition to the oxidative power of coated TiO$_2$ scaffolds in the presence of H$_2$O$_2$, the same samples were also capable of decomposing MB after the pre-treatment with H$_2$O$_2$. This was related to the adsorption and stabilisation of free radicals, presumably O$_2^\cdot$, on the TiO$_2$ surface. It is well-known that the superoxide radical anion can adsorb on metal oxides, such as MgO, ZrO$_2$ or TiO$_2$.\textsuperscript{[134,150]} The decomposition of H$_2$O$_2$ on TiO$_2$ has been described as one mechanism to generate O$_2^\cdot$ with subsequent stabilisation at the metal oxide surface.\textsuperscript{[150]} This has been confirmed by EPR measurements, showing O$_2^\cdot$ radicals coordinated to Ti$^{IV}$ metal centres.\textsuperscript{[136]} Hence, the adsorption of O$_2^\cdot$ results in the formation of metal-dioxygen complexes with distinctly higher lifetime, compared to their free radical counterpart. It is therefore suggested that the formation of metal-dioxygen complexes (superoxo and peroxo) are the reason for the prolonged oxidative power of H$_2$O$_2$ pre-treated scaffolds. This has not been confirmed experimentally in this thesis, and hence, a solid-state EPR study is recommended to verify and identify the presence of adsorbed radicals.

The decrease in the concentration of MB for pre-treated samples could not solely be explained by a higher adsorption capacity for positively charged MB molecules. After the degradation experiments, none of the tested TiO$_2$ scaffolds showed blue or purple appearance due to increased dye adsorption. These results do not support the findings by Zhao \textit{et al}. who observed strong coloration of titanium peroxide.\textsuperscript{[246]} They have related this observation to the adsorption of MB on peroxo surface groups, but not to the active decomposition of MB molecules by the oxidative surface. Therefore, the absence of visible dye adsorption on coated scaffold implies that dye molecules are oxidized at the pre-treated surface. This finding is in agreement with the work of Ogino \textit{et al}. who have reported oxidation of MB for H$_2$O$_2$ pre-treated TiO$_2$ particles.\textsuperscript{[255]} However, the oxidation of MB by pre-treated scaffolds showed distinctly different degradation kinetics compared to the setup in which H$_2$O$_2$ was constantly present. The continuous degradation of MB in the presence of H$_2$O$_2$ was related to simultaneous consumption and new formation of reactive species. For pre-treated scaffolds, oxidative behaviour was only observed within the first 60 min of the experiment. This indicates that active species are consumed and not renewed during the period of the experiments for these samples.
So far, it remains unclear whether the oxidative power of pre-treated scaffolds is due to the presence of stabilised $\text{O}_2^\cdot$ radicals. Alternatively to the adsorption of $\text{O}_2^\cdot$, initially formed $\text{OH}$ radicals could adsorb on the TiO$_2$ surface and cause the oxidative behaviour observed in Paper III. This would confirm the theoretical and experimental studies by Lousada et al., who showed binding of $\text{OH}$ radicals on reduced metal centres following the decomposition of H$_2$O$_2$.\textsuperscript{130} Alternatively, $\text{OH}$ radicals have shown to remove surface H atoms, and thereby form physisorbed H$_2$O on the metal oxide surface.\textsuperscript{130} Hence, the H$_2$O$_2$ pre-treated surfaces in Paper III are not yet sufficiently characterised to explain the oxidative behaviour in dye degradation experiments.

Even though pre-treated surfaces showed some oxidative behaviour, the overall decomposition of MB by pre-treated TiO$_2$ surfaces was significantly lower compared to samples where H$_2$O$_2$ was present during the degradation period. It may be interesting to optimise the oxidative behaviour of pre-treated TiO$_2$ surfaces in the future. One decisive parameter in this context is believed to be the control of oxygen vacancies of the applied coating. These defects have been reported to act as catalytically active sites as described earlier in this thesis. Further, they have shown to provide adsorption sites for free radicals formed during the decomposition of H$_2$O$_2$.\textsuperscript{130,150} A first step to enhance the oxidative power of pre-treated surfaces may be a targeted heat treatment of the deposited film to create a high density of oxygen vacancies. The number of point defects in TiO$_2$ ceramics typically increases for higher temperatures and calcification in oxygen free environments.\textsuperscript{256} Therefore, adjusting the sintering conditions for sol-gel films possibly provides a simple way to enhance oxidative power of TiO$_2$ scaffolds. However, the control of the oxidative power of the applied coating was beyond the scope of this thesis. Rather, it was important to provide a proof of concept whether this surface modification can be used to functionalise TiO$_2$ scaffolds for antibacterial activity.

