Surface functionalization of dental implants for improved biological response and reduced infection risk

A doctoral thesis by
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Oslo, November 2016

Table of contents

List of publications

Paper I

Sebastian Geißler, Hanna Tiainen, Håvard J. Haugen. Effect of cathodic polarization on coating doxycycline on titanium surfaces. *Materials Science and Engineering C* **2016**, 63, 359-366.

Paper II

Sebastian Geißler, Alejandro Barrantes, Pentti Tengvall, Phillip B. Messersmith, Hanna Tiainen. Deposition kinetics of bioinspired phenolic coatings on titanium surfaces. *Langmuir* **2016**, 32, 8050-8060.

Paper III

Sebastian Geißler,[†] Manuel Gomez-Florit,[†] Fernanda C. Petersen, Hanna Tiainen. *In vitro* performance of bioinspired phenolic nanocoatings for endosseous implant applications. *Manuscript* **2016**.

[†] First and second author contributed equally to the study.

1 Introduction

1.1 The dental implant

Teeth are not only important tools for the processing of food, they have also considerable influence on several other factors, such as speech, comfort, facial contour, and esthetics.[1] Patients experiencing tooth loss due to age, injury, or disease suffer often not only from functional constraints, but also from the accompanying psychological and social consequences. Restoration strategies have thus been a subject of intense research in the dental field over the last decades. The replacement of missing teeth is frequently accomplished by inserting single-tooth implants or implant-supported prostheses.[1]

A single-tooth implant is designed to mimic the function of the natural tooth as closely as possible (**Figure 1**). The implant crown is attached via the abutment to the functional anchorage of the implant system. This anchorage, the counterpart to the natural tooth root, is realized by means of a threaded pin which is surgically positioned in the surrounding bone tissue. Titanium and titanium based alloys have established themselves as the state-of-the-art material for such screw-shaped implants. By combining appropriate mechanical properties, high corrosion resistance, and necessary biostability, titanium is highly suitable for long-term implantable devices.[2]

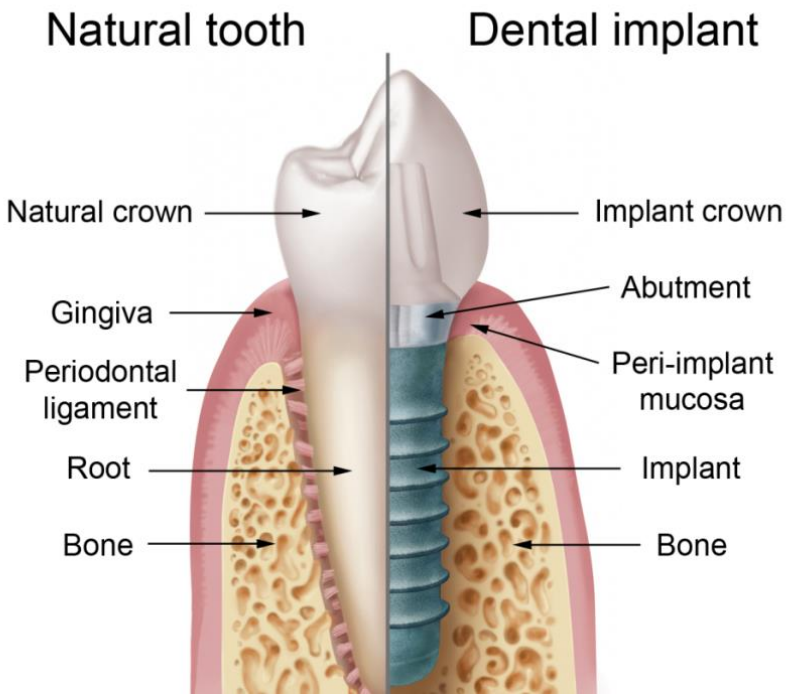

Figure 1. Schematic representation of a natural tooth in comparison to a dental implant (*adapted from Millennium Surgical*[3]*).* While the natural tooth is connected to the surrounding bone by means of the periodontal ligament, the dental implant is in direct contact with bone (osseointegration).

In contrast to the root of a natural tooth, which is connected to the surrounding bone tissue by the periodontal ligament, the titanium implant is in direct contact with bone, a state called osseointegration.[4] The concept of osseointegration is regarded to be a crucial requirement for long-lasting stability and functionality of dental implants.[5-6]

1.2 Peri-implant bone healing

The placement of a dental implant triggers a series of events at the implantation site, and understanding these events is essential for understanding the phenomenon of osseointegration. Every implantation inevitably induces a certain degree of trauma to the bone tissue present at the implantation site. Peri-implant bone healing can therefore be regarded as the key process taking place after implantation. New bone can either form on the old bone surface (distance osteogenesis), or directly on the implant surface (contact osteogenesis).[7] Even though both of these processes occur simultaneously at every peri-implant site, contact osteogenesis is considered to be more crucial with regard to achieving early implant stability.[8]

In principle, peri-implant bone healing involves three mechanisms: osteoconduction, *de novo* bone formation, and bone remodeling.[8] However, there are some earlier processes taking place which set the scene for these mechanisms. The implantation causes damage to blood vessels resulting in hemorrhage and the formation of a hematoma, similar to the situation found at bone fracture sites.[9-10] During the emerging inflammatory response, platelets, macrophages, and other inflammatory cells infiltrate the hematoma, where they cope with infection, secrete cytokines and growth factors, and form a fibrin network which acts as a provisional matrix.[10-12] Macrophages, giant cells, and other phagocytic cells start to migrate toward the implant surface, degrading necrotic tissue and the provisional clot.[10, 13] The resolving clot is then substituted by granulation tissue through angiogenesis and early matrix synthesis, and the resulting vascularized granulation tissue serves as a scaffold for the subsequent osteogenesis.[11-12, 14]

As mentioned above, contact osteogenesis describes the formation of new bone on the implant surface. In order for this to happen, osteogenic cells have to reach the implant surface. The transient matrix resulting from the early coagulation cascade plays therefore an important role as it provides the connection to the implant surface through which the osteogenic cells can migrate.[8] The process of recruitment and migration of differentiating osteogenic cells to the implant surface is called osteoconduction.[15] After this, *de novo* bone formation is initiated with the deposition of a non-collagenous organic matrix that serves as nucleation basis for calcium phosphate mineralization, followed by the deposition of calcium phosphate, crystal growth and collagen fiber assembly which eventually lead to the formation of a collagenous matrix and calcification.[8, 13]

In contrast to slowly developing mature lamellar bone during the normal bone remodeling process, the formation of bone during peri-implant healing is rapid and the newly formed bone exhibits irregular woven microarchitecture.[8, 11] In the final healing phase, the bone remodeling phase, this woven bone is converted into lamellar bone.[9-10]

1.3 Implant success and risk factors

The clinical results for dental implants are influenced by an interplay of various factors related to the peri-implant healing process, the surgical technique, the implant material and surface, the preconditions found at the implantation site, or the subsequent prosthetic design and long-term loading phase.[16] An abundance of studies has been conducted to evaluate the long-term performance of dental implants.[17-18] Commonly applied measures for the assessment of the clinical outcome within these studies are survival and success of the implants. While implant survival only refers to the physical presence of the implant in the mouth, regardless of the occurrence of complications, implant success requires the absence of complications over the entire observation period.[19-20] The interpretation of the study outcomes and the comparison between different studies, however, is in many cases not straightforward, since the terms *survival* and *success* are often not used in the appropriate way.[17] In addition to that, the large amount of existing assessment criteria for implant success and the lack of standardization limit the comparability of different studies.[18-19, 21]

According to the review study by Moraschini et al., the reported survival rates of dental implants are generally high (on average 94.6% with variation from 73.4% to 100% for a minimal follow-up period of 10 years), whereas the success rates vary from 34.9% to 100%, depending on the applied success criteria.[17] Complications, which can lead to reduced success rates or even implant failure (non-survival), can be of biological, mechanical, iatrogenic, or functional origin.[6, 22] The following sections will focus on risk factors that may result in biological failure of the implant, defined as the "inadequacy of the host tissue to establish or to maintain osseointegration".[22]

1.3.1 Risk factor patient

Patient-related factors play an important role in the successful replacement of lost teeth. The demographic changes in our society will increase the age of the population, and therefore also the occurrence of systemic and oral diseases that are more prevalent with age.[23] Existing diseases may compromise the healing phase after implantation and prevent successful osseointegration.[24-25]

Osteoporosis, a skeletal disease characterized by low bone mass and density, can have influence on the bone-to-implant contact and may thus impede implant placement.[26] While some studies reported a correlation between early implant failure and osteoporosis,[25, 27] others did not see a contraindication for the use of dental implants in osteoporotic patients.[24, 26, 28] However, individual evaluation of each case and longer healing periods were recommended in order to achieve implant stability. Furthermore, patients with a history of periodontal disease exhibited a higher risk for implant failure and implant-related complications.[29-30] Diabetes mellitus has often been regarded as a risk factor for successful osseointegration. However, several studies have found no significant effect of diabetes on successful implant osseointegration, provided that the disease was

under medical control.[24-25, 27, 31-32] A further risk factor relates to patients who receive radiation therapy due to tumor treatment. It has been shown that patients with irradiated bone are subjected to a higher risk of implant failure compared to patients with non-irradiated bone.[27, 33] The patient's smoking habits represent another factor that can influence the outcome of implant placement. A significant association has been found between smoking habits and early implant failure.[25, 27, 34]

Even though the influence of certain systemic diseases and other patient-related risk factors on successful implant therapy is sometimes controversial, often also owing to the lack of high-quality long-term studies, adverse effects can often not be excluded. A thorough evaluation of the patient's medical status and history is necessary to select optimal restoration therapy.

1.3.2 Risk factor infection

A risk factor contributing largely to biological failure of dental implants is related to the presence of bacteria at the interface between implant and tissue. Inflammatory lesions affecting the tissue surrounding the dental implant caused by bacterial infection are known as peri-implant diseases.[35] Peri-implant diseases can be classified into peri-implant mucositis and peri-implantitis, depending on the stage of the inflammatory lesion.[35] While peri-implant mucositis describes reversible soft tissue inflammation around a functioning implant, the inflammatory reaction in peri-implantitis is linked to loss of supporting bone around the implant.[35-36] If peri-implant diseases are not diagnosed and treated correctly, they can result in loss of osseointegration and thus eventually lead to implant failure.[36] According to the review study by Zitzmann et al., peri-implant mucositis occurred in 80% of the examined patients and in 50% of the implant sites after a function time of at least 5 years, whereas peri-implantitis was observed in a range from 28% to 56% of patients, and from 12% to 43% of implant sites.[35] Another study reported the occurrence of peri-implant mucositis after at least 5 years of functional loading time in 63.4% of the patients and in 30.7% of the implant sites, whereas peri-implantitis was observed in 18.8% of the patients and 9.6% of the implant sites.[37] It is worth mentioning that peri-implant diseases may be connected to other risk factors, in particular to factors which create favorable conditions for bacteria to colonize the implant surface. In this regard, the occurrence of peri-implant disease was directly associated with poor oral hygiene, a history of periodontitis, or cigarette smoking.[36, 38]

1.4 Race for the surface

Taking the aforementioned biological aspects of peri-implant bone healing and the potential complications associated with it into account, one can generally distinguish between two rivaling mechanisms which will determine the fate of the implant. On one side, there is the host tissue trying to create a functional connection to the implant surface and establish osseointegration. On the other side, there are bacteria, which see the implant

surface as an attractive site for establishing a colony. The battle of these two parties for the implant surface has first been described by Gristina in 1987 as the "race for the surface".[39]

In the early stages of implantation, i.e. during surgery, bacteria can find their way to the implant or the bony socket through the surgeon and health care personnel, the surgical instruments, the air in the operating theater, the patient's saliva and exhaled air, or the peri-oral skin.[40-41] In the fight against such bacterial contamination, the host tissue makes use of its effective defense mechanism: the immune system. However, the immune response of the host defense system, which usually can cope with transient bacterial contamination, is severely impaired in the tissue traumatized by the implantation surgery and in the presence of a foreign body.[41-42] This enables the bacteria to survive at the peri-implant interface and gives them an advantage in the race for the surface.[41]

Once bacteria have successfully attached to the implant surface, they reveal their own powerful defense mechanism: the biofilm. A biofilm is a developed sessile community of bacterial cells that are attached to a surface or interface and embedded in a self-produced extracellular polysaccharide matrix, creating a protective environment for the bacteria.[43-45] In contrast to planktonic bacteria, bacteria in a biofilm exhibit an altered phenotype regarding growth rate and gene transcription.[44] The altered growth mode in a biofilm makes the bacteria less susceptible, or even resistant, to antibiotic therapy and host defense mechanisms, and the bacteria can survive in dormant states for several years before awaking in a more virulent form.[41, 46-47]

The combination of compromised host defense and bacterial contamination in the form of a biofilm represents a significant threat not only for the successful functional integration of the implant, but also for the patient's health. In the United States, for example, biomaterial- and biofilm-associated infections in connection with dental implants have been estimated to occur in 5-10% of implantations.[48] The treatment of such infections involves high effort in order to remove the biofilm and prevent reoccurrence of bacterial contamination, and often surgical intervention or even removal of the implant is necessary.[49-50] In order to prevent biomaterial-associated infections, much research has focused on understanding and influencing the role of the implant surface in such infections, for the purpose of helping the host system to win the race for the surface. In general, this can be achieved by either accelerating and enhancing host tissue integration, or by preventing biofilm formation.