## 5.2 Dark catalysis as antibacterial strategy

The dark catalytic effect of TiO$_2$ shows a range of interesting properties for the potential use in biomedical applications. First of all, the two participants, TiO$_2$ and H$_2$O$_2$, are already widely used in biomedical applications today. TiO$_2$ has shown to be an excellent material in bone tissue applications,\textsuperscript{4} and is one of few metal oxide particles approved by the Food and Drug Administration (FDA) as food additive.\textsuperscript{257} The bactericidal activity of H$_2$O$_2$ has been known for many decades, and ever since H$_2$O$_2$ has been used as an efficient disinfectant in concentrations up to 10\%.\textsuperscript{55,258} Further, oxygen centred radicals, formed by similar reactions than TiO$_2$ dark catalysis, have been shown to possess bactericidal activity against a broad range of pathogens.\textsuperscript{141,208,259} Analogous to TiO$_2$ photocatalysis, no toxic end-products are formed by the chain reaction described in Chapter 1.4.2. Therefore, TiO$_2$ dark catalysis can be considered a safe and environmentally friendly antibacterial strategy. One of the main advantages of TiO$_2$ dark catalysis over classical photocatalysis is the independence from irradiation to form bactericidal radicals. This enables the use of TiO$_2$ dark catalysis in biomedical applications where irradiation is unwanted or not feasible.
5.2.1 $\text{H}_2\text{O}_2$-$\text{TiO}_2$ Suspensions in Peri-implantitis Therapy

The decontamination of dental implants was identified as one potential application for the dark catalytic effect of TiO$_2$. Since H$_2$O$_2$ is already used as a chemical agent in the treatment of peri-implantitis, it has been suggested that the bactericidal effect of H$_2$O$_2$ can be enhanced by the addition of TiO$_2$ particles based on their catalytic activity.$^{113}$ The findings of Paper I confirmed the catalytic activity of TiO$_2$ particles and showed high oxidative power for H$_2$O$_2$-TiO$_2$ suspensions. In a next step, the effect of these suspensions was tested on bacteria which possess a rich arsenal of defence and repair mechanisms to actively cope with ROS attack.$^{127,128}$

The results in Paper II showed that the addition of TiO$_2$ nanoparticles increased the antibacterial effect of H$_2$O$_2$ on $S$. epidermidis biofilms. Control experiments excluded antibacterial properties of TiO$_2$ nanoparticles not related to the interaction with H$_2$O$_2$.$^{260,261}$ Hence, it was suggested that the increase in antibacterial activity of H$_2$O$_2$ was due to the formation of free radicals at the TiO$_2$-liquid interface. In a similar in vitro study, Henderson et al. have reported a positive effect for the addition of TiO$_2$ in H$_2$O$_2$, but only for a high number of replicates ($n = 53$).$^{114}$ In a follow up study, Gustumhaugen et al. have not found significant differences between the decontamination outcome for the treatment with H$_2$O$_2$ and H$_2$O$_2$-TiO$_2$.$^{115}$ Both studies assessed the decontamination outcome by biomass removal after the treatment with chemical agents. The assessment of the total biomass by safranin staining is believed to be the main reason for the low effect of TiO$_2$ particles in H$_2$O$_2$ observed in the two previous studies. Safranin binds to a wide range of negatively charged molecules found in bacteria, but also in extracellular polymeric matrix (EPS).$^{262}$ Hence, the staining technique used in the two previous studies was not suited to detect potential bactericidal activity due to the addition of TiO$_2$ particles. Based on the findings from Paper I, it was assumed that free radicals formed during the interaction of TiO$_2$ and H$_2$O$_2$ possess bactericidal properties according to the damaging mechanism shown in Figure 4. Therefore, a luminescence assay in combination with live/dead staining was used in Paper II providing a sensitive technique to assess viability and follow bacterial re-growth after decontamination.