1.5 The dental implant surface

The important role of the dental implant surface in establishing bone-to-implant contact is well-recognized. When placing an implant, it is the surface of the implant the host tissue will be exposed to first. Consequently, the properties of the surface have a large influence on how the host tissue will react to the foreign body. The inherent surface properties of titanium are one of the main reasons why titanium is used so frequently for endosseous implants in the first place.[2, 51] Upon exposure to air or other oxygen containing

environments, titanium gets instantly covered by a passivating oxide layer typically being a few nanometer thick.[51-52] This passive oxide layer is responsible for the exceptional chemical stability and corrosion resistance of the material in the human body, as it represents a barrier between metal and tissue, and therefore limits the diffusion and release of metal ions from the surface.[52]

A variety of surface properties with respect to both physical and chemical characteristics of the surface are considered to have an effect on the implant-tissue interaction.[51, 53] The physical properties are mainly related to the topography and morphology of the surface. Several studies have shown that increased micro-scale roughness of the surface positively influences bone response to the implant, due to the larger exposed surface area which enhances bone anchorage and biomechanical interlocking between implant and bone compared to smooth surfaces.[54-59] Moreover, surface nanoroughness is regarded to have an effect on the biological response toward the implant.[58, 60-63] Even though the exact mechanisms are unknown, the rationale behind this effect is that nanoscale surface features increase the surface energy, which again increases surface wettability, and therefore the spreading and binding of fibrin and matrix proteins, ultimately leading to enhanced cell adhesion.[58, 64] Beside the physical properties of the surface, also the chemical composition of the outermost surface layers can have an impact on implant-tissue interactions, since surface composition and charge can affect protein adsorption and cell attachment.[53, 65-66]

1.5.1 Established implant surfaces

Modifying the surface properties of dental implants in order to influence the events occurring at the implant-tissue interface has been in the focus of intense research over the last decades. As a result, a large number of different implant systems which feature different implant surfaces are currently available for the dental marked (see **Table 1** for examples).

The most commonly applied surface modification techniques for dental implants involve alterations of the surface topography. Grit-blasting and acid-etching are frequently used methods to create rough surfaces. Wennerberg et al. concluded from their animal experiments that bone response was strongest to moderately rough surfaces with Sa values of approximately 1.5 µm.[55] However, a large variation in surface roughness can be found among the major implant producers, ranging from 0.3 µm to 1.78 µm.[55] It is important to mention that the blasting and etching treatment not only changes the microtopography of the surface, but also the nanotopography and the surface chemistry.[59] The performance of such surfaces is therefore likely to be based on the contribution of multiple factors, and cannot solely be attributed to the change in surface microroughness. Other implant producers focused on changing the properties of the surface oxide layer. By applying anodic oxidation, a porous surface structure with significantly increased oxide layer thickness can be obtained (TiUnite®, Nobel Biocare). This modification has been shown to increase the bone-to-implant contact.[67-69]

Furthermore, several producers offer implants with chemically modified surfaces. A chemically altered surface based on the sandblasted and acid-etched Institute Straumann SLA® surface has been shown to exhibit increased surface free energy and hydrophilicity (SLActive®), mainly due to reduced hydrocarbon contamination.[70] Moreover, calcium phosphate coatings have been applied to implant surfaces in order to enhance the clinical performance of the implants.[71-72] Examples for such surfaces are the NanoTite™ surface (BIOMET 3i) and the MP-1® surface (Zimmer Dental). A further established modification process involves blasting of surfaces with titanium dioxide (TiO_2) particles, followed by etching with hydrofluoric acid (OsseoSpeed™, DENTSPLY Implants). Fluoride-modified surfaces have been shown to exhibit improved biomechanical anchorage and enhanced bone integration.[68, 73-75]

Table 1. Selected examples of available implant surfaces.

Surface	Producer	Modification
SLA®	Institut Straumann	Large-grit sandblasting and acid-etching
SLActive®	Institut Straumann	SLA® + storage in NaCl solution to avoid hydrocarbon contamination
FRIADENT® plus	DENTSPLY Implants	Grit-blasting and acid-etching, followed by a proprietary neutralizing technique
Promote®	CAMLOG	Abrasive-blasting and acid-etching
Laser-Lok®	BioHorizons	Laser-machining to create micro- and nanoscale channels
OSSEOTITE®	BIOMET 3i	Dual acid-etching
NanoTite™	BIOMET 3i	OSSEOTITE® + calcium phosphate particle deposition by Discrete Crystalline Deposition (DCD™)
MTX™	Zimmer Dental	Grit-blasting with hydroxyapatite particles
MP-1®	Zimmer Dental	Hydroxyapatite coating
TiUnite®	Nobel Biocare	Anodic oxidation
OsseoSpeed™	DENTSPLY Implants	TiO_2-blasting and etching in hydrofluoric acid

1.5.2 Toward biofunctional implant surfaces

As illustrated in the previous section, the majority of the implants on the market feature rough surfaces created through physical or chemical modification. The enhanced performance of these implants can mainly be assigned to increased stimulation of bone anchorage, since rough surface topographies have an influence on osteogenic cells and platelet activation.[76-77] However, in situations where exactly these mechanisms are impaired, e.g. at implantation sites with low bone density, low vascularization, or insufficient bone quantity, there is still a potential to improve cell adhesion to the implant

surfaces.[78] Achieving appropriate cell adhesion to the surface is particularly important in order to have the surface occupied by living cells, and therefore make it less susceptible to bacterial colonization.[39]

Therefore, recent trends in the development of modern implant surfaces capable of coping with the challenging situations of compromised host systems and bacterial infection, point toward the addition of a biological component to the existing repertoire of surface modifications. The basic principle of biological and biochemical surface functionalization methods is the attachment of biomolecules which can trigger specific cell and tissue responses to the implant surface.[79] Potential candidates for such biomolecular coatings are for example extracellular matrix proteins, growth factors, short peptides, or antimicrobial agents.[79-80]

Extracellular matrix proteins are involved in diverse processes with respect to cell adhesion, proliferation, and differentiation.[79, 81-83] *In vitro* and *in vivo* studies suggest that implant surfaces functionalized with these proteins can have a positive influence on bone regeneration around the implant.[83] Another approach to influence the processes occurring at the implant-tissue interface is to coat the implant surface with growth factors (such as bone morphogenic proteins, platelet-derived growth factor, or insulin-like growth factor).[79, 84] Such growth factor coatings may be effective in modulating cellular functions, by e.g. attracting circulating osteoprogenitors or promoting the differentiation of stem cells or osteoprogenitors into osteoblasts, and could therefore improve bone repair around implants.[85-86] RGD is an amino acid sequence (Arginine-Glycine-Aspartate) which is recognized by cells through integrin receptors.[87] The functionalization of implant surfaces with RGD-containing peptides represents therefore a further method to influence cell adhesion to the surfaces, thus improving the implant-tissue interactions.[83, 88]

Other strategies encompass the functionalization of the implant surface with the aim of preventing biomaterial-associated infections. Such antibacterial approaches are mainly designed to prevent bacterial colonization of the implant surface before biofilm formation can occur.[89-90] One option to achieve this is the use of antimicrobial peptides. Several studies have reported the immobilization of antimicrobial peptides onto biomaterial surfaces and their antimicrobial activity.[91] Another approach is the release of antibiotics from the implant surface. Various methods have been applied in order to attach antibiotics to implant surfaces, such as calcium phosphate coatings, biodegradable polymer coatings, sol-gel coatings, or loaded nanotubes.[79, 90]

A further method aiming at the incorporation of biomolecules onto implant surfaces has been described by Videm et al.[92] They reported that an electrochemically produced titanium hydride layer could serve as a basis for attaching biomolecules to implant materials. This cathodic polarization process has been employed to bind enamel matrix derivative (EMD) to implant surfaces in order to promote bone regeneration.[93] In addition, the process has been applied for the attachment of doxycycline to titanium based materials.[94] Doxycycline is an antibiotic that belongs to the group of tetracyclines and is effective against both gram-positive and gram-negative aerobic and anaerobic pathogens (**Figure 2**).[95] Surfaces coated with doxycycline by means of cathodic polarization have

been demonstrated to exhibit antibacterial properties and to promote bone formation.[94, 96] However, the mechanisms behind this electrochemical coating process, in particular the role of titanium hydride formation in binding the biomolecules to the surface, remain poorly understood. A reason for this is that the studies used sandblasted and acid-etched surfaces as a substrate for the modifications, comparable to the Institute Straumann SLA® surface.[93-94] The properties of these substrate surfaces conflict with a conclusive surface characterization in two ways: First, the blasting and etching process creates a rough surface topography, which can have an adverse influence on many surface characterization techniques. Second, the acid-etching step already creates a subsurface hydride layer.[97-98] This preexisting hydride layer makes it difficult to evaluate new hydride formation and its effect on biomolecule immobilization.

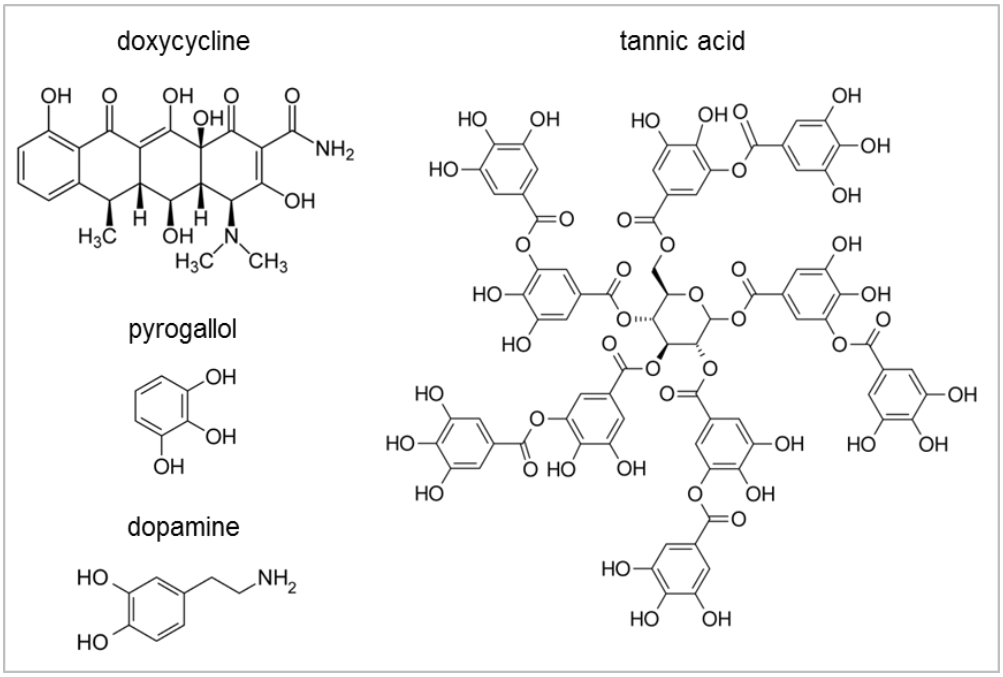

Figure 2. Molecular structures of doxycycline, tannic acid, pyrogallol, and dopamine.

In the quest for new functionalization strategies, multifunctional polymeric coatings have attracted increasing attention in the biomedical field over the last few years.[99] Polydopamine coatings can be stated as the prime example for such coating systems. Inspired by the proteins responsible for the versatile adhesive properties of mussels, dopamine (**Figure 2**) has been reported to form thin polymer coatings on virtually any kind of material through oxidative polymerization processes.[100] Polydopamine-based materials have found various applications in the biomedical field,[101] including the design of cell-adhesive[102] and antimicrobial surfaces,[103-104] or as a scaffold for remineralization applications.[105]

Recently, polyphenols have been identified as a further group of molecules possessing the ability to form material-independent adhesive coatings.[106-107] Polyphenols are secondary metabolites found abundantly in plant tissues, where they are involved in diverse biological functions, such as growth and reproduction, resistance to microbial

pathogens, or protection against radiation damage.[108-110] The variety of polyphenols and phenolic compounds, that has been found to form adhesive surface coatings, increases the functional versatility of such coating systems in comparison to polydopamine coatings.[107] Of particular interest for biomedical applications are the polyphenol tannic acid and the phenolic compound pyrogallol (**Figure 2**). Surfaces coated with these two compounds have been shown to exhibit antioxidant and antibacterial properties,[106] making them promising candidates for the aimed application in this work. In contrast to the polydopamine system, where much research effort has been invested to study the coating mechanisms and potential functional applications, there is still a lack of knowledge in this field with respect to polyphenol coatings.

2 Design of research

2.1 Hypothesis

The studies presented in this thesis focus on surface functionalization of titanium implant materials by *cathodic polarization with doxycycline* and *auto-oxidative surface polymerization of tannic acid and pyrogallol*. The general hypothesis was that these functionalization techniques are appropriate methods to create implant surfaces which can promote bone formation and reduce the risk of infection.

With regard to this hypothesis, the functionalized surfaces were physically, chemically, and biologically characterized according to the design of research displayed in **Figure 3**.

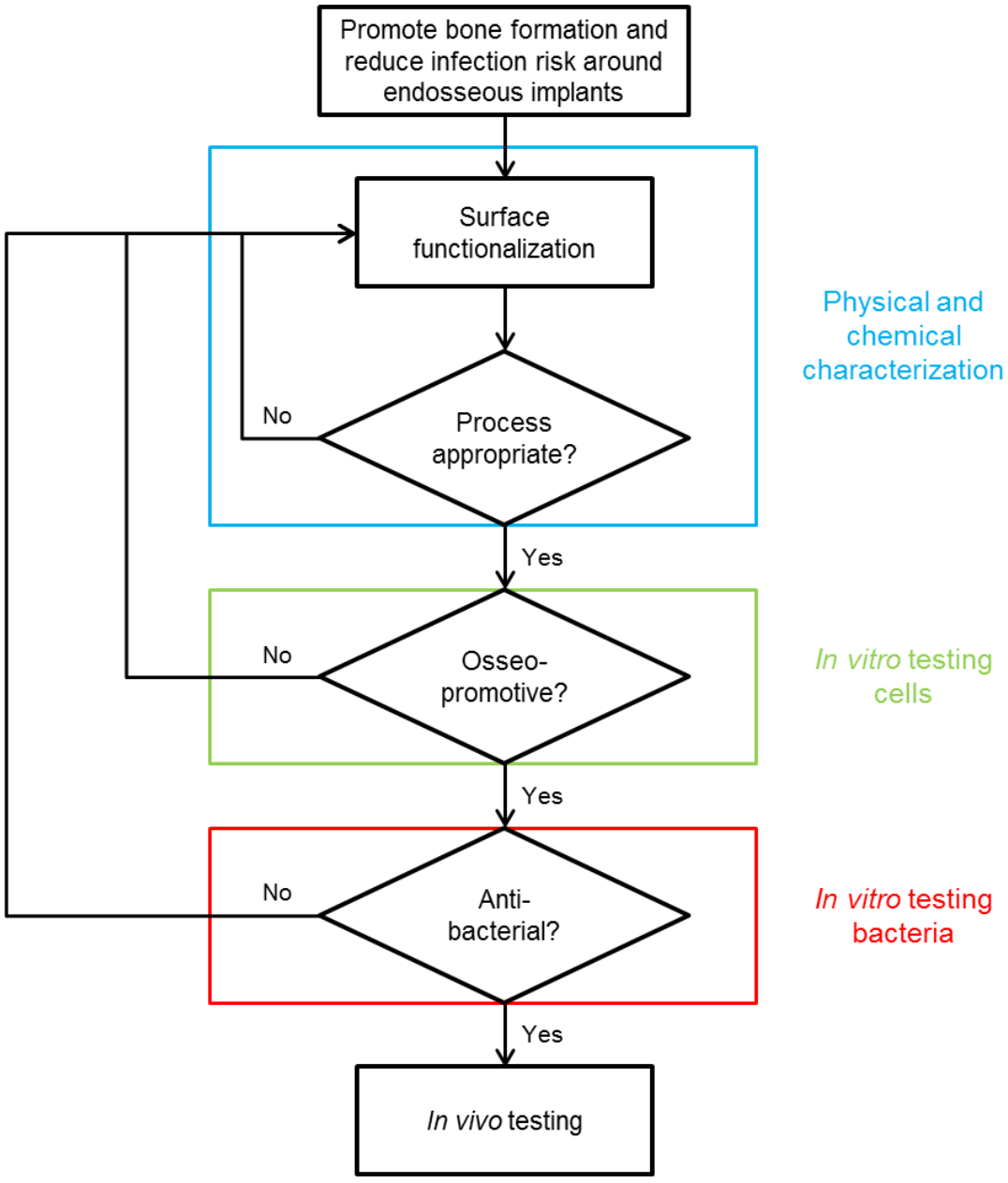

Figure 3. Flow chart illustrating the design of research.

2.2 Specific aims

Cathodic polarization with doxycycline (Paper I)

Cathodic polarization has previously been applied to attach doxycycline to titanium based implant materials in order to promote bone growth and to reduce the risk of infection.[94, 96] Even though the effect of these doxycycline coatings has been tested *in vitro* and *in vivo* in these studies, the changes induced to the substrate's outermost surface layers by the polarization process are poorly understood. Moreover, the proposed role of a formed hydride layer in the binding of doxycycline to the surface remains unclear. Understanding these processes is crucial to understand the biological effect of the coatings. The aims of the first part of this thesis were therefore to:

- Evaluate the cathodic polarization process with regard to the events occurring at the outermost layers of titanium surfaces
- Examine the potential role of hydride formation in binding doxycycline to the surfaces
- Assess the antibacterial properties of the modified surfaces *in vitro*

Auto-oxidative surface polymerization of phenolic compounds (Paper II and III)

Auto-oxidative surface polymerization of the two phenolic compounds tannic acid and pyrogallol was chosen as an alternative method to functionalize titanium surfaces. The simplicity of the coating process, the applicability to a wide range of different materials, and the proposed properties of the coatings make them promising candidates for the targeted application. However, the mechanisms behind the coating formation have not been investigated extensively. Furthermore, the effect of these coatings on cells involved in bone formation has not been studied. The aims of the second part of this thesis were therefore to:

- Investigate the mechanisms behind the coating formation of tannic acid and pyrogallol on titanium surfaces both from a physical and chemical point of view
- Assess the potential of the modified surfaces to promote bone formation *in vitro* using human osteoblasts (hOBs)
- Assess the antibacterial properties of the modified surfaces *in vitro*

3 Methodological considerations

This chapter discusses the methods employed in the present work to reach the aims specified in the previous chapter. It focusses on the appropriateness of the methods and considers the advantages and limitations of the particular techniques. Detailed specifications about used materials, instrumentation, and parameters are provided in the *materials and methods* section of each individual paper.

3.1 Surface functionalization

Disc-shaped grade IV commercially pure titanium samples were used as substrates for functionalization in this study. Prior to modification, the discs were mirror-polished, washed and stored as previously reported.[111] Even though most of the currently available implants feature rough surfaces (see introduction), this mirror-polishing step was chosen to allow the use of more surface specific characterization methods which are limited to surfaces without complex geometries, and to ensure that changes in surface properties caused by the functionalization process can be readily characterized and conclusively distinguished from original surface features.

3.1.1 Cathodic polarization with doxycycline

Ti discs were coated with doxycycline using cathodic polarization which has previously been applied to modify implant materials.[93-94, 96, 112] During this process, the discs acted as the cathode and a platinum ring was used as counter electrode (**Figure 4**).

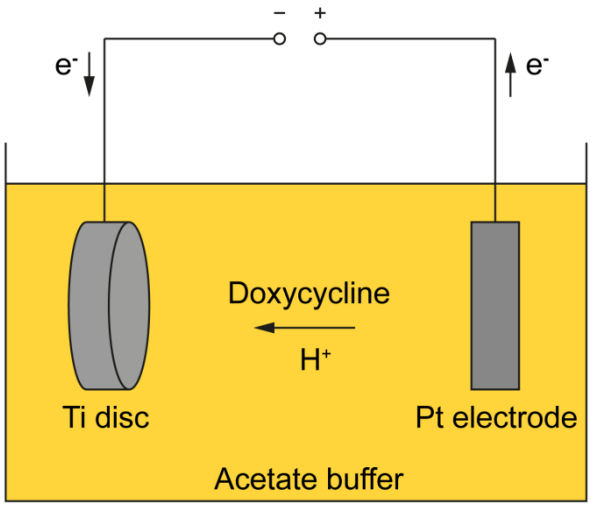

Figure 4. Schematic illustration of the cathodic polarization setup. The negatively charged titanium discs attract hydrogen ions and doxycycline molecules from the acidic coating solution.

During the polarization, the discs get negatively charged and attract hydrogen ions (H^+) from the acidic buffer. With the adsorption of hydrogen ions, the oxide layer on the titanium surfaces starts to dissolve[113] and hydrogen absorption eventually leads to the

formation of titanium hydride on the surface.[114] Simultaneously, the doxycycline molecules, which are positively charged at the used pH,[115] migrate to the Ti surfaces and attach to it.

The process required a custom made setup with individual current output for each mounted Ti disc. Current density was kept constant at 1 mA/cm^2 and was applied for a polarization time of 3 h, similar to the process parameters used in the studies conducted by Walter et al.[94] and Xing et al.[96] The effect of different current densities or polarization times was not investigated in this study, as the aim of this study was not the optimization of the process, but the evaluation of the events occurring at these particular process parameters. Furthermore, degradation of the doxycycline molecules was already observed to some extent with the used parameters, and would probably increase with higher current densities or polarization times (see Supporting Information in **paper I**). The impact of cathodic polarization itself on the titanium surfaces was investigated by applying the process without the addition of doxycycline (hereafter referred to as *polarized* samples).

3.1.2 Auto-oxidative surface polymerization of phenolic compounds

Functionalization of titanium discs with the phenolic compounds tannic acid (TA) and pyrogallol (PG) was performed by means of auto-oxidative surface polymerization as described by Sileika et al.[106] The titanium discs were immersed in glass vials containing the phenolic compounds dissolved in buffered saline solutions at neutral or slightly alkaline pH (**Figure 5**). The glass vials were then agitated on a rocking platform for up to 24 h.

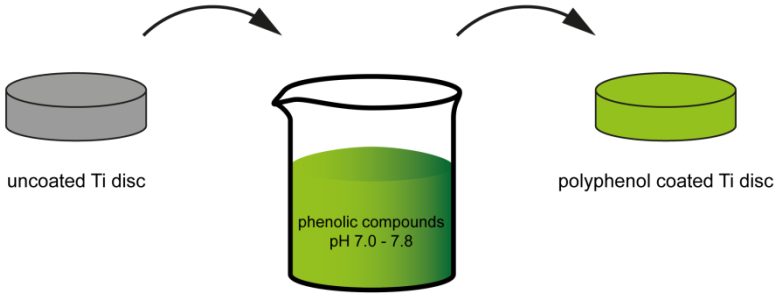

Figure 5. Schematic illustration of phenolic film deposition by dip-coating the titanium discs in buffered saline solution containing tannic acid (pH 7.8) and pyrogallol (pH 7.0).