The results from the luminescence assay and live/dead staining showed a lower number of viable bacteria after the treatment with H$_2$O$_2$-TiO$_2$, compared to the treatment with H$_2$O$_2$. The bactericidal activity of H$_2$O$_2$ itself has been attributed to the penetration of the bacterial envelope. Inside the cell, H$_2$O$_2$ can participate in Fenton reactions, leading to the formation of reactive *OH radicals.$^{123,258}$ These intracellularly formed *OH radicals attack a number of vital cellular components as shown in Figure 4. This damaging pathway of H$_2$O$_2$ is believed to be expanded in the presence of TiO$_2$ particles. The dark catalytic effect of TiO$_2$ provides a potential source of reactive species formed outside the bacterial cell. Membrane damage by lipid peroxidation has been reported to be a relevant damaging mechanism for extracellularly formed radicals.$^{126}$ In lipid peroxidation, polyunsaturated fatty acids are oxidized by free radicals to form a fatty acid radical.$^{263}$ This initial oxidation step induces a chain reaction which eventually leads to disruption of the lipid bilayer, and consequently, cell death.$^{263}$ Both reactive species detected in the H$_2$O$_2$-TiO$_2$ suspensions in Paper I have been shown to contribute to lipid peroxidation in vitro and in vivo.$^{263}$
DISCUSSION

Even though the study in Paper II cannot provide experimental evidence that lipid peroxidation is the main reason for the increase in antibacterial activity of H₂O₂, similar systems for disinfection have been suggested to be linked to this killing mechanism. Maness et al. have attributed the bactericidal activity of TiO₂ photocatalysis to the formation of ROS and subsequent membrane disorder in E. coli. In addition, Ikai et al. have shown an enhanced disinfection efficiency of H₂O₂ under irradiation with near UV-light (photolysis of H₂O₂) for several pathogenic bacteria in a planktonic state (S. aureus, A. actinomycetemcomitans, S. mutans and E. faecalis). The photolysis of H₂O₂ has been shown to generate highly toxic •OH radicals and has therefore been suggested as an efficient disinfection method in the therapy of periodontal diseases. Most notably, this technique has also shown high bactericidal activity in a biofilm model in the same study.

This leads to the important question whether H₂O₂-TiO₂ suspension may be clinically useful for the chemical decontamination of implant surfaces. In a peri-implantitis scenario, bacteria are often protected in a biofilm, and the proposed killing mechanism may be impaired. The lower efficiency of antibiotics and disinfectants for biofilms is primarily based on low metabolic activity of bacteria in biofilms and low diffusion of active molecules through EPS. Thus, the minimum biofilm eradication concentrations (MBEC) of antibacterial agents can be up to 1000 times higher than the minimum inhibitory concentration (MIC) of planktonic bacteria. The antibacterial activity of H₂O₂-TiO₂ suspensions may be particularly affected by the low efficiency of chemical agents in biofilms. Based on the findings from Paper I, the oxidative power of TiO₂ particles is the strongest at the particle surface or in its proximity. It is assumed that the incorporation in EPS shields bacteria from a direct contact with the oxidative surface, thereby attenuating the synergistic effect of TiO₂ particles in H₂O₂ suspensions.

One interesting observation in Paper II was that the treatment with H₂O₂ or H₂O₂-TiO₂ removed a substantial amount of the original biofilm from the implant surface. This was correlated to the formation of bubbles, possibly due to oxidation of the Ti substrate by H₂O₂. The development of gaseous oxygen may partly be responsible for the removal of biofilm mass, either by mechanical means or by the creation of high oxygen concentrations at the Ti-biofilm interface. Alternatively, the extensive biofilm removal for H₂O₂-based treatment regimens may be linked to the decomposition of EPS by H₂O₂ as reported previously. This effect may be even more pronounced in a clinical situation, where the implant surface is covered in blood. Blood is rich in catalase which readily decomposes H₂O₂ to water and oxygen. The formation of oxygen is believed to further contribute to the eradication of biofilms for H₂O₂-based treatments. The influence of blood on the treatment with different chemical agents was not represented in the conducted in vitro study. This may have led to an underestimation of the antibacterial efficiency of H₂O₂ and H₂O₂-TiO₂ suspensions compared to the treatment with chlorhexidine solutions.
Chlorhexidine (CHX) was tested to compare the treatment with H$_2$O$_2$ and H$_2$O$_2$-TiO$_2$ to another chemical agent used in peri-implantitis therapy.$^{100,270}$ CHX showed high bactericidal activity and prevented bacterial regrowth after decontamination in Paper II. This is in good agreement with work of several other groups who have reported a strong bactericidal effect of CHX solutions on staphylococcal biofilms.$^{271-273}$ Contrary to H$_2$O$_2$-based treatments, the decontamination with CHX was unable to remove biofilm mass from the implant surface. This qualitative observation was supported by the quantitative assessment of biomass removal by Henderson et al.$^{114}$ In this study, the treatment with CHX showed no significant reduction in biomass compared to controls. On the other side, the treatment with H$_2$O$_2$ and H$_2$O$_2$-TiO$_2$ showed a high capacity to remove biofilm from the surface possibly due to the formation of oxygen as described above. Therefore, the treatment outcome strongly depended on the antibacterial mode of action, and the use of complementary methods was found crucial to assess the decontamination of different chemical agents.

Under the test conditions in Paper II, a few viable bacteria survived the treatment with H$_2$O$_2$-based agents and formed a new biofilm during re-incubation. In contrast, no bacterial regrowth was observed when contaminated Ti surfaces were treated with CHX. Therefore, one may argue that the treatment with CHX solutions showed the greatest potential as antibacterial agent for peri-implantitis therapy. This neglects the fact that the ultimate goal in peri-implantitis treatment is the re-osseointegration of the decontaminated implant. Thus, the antibacterial agent used for implant decontamination must provide high antibacterial activity, and at same time it should not impair wound healing. The use of CHX in open wounds is controversial, since cytotoxic effects of this agent have been reported on different cell types.$^{274,275}$ Further, CHX has been reported to adsorb on titanium and thereby exhibiting prolonged activity after treatment.$^{213}$ This is particularly concerning since a slow release of CHX from the implant surface may contribute to CHX resistance of pathogens. CHX resistance has already been reported for several strains, including methicillin resistant *S. aureus* (MRSA).$^{276}$ H$_2$O$_2$ on the other hand, is generally considered a safe disinfectant with a high activity against a broad range of Gram-positive and Gram-negative bacteria and little or no adverse effects on wound healing.$^{108,258}$ Pan et al. have even reported a positive effect for low level H$_2$O$_2$ exposure on epithelial wound healing.$^{277}$ Nevertheless, the SEM and confocal images in Paper II revealed the presence of TiO$_2$ agglomerates remaining on the Ti surface after the treatment with H$_2$O$_2$-TiO$_2$ suspension. In a clinical situation, these agglomerates would remain in the body and potentially cause cytotoxic effects, particularly if broken up into TiO$_2$ nanoparticles.$^{278}$ Hence, a toxicity screening is required to verify the safe use of H$_2$O$_2$-TiO$_2$ suspensions in implant decontamination.

To this date, only few *in vivo* studies have been published on the chemical decontamination of implant surfaces in the surgical treatment of peri-implantitis.$^{279}$ There is an overall consensus that chemical decontamination should be used in addition to mechanical debridement, but no recommendation has been established on which chemical agent should be used.$^{99,107}$ A large number of *in vitro* studies has been conducted with the aim to identify the most potent biocide for surface decontamination.$^{106,114,115,280,281}$ The results of these studies are often in disagreement with each other, and so far, no conclusion on which chemical agent that may be
most suitable for the treatment of peri-implantitis can be drawn from these in vitro studies. One reason for the high level of disagreement is based on the specific test conditions used in each of these studies\(^ {279}\). Important experimental parameters that have been shown to influence the antibacterial efficiency of agents in vitro are chemistry and topography of the tested surface, composition and age of the biofilm, as well as biocide concentration and exposure time.\(^ {279}\) However, the different conclusions drawn in these studies also imply that there is no “one-size-fits-all” chemical agent for efficient implant decontamination. Hence, a combination of antibacterial agents with different modes of action, such as H\(_2\)O\(_2\) (biofilm removal) and CHX (bactericidal), was suggested in Paper II. A combined approach of two or more chemical agents may have synergistic effects, and therefore improve the decontamination outcome and lower the risk of re-infection.