At the used pH range, polyphenols composed of one or more *ortho*-di or tri-hydroxy phenyl groups undergo auto-oxidation,[110] and the formed species react with each other in solution and with the titanium substrate, forming a polyphenolic coating on the discs. Initial coating conditions were selected according to the study by Sileika et al. to 0.1 M bicine buffer containing 0.6 M NaCl, and the buffer was adjusted to pH 7.8.[106] Studies on PG coating formation showed that thicker coatings could be produced by lowering the pH to 7.0, while comparable coatings could be obtained with lower concentrations of $MgCl_2$

compared to NaCl.[116] Therefore, PG coatings were produced in 0.1 M bis-tris buffer containing 0.1 M $MgCl_2$, and the buffer was adjusted to pH 7.0.

The dip-coating process has the advantage of being simpler and more practical in its applicability compared to the cathodic polarization process. In contrast to cathodic polarization, the dip-coating process requires only standard laboratory equipment, and sample handling is much easier as the Ti discs do not have to be mounted to a set of electrodes. Moreover, only 10 ml of coating solution are needed to produce a batch of coated discs, as opposed to 200 ml for one polarization process. All these factors can have an immense influence with regard to a potential industrial up-scaling of the functionalization process.

3.2 Surface visualization

In the present work, several techniques were employed to visualize the mirror-polished control surfaces, the surfaces after cathodic polarization both with and without doxycycline, and the polyphenol coated surfaces. In general, surface features can be visualized by either optical (e.g. scanning electron microscopy, laser profilometry, confocal laser scanning microscopy) or stylus techniques (e.g. stylus profilometry, atomic force microscopy). The limitations of each method originate mainly from their lateral and vertical resolution as well as from the accessibility of surface features.[117]

The Ti discs modified by cathodic polarization were visualized by means of a field emission scanning electron microscope (FE-SEM; **paper I**). This method allows fast imaging of the surface at high magnification with a resolution that can approach a few nanometers. The high resolution was the deciding factor for choosing SEM over laser beam techniques, as the surface features were expected to be in the nanometer range. SEM is based on the interactions of a focused primary electron beam, which is scanned across the specimen, with the surface-near area of the sample. The primary electrons penetrate the surface and are scattered both elastically and inelastically. The penetration depth (interaction volume) of the electrons into the sample is dependent on the beam energy and increases with increasing acceleration voltage. SEM can detect several signals deriving from different depths within the interaction volume, including secondary electrons (SE), backscattered electrons (BSE), and characteristic X-ray photons (**Figure 6**).[118] In order to image the sample surface as detailed as possible, this study employed low acceleration voltages (5 kV) in combination with the detection of secondary electrons, which are emitted from a volume closer to the surface compared to backscattered electrons. The Ti discs were sputter-coated with platinum in order to avoid sample charging. Focusing the sample surfaces was challenging for the mirror-polished control surfaces due to the absence of very distinct surface features. For this reason, small surface defects such as scratches had to be found to perform accurate focusing.

Atomic force microscopy (AFM) was chosen as a complementary method to SEM (**paper I & II**). In AFM, a stylus with a sharp tip (cantilever) is applied to raster-scan the sample surface, and forces between cantilever and surface lead to a deflection of the

cantilever. Using a laser beam that is reflected from the backside of the cantilever, the deflection of the cantilever is detected (**Figure 6**). In contrast to SEM, where information is collected from a certain volume ranging into the material, AFM directly probes the contours of the surface, and depending on the measurement conditions, sample, and instrumentation, high resolution up to atomic resolution can be obtained.[117] The acquired information can be used to create two- and three-dimensional topographical images of the surface. Samples can be measured in contact mode, tapping mode (AC mode), or non-contact mode. The mirror-polished control surfaces and the polarized surfaces were scanned in contact-mode due to their low roughness. However, surfaces with more complex surface structures and increased roughness (such as the doxycycline and polyphenol coated surfaces) have to be scanned in AC mode to avoid shear force induced damage to the cantilever and sample surface, which can cause scanning artefacts. Beside this potential occurrence of artefacts, the limitations of AFM are the confined lateral and vertical range, low scanning rates, and problems of the cantilever to follow surface features with high aspect ratio.[117] Thus, achieving high quality scan results can be a time consuming task, especially when scanning large surface areas. An advantage compared to SEM is that AFM allows for the readout of surface-specific parameters such as roughness values. Making use of this, changes to the surfaces induced by the functionalization method can be quantitatively examined (changes in surface roughness in **paper I**). Furthermore, AFM does not require vacuum, making the characterization conditions more realistic.

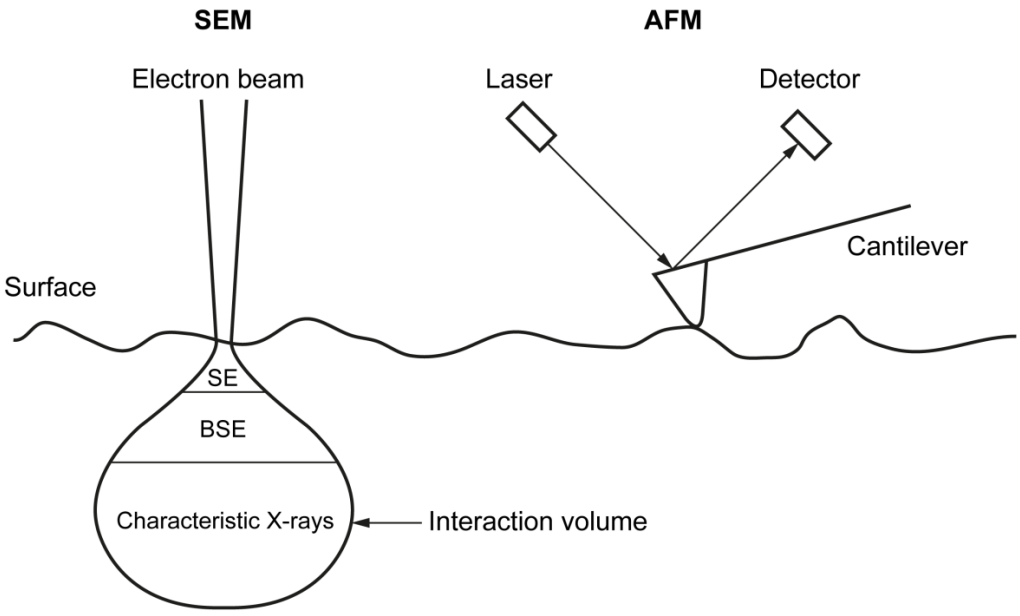

Figure 6. Schematic illustration of the measurement principles of scanning electron microscopy (SEM) and atomic force microscopy (AFM). The SEM signal derives from a certain interaction volume within the material. It includes secondary electrons (SE), backscattered electrons (BSE), and characteristic X-ray radiation, which originate from different depths of the interaction volume. The size of the volume is dependent on the energy of the incident electron beam. In contrast, AFM directly probes the surface by scanning it with a sharp tip cantilever.

In the case of cathodically polarized Ti discs, it was not sufficient to evaluate the modified surfaces from top view. As the polarization process was proposed to induce changes to the oxide layer and create a titanium hydride layer, it was desirable to visualize these features within the surface-near region. This is usually done by means of transmission electron microscopy (TEM) on cross sections obtained from the surface area. The image quality of this method is highly dependent on the quality of the specimens. Cross sections are commonly produced by mechanical abrasion or ion milling techniques, and need to be uniform and very thin in order to allow the electron beam to penetrate. Specimens of the mirror-polished control samples and the polarized samples were first mechanically polished down to a thickness of 5-10 µm by tripod wedge polishing.[119] However, this mechanical polishing step induced defects to the samples due to smearing effects. For this reason, the final thinning of these samples and the complete preparation of the doxycycline sample was performed by means of a focused ion beam (FIB) scanning electron microscope to avoid artifacts created by mechanical interactions with the samples. The dual beam instrument was equipped with an ion gun and an electron gun, allowing SEM visualization during sample preparation by ion milling. It is therefore an efficient and versatile method for making site-specific samples also from complex geometries.[120]

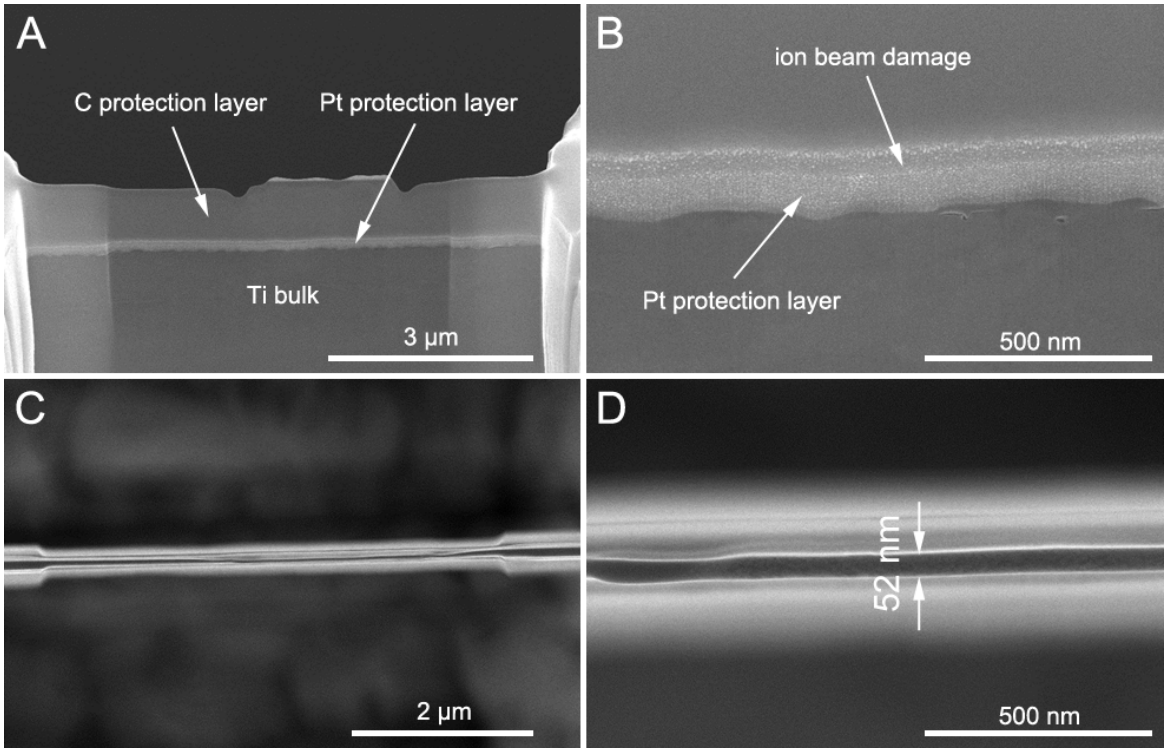

Figure 7. SEM images of a TEM cross section sample prepared by FIB. (A) The side view of the TEM sample shows the carbon (C) and platinum (Pt) protection layers covering the bulk material. (B) High magnification image revealing ion beam damage in the upper part of the Pt protection layer. (C) Top view of the TEM sample. (D) The final thickness of the cross section was approximately 50 nm.

The problem with ion beam techniques in general is that these methods can introduce ion beam damage in form of an amorphous surface layer which can impair the imaging quality.[121] To avoid extensive damage, the sample surfaces were covered with protective layers of carbon and platinum. Such layers can be produced by either electron beam assisted or ion beam assisted deposition. First, a platinum protection layer was deposited with electron beam assisted deposition to avoid ion beam damage. Then, Ga^+ ion beam assisted deposition was applied to deposit further platinum and carbon protection layers (**Figure 7 A**). Beam damage caused by the ion beam assisted deposition of the protective layers could be seen in the upper part of the platinum protection layer (**Figure 7 B**). The samples were first thinned by the Ga^+ ion beam at an acceleration voltage of 30 kV. The final thinning step was performed at 5 kV ion beam acceleration voltage, as reducing the incident ion beam energy has been shown to significantly reduce beam damage.[122] The results of the sample preparation were approximately 5 µm wide cross sections with a thickness of approximately 50 nm (**Figure 7 C, D**).

3.3 Real time monitoring of phenolic coating deposition

Studies on thin film deposition to surfaces require instrumentation with high sensitivity to follow adsorption processes down to the molecular level. Moreover, when the dynamic processes of the film deposition are to be investigated, techniques which allow real time monitoring of the deposition are necessary. These requirements limit the range of appropriate analytical methods mainly to the following: optical waveguide lightmode spectroscopy (OWLS), surface plasmon resonance (SPR), in situ ellipsometry, and quartz crystal microbalance with dissipation monitoring (QCM-D).[117] All of these methods can detect mass adsorbing to the surfaces with sensitivity down to 1 ng/cm^2.[117] SPR, however, is restricted to noble metal surfaces and was thus not regarded in this work. The advantage of QCM-D compared to the other methods is that QCM-D can also be used to obtain information about structural properties of the deposited thin film.[123] Therefore, QCM-D was the method of choice to study the formation mechanisms of the phenolic coatings. Since the interpretation of results obtained by QCM-D is not as straightforward as for example for imaging techniques, this section will discuss the physical principle of QCM-D, the design of the study, and the interpretation of the data in more detail.

3.3.1 Background of QCM-D

The quartz crystal microbalance with dissipation monitoring is a piezoelectric sensor that uses acoustic waves to study thin films on surfaces in terms of adsorption and desorption processes, molecular interactions, and structural properties. Its physical concept is based on the inverse piezoelectric effect, in which a shear deformation is induced to a thin crystalline quartz disc by applying a voltage to gold electrodes deposited on each side of the disc (**Figure 8**).[124] Upon application of an alternating voltage to the electrodes, the

quartz crystal starts to oscillate at its fundamental resonant frequency, and an acoustic wave is generated propagating perpendicular to the crystal surface.[125]

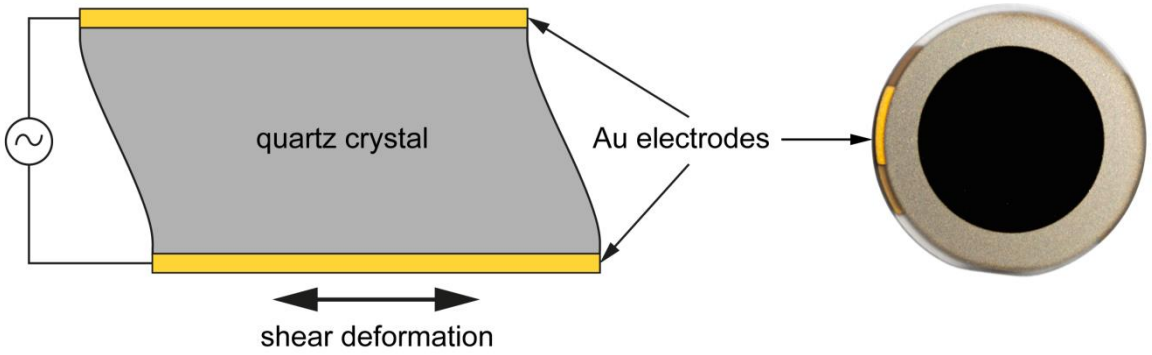

Figure 8. The QCM-D sensor consists of a quartz crystal sandwiched between two gold electrodes. When an alternating voltage is applied to the electrodes, the induced shear deformations make the quartz crystal oscillate at its resonance frequency.

As the use of the term "balance" in the instrument's name already indicates, the technique is applied to detect mass on the quartz crystal. The concept was first described by Sauerbrey in 1959, who demonstrated that the frequency change of the oscillating quartz crystal can be related to the mass adsorbing to its surface:[126]

$$\Delta m_l = -\frac{\rho_q h_q}{f_0}\frac{\Delta f}{n} = -C\frac{\Delta f}{n} \qquad \textbf{Equation 1}$$

where Δm_l is the mass change of the adsorbed layer per unit area, ρ_q and h_q are the density and thickness of the quartz crystal, respectively, f_0 is the fundamental resonant frequency, Δf is the frequency shift caused by the adsorbing layer, n is the overtone number, and C is the mass sensitivity constant (= 17.7 ng/(cm^{2}Hz) for a 5 MHz quartz crystal). The Sauerbrey relation assumes that the adsorbed mass is uniformly distributed, much smaller than the weight of the quartz crystal, and rigidly attached with no motion relative to the crystal.[127] However, when a non-rigid (viscoelastic) layer is deposited on the quartz crystal surface and the oscillation is damped to a large extent by this adsorbed layer, the relation between mass and frequency change can no longer be regarded as linear.[125] To address these viscoelastic properties of the adsorbed layer, a further parameter can be monitored in addition to the frequency: the dissipation factor. The dissipation factor D is a measure for the energy loss-rate of an oscillating system and is defined as the reciprocal of the quality factor Q:[127-128]

$$D = \frac{1}{Q} = \frac{E_{dissipated}}{2\pi E_{stored}} \qquad \textbf{Equation 2}$$

where $E_{dissipated}$ is the energy dissipated during one oscillation, and E_{stored} is the energy stored in the oscillating system. Changes in the dissipation factor can be measured by switching the driving voltage periodically on and off, and fitting the oscillation decay.[127]

Combining the detection of changes in frequency and dissipation factor, the QCM-D system can provide valuable information on interactions between surfaces and biomolecules, polymers, nanoparticles, cells and bacteria, etc. It is therefore a powerful characterization technique for biomaterial, sensor, polymer, or pharmaceutical applications. The standard gold substrate can be varied by coating the electrodes with a diverse range of materials such as titanium, silica, stainless steel, or hydroxyapatite.

3.3.2 Design of the QCM-D study

The QCM-D setup employed in this work was designed to represent the real coating conditions described in section 3.1.2 as closely as possible. A continuous flow system was applied to monitor the adsorption processes of the phenolic compounds in real time over 24 h. Quartz crystals which had been coated with a 120 nm thick layer of titanium (Q-Sense QSX 310) were used as coating substrates. They were mounted in flow chambers with a temperature stabilization loop to allow solution flow over the crystal at controlled temperature. TA and PG were dissolved in the coating solutions and the solutions were stirred in glass beakers outside the QCM-D system. At the same time, a peristaltic pump combined with a PTFE tubing system was utilized to pump the coating solutions into the flow chambers and over the quartz crystal (**Figure 9**). The setup consisted therefore of two separate systems: the reaction of the phenolic solutions outside the chambers, and the interactions of the solutions with the titanium coated quartz crystal inside the chambers.

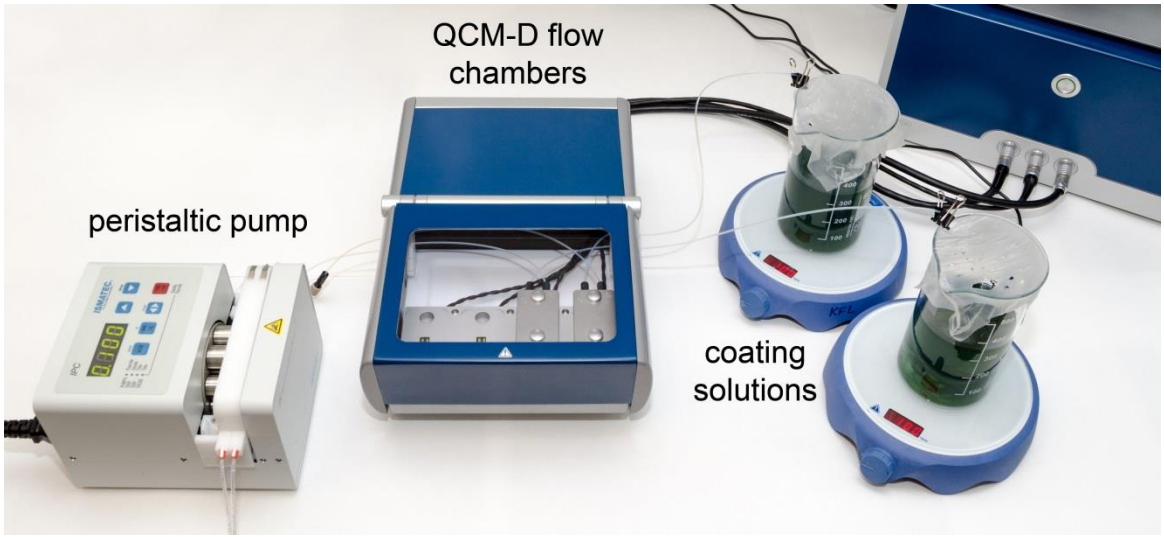

Figure 9. QCM-D setup used in this study. The coating solutions were stirred outside the QCM-D system and pumped into the flow chambers by means of a peristaltic pump.

The translation of the real coating conditions into the QCM-D setup brings along potential sources of error. In the real coating setup, the reactions of the phenolic compounds in the solutions and the adsorption process to the substrate occurred simultaneously in the glass vials on the rocking platform. The separation of these

processes in the QCM-D setup introduced a delay in the start of the coating formation on the quartz crystals, since the coating solutions first had to be pumped into the chambers. As the initial adsorption processes were very slow for PG coatings, this delay was considered to have a slight effect only on the early TA coating formation.

Moreover, the agitation of the rocking platform, which is necessary to ensure sufficient oxygen supply and guarantee adequate contact between the reactive molecules, was mimicked by stirring the coating solutions outside the QCM-D system. The selection of the stirring speed might be a further source of deviation from the real setup, because too fast or too slow stirring could speed up or slow down the reactions in solution, respectively. In the present study, the stirring speed was chosen to 100 rpm. This speed seemed to induce similar agitation of the solutions compared to the rocking platform, while not swirling up the large particles that precipitated to the bottom of the glass beakers during TA reaction (see video in the supporting information of **paper II**). Clogging of the tubing system by these particles could lead to unstable solution flow or even create vacuum in the system, which can eventually break the quartz crystals. In addition, the flow rate of the peristaltic pump had an influence on the coating deposition (see Supporting Information in **paper II**). A flow rate of 100 µl/min was used for the experiments, as the coating kinetics obtained with this flow rate fitted best with the observations made by other analytical methods (ellipsometry and AFM).

The high reactivity of the phenolic compounds and the fact that they interact with a wide range of different materials limited the reuse of the QCM-D equipment, since coating deposition also occurred on the inside of the PTFE tubing and the walls of the flow chambers. To ensure reproducibility of the measurements, the tubing had to be exchanged, and the chambers had to be disassembled and cleaned thoroughly on a regular basis.

3.3.3 Interpretation of QCM-D data

As stated earlier, QCM-D monitors the changes in two parameters over time: the frequency and the dissipation. The frequency shift gives information about the adsorbed mass. According to the Sauerbrey relation (**Equation 1)**, decreasing frequency indicates gain of mass on the crystal surface, while increasing frequency indicates mass removal from the surface (**Figure 10 A**). The dissipation factor on the other hand tells more about the structure of the phenolic films. If the layer adsorbing to the surface is thick, soft, or loose, the energy of the quartz crystal dissipates rapidly and the values for the dissipation factor are high.[129-130] In contrast, a compact and rigid layer does not damp the oscillation to such an extent, and the values for the dissipation factor remain low (**Figure 10 B**).[129-130]

Plotting the changes in dissipation factor versus the frequency shifts can be useful for interpreting the obtained QCM-D results. *ΔD-Δf*-plots show the dissipation per coupled unit mass and highlight structural changes in the adsorbing layer and mechanistic processes occurring during the experiment.[129] By means of **Figure 10 C**, the *ΔD-Δf*-

curves in **paper II** can be analyzed in terms of such processes. This is exemplarily demonstrated for a typical *ΔD-Δf*-plot of PG coating deposition (**Figure 10 D**). Starting from the origin of the plot (*ΔD* = *Δf* = 0) and following the curve, every direction change of the curve indicates a different process taking place at the quartz crystal surface. The plot starts with a steep curve pointing toward positive dissipation and negative frequency values (arrow 1), indicating that *more mass* is adsorbing to the crystal while the layer becomes *less rigid*. After that, the curve changes direction and remains stable until the end of the experiment (arrow 2). This second process represents a further gain in mass while the loss in rigidity is less pronounced compared to the first process. The deposition of PG to the titanium coated quartz crystal can therefore be characterized as a biphasic process.