5.2.2 TiO\(_2\) bone scaffolds with antibacterial properties

The findings from Paper II showed that H\(_2\)O\(_2\)-TiO\(_2\) suspensions can be used to efficiently kill bacteria with implications in the decontamination of implant surfaces. In Paper III, the catalytic activity of particle suspensions was successfully translated to TiO\(_2\) scaffolds by applying a thin TiO\(_2\) coating. Coated scaffolds showed oxidative behaviour in the presence of H\(_2\)O\(_2\), but also when pre-treated with high concentration H\(_2\)O\(_2\). Hence, it was interesting to investigate the biological response towards modified TiO\(_2\) scaffolds designed to guide bone repair in critical size defects.

Bone repair by TiO\(_2\) scaffolds and peri-implantitis treatment with H\(_2\)O\(_2\)-TiO\(_2\) suspension represent two fundamentally different scenarios where TiO\(_2\) dark catalysis could be used. In peri-implantitis, bacteria have already won the race for the surface, and the aim is to re-establish a situation in which cells can have a second chance.\(^ {99}\) Therefore, the in vitro study in Paper II was designed to investigate the effect of radicals on an already existing biofilm. This differs from the in vitro studies in Paper III, resembling the situation of a primary implant patient. In this scenario, host tissue cells will compete with bacteria to occupy the surface of the freshly inserted device.\(^ {1,2}\) Therefore, it was necessary to test the modified TiO\(_2\) surfaces on both bacteria and tissue cells. Contrary to the experimental setup in Paper II, the oxidative effect of TiO\(_2\) surfaces on cells could not be assessed when the catalyst and H\(_2\)O\(_2\) were present at the same time. This was due to the fact that H\(_2\)O\(_2\) itself shows toxic effects on MC3T3 cells already at micromolar concentrations.\(^ {282}\) Instead, TiO\(_2\) discs pre-treated with H\(_2\)O\(_2\) were used as samples to assess the biological performance of the developed surface modification.

The results from the luminescence assay in Paper III demonstrated that the pre-treatment with H\(_2\)O\(_2\) resulted in a delay of bacterial growth. This was independent of whether samples were sol-gel coated or not. This finding is partly in conflict with the results from CFU counting and live/dead staining. These two methods could confirm the antibacterial effect for sol-gel coated samples but not for uncoated samples. This discrepancy between the complementary assays could be related to the limitation of luminescence as a marker for viability in the experimental setup used in Paper III (Figure 21). As described in Chapter 3.6.3, free radicals may interfere with the bacterial luciferase cascade, for example by oxidising one of the substrates for this reaction. Hence, the linear correlation between luminescence and viability
shown in Figure 22 may be invalid in the presence of pre-treated surfaces which potentially act as a source for free radicals. Nevertheless, luminescence was useful to continuously follow bacterial growth and select time points for further analysis by CFU counting and imaging techniques.

While the results for uncoated surfaces are partly conflicting, all in vitro assays conducted in Paper III showed a clear antibacterial effect on sol-gel coated TiO$_2$ discs after the pre-treatment with H$_2$O$_2$. The observed antibacterial effect suggested the presence of an oxidative layer formed during the 1 h exposure of the coating to H$_2$O$_2$. As described earlier in this thesis, this layer may be formed during the catalytic decomposition of H$_2$O$_2$ and the stabilisation of radicals on the metal oxide surface. Only a few studies have investigated the antibacterial effect of such surface bound radicals on Ti and TiO$_2$. Unosson et al. have reported an inherent antibacterial effect for H$_2$O$_2$ treated Ti discs on *S. epidermidis* and *S. mutans*. They have previously shown that the applied H$_2$O$_2$ treatment of Ti surfaces resulted in a nanoporous anatase layer with oxidative behaviour. One difference between the experimental setup of Unosson et al. and the in vitro study conducted in Paper III was the time points chosen to determine bacterial viability after seeding. While Unosson et al. investigated the immediate response to the surface 10 and 20 min after seeding, considerably longer time points were selected in Paper III. The viability was assessed after 4 h, since this time point referred to the beginning of exponential growth according to the acquired luminescence curves. Further, bacterial viability was assessed after the termination of the experiment at 12 h. Interestingly, live/dead images showed a high number of dead bacteria after 4 and 12 h for coated TiO$_2$ discs that had previously been treated with H$_2$O$_2$. This indicated that the applied surface modification may have a prolonged bactericidal activity, although, biofilm formation could not be prevented as shown by SEM. With respect to the prolonged activity and the short half-life of free radicals, it can be assumed that the antibacterial effect is not due to the direct interaction of bacteria and released radicals. It is more likely that adsorbed radicals show contact killing behaviour or contribute to the formation of more stable ROS such as H$_2$O$_2$.

One interesting observation in the study by Unosson et al. was that the antibacterial effect of the tested oxidative surface was stronger on *S. mutans* than *S. epidermidis*. This may be due to the different enzymatic defence mechanisms of these strains to cope with oxidative stress. While both strains are superoxide dismutase (SOD) positive, only *S. epidermidis* is catalase positive. Catalase is an important intracellular enzyme that converts H$_2$O$_2$ to oxygen and water. Hence, this observation further supports the hypothesis that pre-treated TiO$_2$ surfaces, similar to the one studied in Paper III, kill bacteria via a ROS-mediated damaging pathway (Figure 4). In a similar study, Cai et al. have investigated the antibacterial activity of TiO$_2$ nanocomposites shortly after UV irradiation. They have also related the antibacterial effect to adsorbed superoxide species, but found no differences in the antibacterial efficiency between *S. mutans* and *S. epidermidis*. However, *E. coli* showed a distinctly higher tolerance against the oxidative surface than *S. mutans* and *S. epidermidis* in this study. This may indicate lower antibacterial efficiency of the oxidative surface on Gram-negative bacteria than Gram-positive bacteria. Similar results have been obtained for the efficiency of TiO$_2$ photokilling, and they have been related to the different cell wall structure of Gram-positive
and Gram-negative bacteria\textsuperscript{286} These results underline the shortcoming of the experimental setup in Paper III, in which the antibacterial effect of modified TiO\textsubscript{2} surfaces has only been tested on one bacterial strain. Although the strong biofilm former \textit{S. epidermidis} was found an adequate model strain to test antibacterial surfaces, further studies are required to confirm the antibacterial effect on other species, particularly Gram-negative bacteria.

In comparison to other anti-infective implant surfaces based on the formation of ROS,\textsuperscript{287,288} the presented TiO\textsubscript{2} surface showed a relatively low reduction in bacterial viability. It is generally assumed that log-reduction in bacterial viability is required for the development of infection-resistant biomaterials. The surface modification tested in Paper III showed a viability reduction of 43\% for \textit{S. epidermidis} compared to the control. Hence, it may be necessary to enhance the bactericidal activity of the presented TiO\textsubscript{2} surface to prevent BAI \textit{in vivo}.

The antibacterial activity of the applied sol-gel coating may be enhanced by increasing the oxidative power of this surface. One measure to achieve high oxidative power could be the optimisation of the coating for a high density of oxygen vacancies as described in Chapter 5.1.2. However, the presented surface modification may even exhibit enhanced antibacterial activity \textit{in vivo} without further surface modification. Tengvall \textit{et al.} have shown that various reactive species are formed during the degradation of a Ti-peroxy gel.\textsuperscript{156} While degradation products showed no inherent antibacterial effect, the addition of peroxidase and a halogen resulted in strong bactericidal activity on \textit{E. coli}.\textsuperscript{124} This has been related to the release of H\textsubscript{2}O\textsubscript{2}, possibly via superoxide and peroxide species coordinated to Ti\textsuperscript{IV}.\textsuperscript{124} In the presence of halogens, functionally available H\textsubscript{2}O\textsubscript{2} can be enzymatically converted to highly bactericidal hypohalous acids, such as hypochlorous acid (HOCl) or hypobromous acid (HOBr).\textsuperscript{117} The strong bactericidal activity of hypohalous acids is based on their intra- and extracellular damage to biomolecules, particularly thiol containing compounds.\textsuperscript{117,124} The described conversion of ROS into hypohalous acids is a substantial part of the killing mechanism of inflammatory cells which are rich in peroxidases.\textsuperscript{119} Hence, it is possible that the applied surface modification provides functionally available H\textsubscript{2}O\textsubscript{2} during inflammation, and thereby, supports phagocytosis \textit{in vivo}.