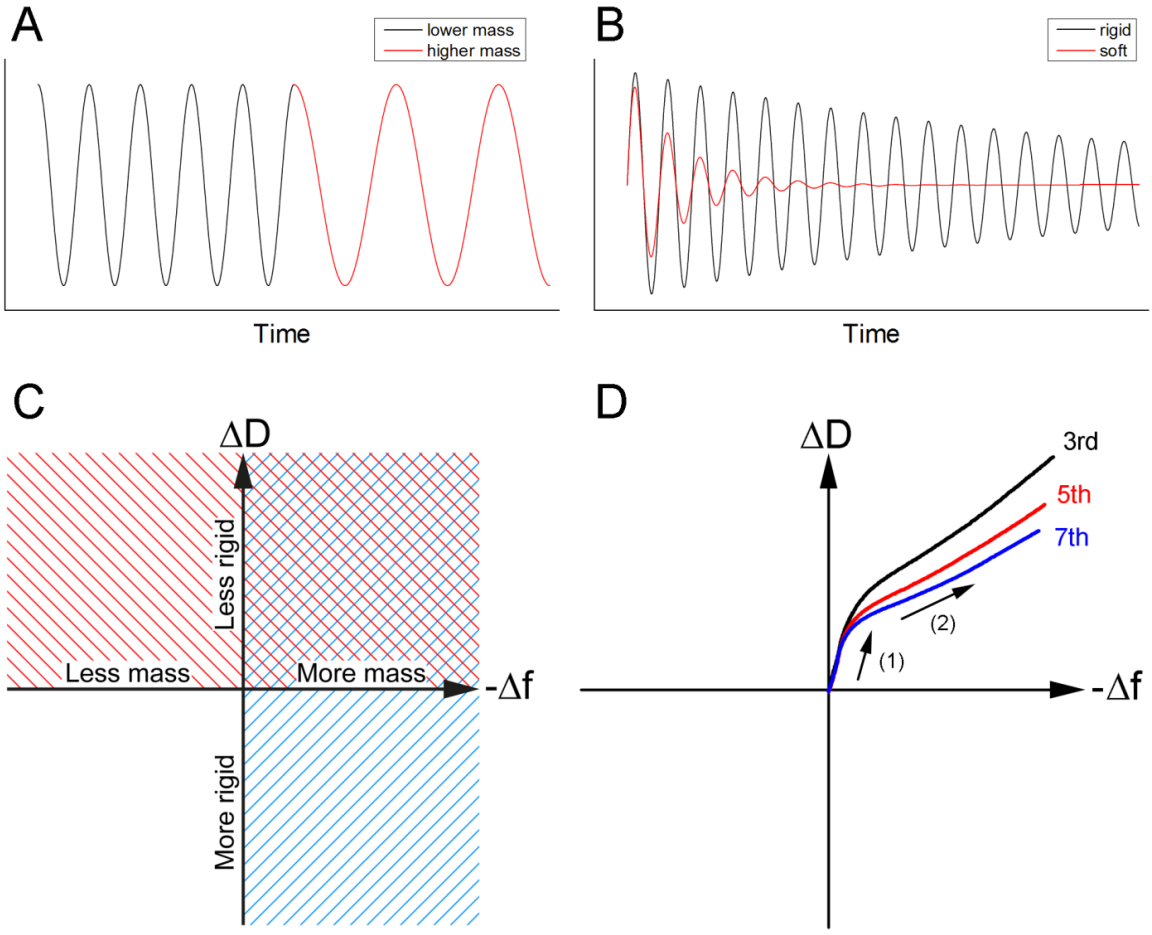

Figure 10. (A) Relation between resonance frequency and adsorbed mass. (B) Damping of the oscillation due to different structural properties of the adsorbed layer. (C) Schematic illustration for ΔD-Δf-plot interpretation *(adapted from McCubbin et al.[129])*. Negative frequency shifts imply mass adsorption (blue hatching). Positive shifts in dissipation factor indicate loss of rigidity of the adsorbing layer (red hatching). (D) Exemplary ΔD-Δf-plot for PG coatings. By drawing arrows starting from the origin of the plot, different mechanistic processes during coating formation can be distinguished and interpreted according to (C). For PG coating deposition, two different processes with mass adsorption in a viscoelastic way could be observed.

Furthermore, monitoring the different harmonics of the oscillation can provide additional information about the coating structure as a function of distance from the quartz crystal surface.[131] The higher the harmonic, the closer it senses to the surface of the crystal.[129] Therefore, the fundamental frequency probes furthest away from the surface and mainly the solution in the flow chamber. It is very sensitive to changes in the solution and usually gives unstable signal.[131] For this reason, only the third, fifth, and seventh harmonic were analyzed in this work. An overlapping of the signal of the different harmonics implies that the adsorbed layer is homogeneous throughout the coating thickness (process 1 in **Figure 10 D**). In the second process, however, the curves for the different harmonics split. The third harmonic, which is the one sensing furthest away from the crystal surface, exhibited the highest dissipation values. This indicates that the PG coating was less rigid (more viscoelastic) at the outer layers compared to the layers closer to the quartz crystal.

3.4 Analysis of coating thickness

The thickness of the doxycycline coatings on titanium discs was determined by means of the obtained TEM cross sections (**paper I**). This had the advantage that the thickness of the oxide and hydride layers of the samples could be evaluated at the same time. However, due to the time consuming and expensive cross-section preparation, only a limited number of samples could be analyzed. The thickness determination was thus rather qualitative than quantitative. Due to the fact that the doxycycline coating observed by TEM was very inhomogeneous and the substrate's oxide layers changed in an uncontrollable manner during the polarization process, no further methods to detect the coating thickness were considered in this work.

The thickness of the phenolic coatings in **paper II** was analyzed by means of ellipsometry. Ellipsometry is an optical method commonly used to measure thin film properties. Its physical concept is based on the change in polarization state of light when interacting with a surface.[123, 132] Elliptically polarized light is directed to and reflected from the sample surface. The adsorbed thin film on the surface changes the phase and amplitude of the reflected light, and by detecting the changes in the ellipsometric angles, the refractive index and the thickness of the film can be determined.[117, 123] To do this, an optical model has to be applied and the model parameters have to be fitted to the measured data. Even though ellipsometry is a fast measurement technique and allows therefore the analysis of a large number of samples within relatively short time, the modeling of the experimental data can be a complex and time consuming task. This was in particular a problem when performing ellipsometry measurements on the coated titanium discs. Due to high variations of the optical constants of the titanium substrate, the modeling outcome was highly variable and no conclusive determination of the coating thickness was possible. For this reason, silicon wafers were chosen as coating substrate. Silicon substrates are frequently used for ellipsometric studies, as they represent favorable model surfaces. The surfaces are very flat and thus minimize the effect of roughness on

the measurement. Moreover, the refractive index of silicon is high, providing a large optical contrast to organic layers and making the measurements highly sensitive with high thickness resolution.[133] To ensure that the coating deposition of the phenolic compounds was comparable on titanium and silicon substrates, QCM-D experiments were repeated on silicon dioxide (SiO_2) coated quartz crystals (Q-Sense QXS 303). The results confirmed similar coating deposition kinetics for both phenolic compounds. The performance of ellipsometry measurements on silicon wafers had the advantage that a system that was optimized for measurements on Si/SiO_2 in air could be used for the analysis (Auto-EL III, Rudolph Research). However, a limiting factor was that the refractive index of the coatings had to be known in order to be able to calculate the coating thickness from the measured data. The refractive index was assumed to be 1.465 since this value has previously been used to measure the thickness of polydopamine layers.[134] This was only an approximate value and might therefore represent a source of error in the thickness determination. A further limitation of the method is that it averages the thickness over a large surface area (approximately 0.6 mm^2). Thickness variations on the sample surface are therefore not detectable.

For this reason, AFM was employed as an additional method to determine the thickness of the phenolic coatings. Scanning was performed on partially coated silicon wafers, and the scan profile was used to measure the step between coating and bare substrate. The measured thickness for TA coatings was similar for both ellipsometry and AFM, confirming that the assumed refractive index for the ellipsometry measurements was a good approximation. For PG coatings, the thickness measured by ellipsometry was higher than the one determined by AFM. The reason for this could on the one hand be that the refractive index might be different for PG coatings. On the other hand, AFM images also revealed that PG coatings were not as homogeneous as TA coatings. This could explain the observed differences between the two methods, since ellipsometry averages over large surface areas, whereas AFM only measures on certain spots on the substrate.

In addition to ellipsometry and AFM, the obtained QCM-D data can also be used to gain information about the thickness of the coatings. For thin, rigid, and evenly distributed films, the adsorbed mass can be calculated from the measured frequency shift by means of the Sauerbrey relation (**Equation 1**), whereupon the thickness d of the adsorbed film can be calculated:

$$d = \frac{\Gamma}{\rho} \qquad \textbf{Equation 3}$$

where Γ is the mass per unit area of the substrate and ρ is the density of the adsorbed film. However, both TA and PG coatings also showed noticeable shifts in the dissipation factor, so that only the initial TA film could be regarded to fulfill the Sauerbrey assumption of a rigid film. The limitation of calculating the thickness by means of **Equation 3** is that the density of the deposited film has to be known. In this work, the density of the phenolic

films was assumed to be 1.2 g/cm^3. This value has been used in other studies to calculate the thickness of polydopamine films,[134] and the thickness of TA adsorbed on silica and gold.[135] The calculated thickness for the TA film deposited after 60 min coating time was approximately 28 nm, which was double the thickness measured by ellipsometry. To find an explanation for this discrepancy, not only potential errors in the density assumption, but also the main difference between the applied measurement techniques have to be considered: while ellipsometry and AFM probe the dry mass of the coatings (the coated substrates were dried with nitrogen after the coating process), QCM-D measures both the dry mass and the water that is coupled to the adsorbed mass.[136] Direct hydration, viscous drag, or water entrapment in cavities of the adsorbed film can increase the additional mass and can therefore have a large effect on the measured frequency shifts.[123, 129, 136] Thus, combining QCM-D with either ellipsometry or AFM provides also information about the hydration state of a deposited thin film.

The significant increases in dissipation factor for PG coatings and for longer times of TA coating deposition did not allow the use of the Sauerbrey relation. For obtaining the thickness of such viscoelastic layers, a continuum mechanics model represented by an elastic component (spring) in parallel with a viscous component (damper) can be applied (**Figure 11 A**).[137] By using this Voigt model to fit both the frequency and the dissipation shifts, several parameters such as the thickness d, the elastic shear modulus μ, or the shear viscosity η of the phenolic film can be obtained. In this study, the model system consisted of a single coating layer which was covered by bulk fluid (**Figure 11 B**).

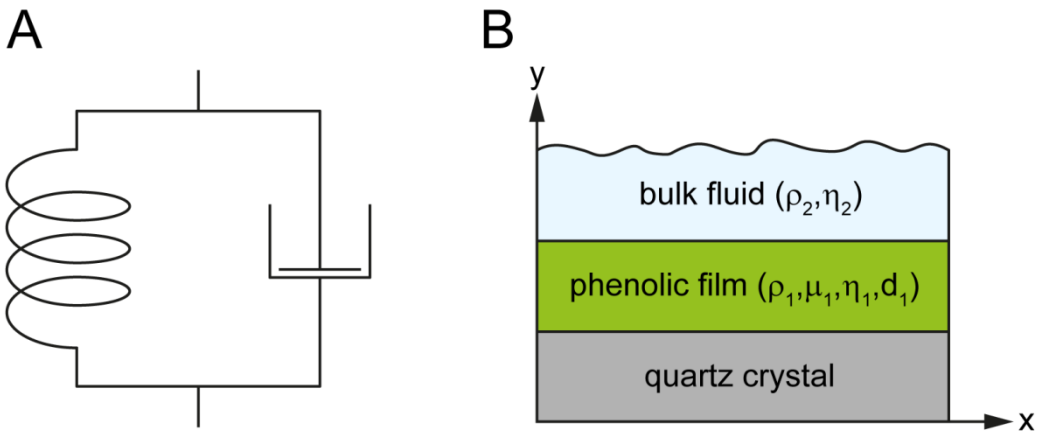

Figure 11. (A) The Voigt viscoelastic element consisting of an elastic component in parallel with a viscous component. (B) Schematic illustration of the model system used for this study. It is represented by a single coating layer which covers the quartz crystal and which is covered by bulk fluid.

Modeling of the third, fifth, and seventh harmonic was performed using QTools software. While a good fit between measured and modeled data was obtained for PG coatings, the frequency and dissipation curves for TA coatings could not be modeled. The reason for this might be explained by the main limitation of the model system: the assumption of a homogeneous layer covering the quartz crystal. The observed growth of

TA particles in the coating solutions already suggests that the deposited layer might not be homogeneous. While only small TA particles adsorb to the crystal in the beginning of the coating, the size of the adsorbing particles increases with increasing coating time. Therefore, it is likely that the coating structure exhibits a gradual change from the crystal surface toward the outermost coating layer. Varying densities within the coating would conflict with the initial model assumptions. The particle growth was not as fast and pronounced for PG coatings, suggesting that the coating structure might be more homogeneous in comparison to TA coatings. The PG coatings might thus have been better represented by the applied Voigt model.

3.5 Analysis of coating chemistry

Several methods, such as X-ray photoelectron spectroscopy (XPS), secondary ion mass spectroscopy (SIMS), and Fourier transform infrared spectroscopy (FTIR), were used in this work to characterize the chemical properties of the modified surfaces. XPS is a powerful technique to analyze the elemental composition of the surface coatings and to get information about the chemical binding states of the elements on the sample surfaces. In XPS, the sample is irradiated with photons (monochromatic Al Kα radiation), and photoelectrons which were ejected from the sample surface are detected. The kinetic energy of the photoelectrons is the difference between the incident photon energy and the binding energy of the electrons, and by detecting the number and kinetic energy of the electrons, a spectrum of binding energies can be obtained.[138-139] Each binding energy is characteristic for a specific atom within the analyzed surface, and the spectra can thus be used to get quantitative information about the elemental composition of the surface. The depth from which the photoelectrons are emitted is limited to 1-10 nm due to the short free mean path of the electrons in a solid sample.[138] Therefore, special attention had to be paid to sample handling between the modification process and the measurements in order to avoid excessive sample contamination from the atmosphere. This was also the reason why the PG precursor powder could not be analyzed in **paper II**. Oxidation due to the high reactivity of the powder had too much influence on the measured outermost layers and led to unreliable results.

From the recorded detail spectra of the expected elements on the surface, it is possible to get useful information about the binding state of the respective element. However, the identification of the different components which contribute to a certain peak in a detail spectrum can sometimes be a challenging task. If a molecule with a known chemical structure is adsorbed on a surface, the analysis of the detail spectra is relatively straightforward and the presence of the molecule on the surface can therefore be assessed. In contrast, if the adsorbed coating is an unknown reaction product of the precursor molecules, as it was for the phenolic compounds in **paper II**, the analysis can be much more difficult. This can in particular be seen for the analysis of the oxygen peak of the phenolic coatings and particles. Due to the fact that multiple components can be found

within a small binding energy region,[140] and that peak shifts caused by hydrogen bonds can occur,[141] it was not possible to identify the exact peak composition.

In addition to standard XPS, angle-resolved XPS (ARXPS) was applied in **paper I** to analyze the changes introduced to the oxide layer of the titanium discs during the polarization process. ARXPS makes use of the relation between the electron escape depth and the angle at which the emitted electrons are detected.[139] The larger the detection angle with regard to the surface normal, the smaller is the depth from which the photoelectrons originate. In the case of a thin oxide layer covering the bulk titanium material, electrons detected at large angles are therefore mainly emitted from the oxide layer, while electrons detected at normal angle also originate from the bulk. By varying the detection angle, a depth profile of the outermost surface layer can thus be obtained. In conventional ARXPS, this is done by tilting the sample. The Thetaprobe spectrometer used in this study, however, allowed parallel acquisition of different angles without tilting the sample. This has some advantages compared to the conventional method. It can be applied to large samples which are difficult to tilt; measurements of specific surface features are easier, since the analysis position and area do not have to be adjusted after every tilting step; the charge compensation conditions which change when tilting the sample do not need to be adjusted.[142] ARXPS is limited to very flat surfaces and thus only the polarized samples could be analyzed and compared to the control sample. An advantage of obtaining depth profiles by ARXPS compared to commonly applied sputtering techniques is that it is a non-destructive method and can therefore also be used to determine the chemical state of the sample.[142] The results obtained by ARXPS confirmed the growth of the oxide layer on the polarized samples, which was observed on the TEM cross sections.

XPS is not suitable to detect hydrogen and helium.[139, 143] Due to the proposed attraction and incorporation of hydrogen during the cathodic polarization process, however, it was also desirable to assess the hydrogen content in the outermost surface layers. In previous studies, hydrogen was detected by means of secondary ion mass spectroscopy.[94, 112] SIMS is based on the bombardment of the sample surface with primary ions. The sample is sputtered by the primary ions which penetrate the surface and interact with the lattice atoms.[144] These interactions result in the emission of positively and negatively charged atoms (secondary ions) which can be detected. SIMS was used in **paper I** to analyze the content of the isotopes ^{1}H and ^{12}C.

The modified surfaces were furthermore characterized by means of Fourier transform infrared spectroscopy. FTIR is a powerful analytical tool for detecting functional groups of a sample. Its concept is based on the vibrations of the atoms of a molecule when infrared light is absorbed.[145] There are two types of vibrations: stretching vibrations (bond-length variations) and deformation vibrations (bond-angle variations) of the molecule.[146] The infrared absorption occurs at specific frequencies for a particular functional group, and the absorption bands in the infrared spectrum can therefore be used to determine the functional groups present in a sample.[146] For many molecules or compounds, reference spectra exist in literature which help identifying and assigning the specific absorption bands. To confirm the presence of a molecule that is adsorbed on a

sample surface, the infrared spectrum of the sample can be compared to the spectrum of the molecule itself. However, obtaining infrared spectra of good quality from a thin film adsorbed on a surface is not straightforward. For solid surfaces which can not be analyzed with conventional transmission methods, reflectance methods have to be applied. However, with the diffuse reflectance accessory used in this work for measuring the doxycycline and polyphenol coatings on titanium discs, only a very weak signal could be detected. The reason for this might be the large penetration depth of the infrared beam (typically in the µm range), making the detection of nanometer scale coatings difficult. The presence of doxycycline on the sample surfaces could thus not be confirmed by FTIR. In **paper II**, the evaluation of the coating chemistry was therefore conducted by analyzing the phenolic particles that were formed in the coating solutions. After filtering the coating solutions, the obtained phenolic particles were dried and infrared spectra were acquired by means of an attenuated total reflectance (ATR) accessory. The detected signal in these spectra was of much better quality and could be used for chemical assessment of the reaction products. Similar to XPS analysis, however, conclusive assignment of the infrared bands is challenging when the chemical or physical interactions during the reaction of the precursor molecules are unknown.

Moreover, UV/Vis spectroscopy was applied to measure the absorbance of light by the formed phenolic particles. The coating solutions were filtered to reduce scattering due to large particles, and shifts in the absorbance peaks for different coating times was assessed to reveal differences in particle growth for the phenolic compounds.

3.6 Analysis of coating stability

Doxycycline has previously been shown to be released from the coated surfaces in two studies. Walter et al. immersed the samples in a solution of acetonitrile and trifluoroacetic acid (ACN-TFA) and performed UV/Vis measurements to detect released doxycycline in the solution.[94] However, this was done to determine the total amount of doxycycline on the modified surfaces and not to investigate the release kinetics of the biomolecule. Xing et al. tested the release under more physiological conditions in phosphate buffered saline (PBS) at 37 °C by means of high-performance liquid chromatography (HPLC).[96] They observed a burst release of doxycycline within the first 6 h. In the present work, a simple coating stability test was performed by immersing the samples in water for 10 h (**paper I**). Release of doxycycline was confirmed by comparing XPS spectra obtained before and after the immersion. In addition, loss of fluorescence of the sample surfaces was a sign for the release of the biomolecule. The main aim of this release experiment was to investigate the potential long-term antibacterial effect of the coating which remained on the surfaces after the immersion (see section 3.7.2).

The stability of the polyphenol coated surfaces was investigated by rinsing coated quartz crystals in the QCM-D system with PBS at 37 °C for 12 h, and monitoring the frequency changes during rinsing (**paper III**). An increase in resonance frequency was related to the release of phenolic compounds from the surface. The rinsing time was

chosen to 12 h, as this was the time frame used for the bacteria study (section 3.7.2). Investigating the coating stability by means of QCM-D had the advantage that coating formation and coating removal could be monitored in one experiment. However, frequency shifts can not only be associated with the release of phenolic compounds from the surface, since also dehydration processes (water loss) could affect the frequency of the quartz crystal. Methods for direct determination of released phenolic compounds, such as UV/Vis or liquid chromatography-mass spectrometry (LC-MS), were not considered in this work, as the composition of the released compounds was unknown and thus no reference standard could be obtained.

3.7 *In vitro* testing

Before the performance of a biomaterial is tested *in vivo*, a screening regarding the response of a biological system to the material is commonly conducted *in vitro*. *In vitro* tests provide first insights in biological events occurring in the presence of a biomaterial, without the need to take ethical considerations into account that come along with animal experiments. They are fast and cost-effective screening methods allowing the reproducible evaluation of biomaterials in a controlled environment.[147] The main concern with respect to *in vitro* models is their limited representation of *in vivo* situations. The largely reduced complexity of *in vitro* models, such as the use of mostly only one cell type in static two-dimensional culture conditions with optimized culture media, make an extrapolation to the *in vivo* scenario very difficult.[147-148] However, results obtained by *in vitro* tests are necessary in order to detect potential negative effects (such as cytotoxicity) at an early stage before performing animal studies. Since the aim of the present work was to produce implant surfaces that can promote bone formation and reduce the risk of bacterial infection, *in vitro* tests were conducted both with cells and with bacteria.

3.7.1 Cell study

The cellular response to polyphenol coated titanium discs was investigated in an *in vitro* cell study. The surfaces modified by cathodic polarization with doxycycline were not tested, as they have already previously been analyzed *in vitro* using an osteoblastic cell line (MC3T3-E1), as well as *in vivo* in a rabbit study.[94] The present work employed a primary cell culture which means that the cells were isolated directly from the tissue of origin before the culture. Often, cell studies are also conducted using cell lines which are derived and subcultured from primary cells. Even though cell lines have some advantages compared to primary cell cultures (such as the longer life span due to immortalization and a higher degree of standardization), the *in vivo* situation is better represented by the use of primary cells.[149] Primary human osteoblasts (hOBs) were used for the cell study in **paper III**, being the cells responsible for bone formation, i.e. the synthesis and deposition of the bone extracellular matrix, and thus representative for the target tissue.[150] Even though the surface roughness of the Ti discs can have an influence on the interactions between the

cells and the surfaces, mirror-polished surfaces were used for the cell study in order to only investigate the effects of the coatings.

Cell morphology was analyzed after two days of culture by means of confocal laser scanning microscopy (CLSM). The advantage of CLSM compared to conventional microscopes is the capability of scanning within only one focal plane while eliminating the influence of light from outside this focal plane. Images from different sample depths can then be overlaid to create high resolution image stacks. The equipment with multiple lasers allows excitement of the sample at different wavelengths, which is commonly used to simultaneously visualize different cell compartments that were stained with different fluorescent dyes. In this study, cell staining was performed using phalloidin Alexa Fluor 488 (green fluorescence) for actin cytoskeleton and DAPI for cell nuclei (blue fluorescence), and the obtained images were used to analyze cell spreading and morphology.