The response of inflammatory cells to modified TiO\textsubscript{2} surfaces is indisputably an interesting research subject. Larsson \textit{et al.} have reported anti-inflammatory activity of Ti-peroxy gels based on \textit{in vitro} results with activated polymorphonuclear granulocytes (PMNGs).\textsuperscript{289} Hence, the developed surface modification for TiO\textsubscript{2} scaffolds may offer an opportunity to modulate inflammation for optimal bone repair. However, with respect to the early stage of this project, it was found more relevant to exclude potential cytotoxic and genotoxic effects of the developed surface modification. Radicals are highly reactive to a number of biomolecules present in both cells and bacteria (\textbf{Figure 4}). This is one major difference between ROS-based and other antibacterial strategies, such as antibiotic loaded surfaces, which typically target biochemical processes specific to bacteria.\textsuperscript{76} Hence, the developed coating could exhibit toxic effects on tissue cells, and thereby, weaken the potential as an anti-infective surface in bone tissue regeneration.
The toxicity screening performed in Paper III showed no signs of cytotoxic effects on murine pre-osteoblasts by coated surfaces that had been pre-treated with H₂O₂. Based on confocal images, these surfaces provided an excellent substrate for attachment and spreading of MC3T3 cells. Unfortunately, the performed genotoxicity assay could not provide conclusive evidence that the pre-treatment of coated TiO₂ surfaces possesses no genotoxic effects on the same cell line. A high baseline genotoxicity was observed for all groups, including controls (Appendix A). This was probably due to methodological problems, such as unwanted DNA damage during sample preparation. DNA damage has been described as one of the most potent damaging mechanisms of ROS. The attack by ROS can cause DNA strand breakages and chemical modification of DNA bases. Potential consequences of DNA damage by ROS are cell death but also gene mutations involved in cancer development. With respect to the severe consequences of DNA damage, it is of utmost importance to clarify the ambiguous results from the comet assay in Paper III. Nevertheless, with exception of the unclear effect on DNA, no toxic effects by the modified surface were observed on the tested cell line.

The combination of antibacterial activity without adverse effects on tissue cells is a fundamental requirement in the development of anti-infective surfaces for permanent implants. This absence of adverse effects on tissue cells distinguishes the presented TiO₂ surfaces from many ROS-releasing scaffolds intended for tissue regeneration. For example, Wang et al. have investigated the biological response of bacteria and human osteoblasts (hOBs) to nanofiber scaffolds releasing H₂O₂ and calcium hydroxide (Ca(OH)₂). These scaffolds showed high bactericidal activity against S. epidermidis and E. coli, but also revealed distinct cytotoxic effects on hOBs during first 24 h of culture. Interestingly, in this study, hOBs recovered a healthy status for longer time points of culturing. This implies that a burst effect due to high levels of ROS may not cause long-term cytotoxicity in tissue cells, but could be efficient in killing pathogens during early stages of implantation.

This leads to the final question: where do the oxidative TiO₂ surfaces rank among the different approaches for infection-resistant biomaterials? The findings from Paper III represent a proof of concept that TiO₂ scaffolds can be surface modified for antibacterial activity without harming tissue cells. Compared to many other approaches, antibacterial properties were achieved by a fairly simple surface modification. TiO₂ scaffolds, which have already been shown to be suitable for guided bone repair, can be functionalised by sol-gel coating and H₂O₂ treatment. The resulting oxidative surface showed bactericidal activity, particularly at early stages of biofilm development. It is commonly accepted that the race for the surface due to peri-operative contamination is decided within hours after implantation. Thus, the presented surface modification is of great interest in the prevention of early bacterial colonisation of implant surfaces.

Like any other proof of concepts, the in vitro studies conducted in Paper III leave many questions regarding the potential use of modified TiO₂ scaffolds unanswered. Yet, the positive findings from this study are encouraging to further investigate TiO₂ dark catalysis as an antibacterial strategy.
6 CONCLUSION

The dark catalytic effect of TiO₂ particle suspensions and modified TiO₂ scaffolds was investigated with reference to biomedical applications. High catalytic activity of Degussa P25 nanoparticles was observed in the presence of H₂O₂. This oxidative behaviour could be related to the formation of oxygen centred radicals at the TiO₂-liquid interface, namely HO₂*/O₂* and •OH. The decomposition of MB occurred at the liquid-particle interface, or in its proximity rather than in solution. There was strong evidence that O₂* plays an important role in the decomposition of the tested model dye, potentially by adsorbing on the metal oxide surface.

In a follow up study, the potential of H₂O₂-TiO₂ suspensions for implant decontamination was assessed in vitro. The addition of TiO₂ nanoparticles was shown to enhance the antibacterial effect of H₂O₂ against S. epidermidis biofilms. While chlorhexidine showed higher bactericidal activity than H₂O₂-TiO₂ suspensions, only the treatment with H₂O₂ and H₂O₂-TiO₂ suspensions actively removed biofilm from the implant surface. This ability was correlated to the formation of bubbles during the treatment, and is believed to be further enhanced in the presence of blood. Hence, the eradication of biofilms with H₂O₂-TiO₂ suspensions is a promising approach for the chemical decontamination of implant surfaces in peri-implantitis therapy.

The catalytic activity of TiO₂ particles in the presence of H₂O₂ was successfully translated to TiO₂ surfaces by applying a thin anatase coating to ceramic scaffolds for bone repair. The open-porous architecture of the original scaffolds remained intact after sol-gel dip coating. The applied TiO₂ layer maintained oxidative behaviour when pre-treated with H₂O₂. This oxidative layer showed prolonged bactericidal activity against S. epidermidis biofilms and no cytotoxic effects on MC3T3 cells. Therefore, the dark catalytic effect of TiO₂ provides a simple method to functionalise biomaterials for antibacterial properties in tissue engineering applications.
7 OUTLOOK

This thesis provides a proof of concept that TiO\textsubscript{2} dark catalysis has potential in biomedical applications. This approach limited the exploration of some key findings, and surprising observations along the way could not always be followed up on. Thus, a few suggestions are given in which direction further research could be conducted.

It is necessary that future work on the dark catalytic effect of TiO\textsubscript{2} is driven by both, basic and applied research. A better understanding of the molecular mechanisms behind TiO\textsubscript{2} dark catalysis is believed to be crucial to tailor these systems for biomedical use. This includes the identification of material properties for high catalytic activity, the adsorption of surface bound reactive species and their stability in different environments.

A deeper understanding of these topics is necessary to tune the dark catalytic effect of TiO\textsubscript{2} for the demand in different biomedical applications. Simultaneously, TiO\textsubscript{2} scaffolds and H\textsubscript{2}O\textsubscript{2}-TiO\textsubscript{2} suspensions should be further assessed in experimental setups with higher relevance for the intended application. For the use of H\textsubscript{2}O\textsubscript{2}-TiO\textsubscript{2} suspensions to eradicate biofilms, this means that the antibacterial activity should be confirmed on other strains than \textit{S. epidermidis} and for at least one Gram-negative strain. It will also be necessary to look into potential cytotoxic effects of the TiO\textsubscript{2} agglomerates which remained on the implant surface after treatment.

Analogous, the absence of toxic effects on TiO\textsubscript{2} surface coatings has to be confirmed for human cells. As pointed out in this thesis, this includes the thorough investigation of potential genotoxic effects by the developed surface modification. Further, it remains unclear how the effect of this surface modification is influenced by the interaction with blood. This should be tested in a corresponding \textit{in vitro} study. With respect to the intended use as scaffold for bone repair, the influence of the functionalisation on inflammation and bone formation is of particular interest. The relevant \textit{in vitro} studies may increase the probability for adequate biological response in co-culture systems, and eventually \textit{in vivo}.

The applications suggested in this thesis are closely related to dental implants and bone repair in the maxillofacial region. This should by no means exclude the possibility that the dark catalytic effect can improve the performance of biomaterials in other parts of the body as well.
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APPENDIX

Appendix A: Results of the comet assay control groups. Negative control refers to MC3T3 cells cultured on tissue culture plastic for 24 h. Positive controls were cultured identically but exposed to 3% H$_2$O$_2$ for 1 h before electrophoresis was performed (mean ± SD, n = 300)

Appendix B: EPR spin trapping experiments verifying the presence of 'OH radicals in the control sample. (Left) 3% H$_2$O$_2$ + 200 mM DMPO. (Right) 3% H$_2$O$_2$ + 200 mM DMPO + 0.3 mM ethanol. The times refer to the recording of EPR spectra after mixing the ingredients.
Appendix C: Preliminary results for MB degradation by ALD coated scaffold in comparison to sol-gel coated scaffolds in the presence of 3% H2O2.