At this time point, also the cytotoxic effect of the modified surfaces was investigated by measuring the activity of lactate dehydrogenase (LDH) as an index of cell death. LDH is a stable cytoplasmic enzyme that catalyzes the conversion of lactate to pyruvate via reduction of NAD+ to NADH.[151] Diaphorase then uses NADH to reduce light yellow tetrazolium salt (INT) to red formazan salt, the absorbance of which can be quantitatively measured at 492 nm.[151-152] This coupled reaction is the basis for determining the activity of LDH which is released in the cell media upon damage of the cell membranes. The LDH activity measured from the supernatant of the tested samples was related to the LDH activity in the medium of cells seeded on unmodified titanium coins (low control) and on empty wells after adding 1% Triton X-100 to cause 100% cell death (high control). Several other methods exist to analyze cell viability or cell death, such as trypan blue dye exclusion,[153] the neutral red assay,[154] the MTT assay,[155] and many more.[151] Depending on the working principle of each method, there is a risk for over- or underestimation of the cytotoxicity, and the combination of multiple assays should be considered in order to increase the reliability of the results.[156]

The gene expression of certain target genes was analyzed by means of real-time reverse transcription polymerase chain reaction (real-time RT-PCR). After total RNA was isolated from the samples, the RNA was transcribed into complementary DNA (cDNA) by reverse transcriptase, and the cDNA was amplified using real-time PCR with gene specific primers. Real-time RT-PCR is commonly used as it is a cost-effective, fast, accurate, sensitive, and quantitative method to detect specific RNA sequences.[157] However, the widespread use of the technique and its relatively simple application have given rise to concerns regarding the standardization of experiments and the reproducibility of results.[158] A thorough experimental design, high sample quality, sensible selection of reference genes and primers, and thorough following and reporting of applied protocols are necessary requirements to ensure high quality and reliability of the obtained results.[157] In the present work, the gene expression of type I collagen (COL1A1), alkaline phosphatase (ALP), osteocalcin (OC), and interleukin-6 (IL6) was analyzed. Collagen, ALP, and OC are differently expressed during osteoblast

development and can therefore be used as fingerprint genes in the determination of the temporal stage of an osteoblast (**Figure 12**). Generally, three time-dependent stages can be distinguished:[159]

- *Proliferation*: The osteoblasts expand and produce bone extracellular matrix. Type I collagen is one of the earliest osteoblast markers and is highly expressed at this stage.
- *Matrix maturation*: In preparation for mineralization, maturation and organization of the extracellular matrix take place, characterized by upregulation of ALP.
- *Mineralization*: The terminal stage in osteoblast development is represented by high expression of genes related to the ordered deposition of hydroxyapatite, such as OC.

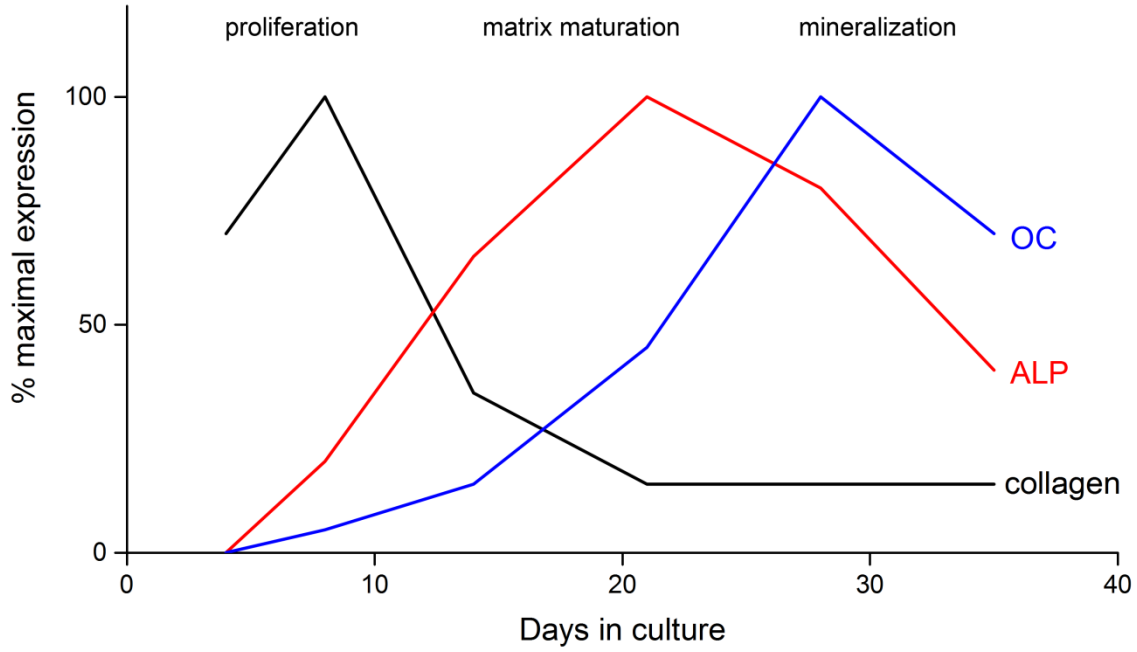

Figure 12. Sequential expression of osteoblast-related genes collagen, ALP, and OC during 35 days of culture *(adapted from Stein et al.[159])*. While type I collagen shows highest expression in the proliferation stage, ALP expression peaks in the matrix maturation stage. The expression of OC is highest during the mineralization stage.

The expression of these three genes was therefore used to obtain information about the influence of the modified surfaces on the developmental stage of hOBs in comparison to unmodified titanium surfaces. In addition, IL6 expression was determined as a marker for inflammation, since IL-6 is a cytokine involved in the regulation of the immune response and the acute phase reaction to inflammatory stimuli or tissue injury.[160-161]

At the end of the cell culture after 21 days, ALP activity and calcium content were measured. ALP is an enzyme attached to the outer face of the plasma membrane playing a

key role in bone calcification,[162] and its activity was determined by measuring the cleavage of p-nitrophenyl phosphate in a soluble yellow end product that can be detected spectrophotometrically. The calcium content was quantified by means of an atomic absorption spectrometer.

3.7.2 Bacteria study

The antibacterial properties of the functionalized titanium discs were tested by culturing *Staphylococcus epidermidis* Xen43 on the sample surfaces. *S. epidermidis* is a Gram-positive bacterium found in the normal bacterial flora of the human skin and mucous membranes.[163] When skin or mucosa is injured (e.g. during an implantation surgery), *S. epidermidis* can enter the wound site, colonize the implant surface, and cause infection.[164] In fact, together with *Staphylococcus aureus, S. epidermidis* is the most common cause of biofilm-associated infections,[163-165] and has been described to create pathogenic biofilms on implant surfaces.[166-167] The strain is one of the initial colonizers during the multistage dental plaque formation process,[168] preparing a favorable environment for late colonizers. *S. epidermidis* is relatively innocuous compared to other bacterial strains commonly used as model strains for biofilm formation,[163] and therefore represents a suitable and safe biofilm model to investigate the antibacterial properties of the modified surfaces.

Xen43 is a bioluminescent strain created by inserting the *lux* genes into the bacterial genome of the biofilm-forming *S. epidermidis* 1457.[169] As a result, metabolic activity of the bacteria is accompanied by the emission of visible light (i.e. bioluminescence),[169] which can be measured by means of a multi-detection plate reader. The metabolic activity of the bacteria in the presence of the functionalized surfaces could therefore be monitored in real-time, which was one of the main advantages of this *in vitro* method. The number of viable bacteria, as determined by the number of colony-forming units (CFUs), can be linearly correlated with the detected luminescence until bacteria growth reaches the stationary phase (**Figure 13 A**).[170] After that, luminescence could no longer be correlated with the number of cells, and the observed decrease in luminescence could not be related to the death of bacteria, but rather to decreased metabolic activity due to the lack of nutrients and oxygen in the sealed well plates.[171] In **Figure 13 B**, representative luminescence profiles for normal bacterial growth (in presence of a polarized titanium disc) and for inhibited bacterial growth (in presence of a doxycycline coated disc) are shown.

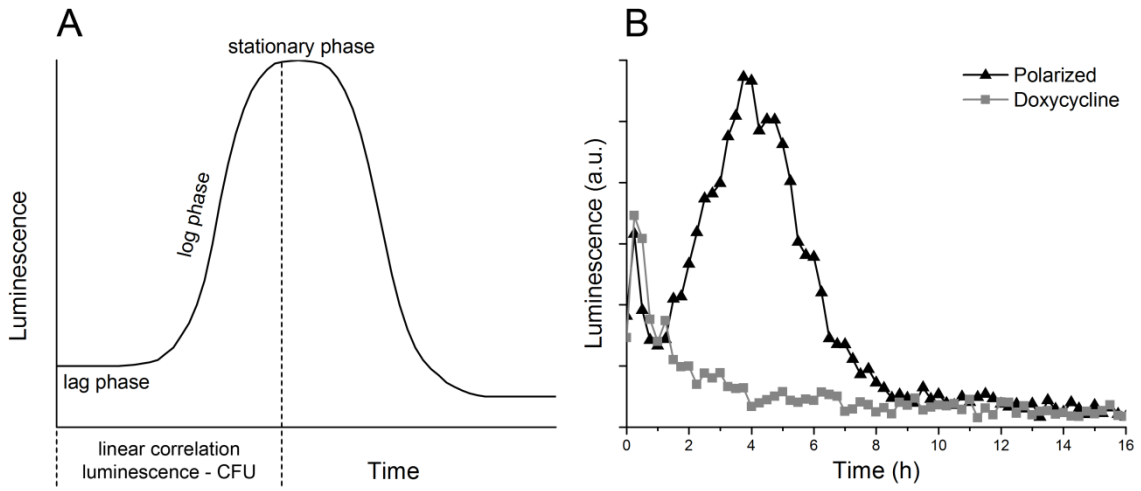

Figure 13. (A) Schematic representation of the correlation between bacterial growth phases and detected luminescence. Luminescence is linearly related to the colony-forming units (CFU) up to the time point where bacteria growth reaches the stationary phase. (B) Exemplary plot of luminescence profiles for bacteria cultured on polarized and doxycycline coated titanium discs. While bacteria showed typical phases for bacterial growth in the presence of the polarized sample, no significant luminescence could be detected in the presence of doxycycline coated coins.

It is worth mentioning that by using this bioluminescence bacteria assay, the luminescence of both the planktonic bacteria in the medium and the bacteria colonizing the disc surfaces were measured. In case of the doxycycline coated samples in **Figure 13 B**, the absence of significant luminescence was thus a sign for release of the antibiotic from the surface, influencing also the planktonic bacteria growth. In contrast, the presence of luminescence alone can not conclusively be used as an indicator for biofilm formation, as the coatings might also prevent biofilm formation by inhibiting bacterial adhesion, or by killing only bacteria in contact with the coating and not in the medium.

For this reason, the samples were additionally analyzed for biofilm formation by means of SEM visualization. Imaging of bacteria adhering to the Ti disc surfaces was performed using a tabletop SEM detecting backscattered electrons (**paper I & III**). Even tough the tabletop SEM has a lower resolution compared to the FE-SEM, image quality and achieved magnifications were sufficient to get an overview of bacterial colonization and biofilm formation on the sample surfaces. Furthermore, the simple applicability of the tabletop SEM made it convenient to analyze a large number of samples without being very time-intensive. Prior to visualization, the bacteria had to be fixed on the disc surface, dehydrated with ethanol, and the discs were metal sputtered. SEM imaging could at the same time be used to investigate the shape of the bacteria on the surfaces, so that e.g. collapsed and differently sized bacteria could be detected on the doxycycline coated surfaces. However, the shape of the bacteria might also have been influenced by the sample preparation (dehydration) or by the vacuum used in SEM. Thus, in most cases, no conclusive distinction could be made if the bacteria were dead or alive prior to imaging. For the *in vitro* analysis of the doxycycline coatings in **paper I**, however, the measured luminescence correlated well with the observed bacterial colonization of the surfaces. In

this study, also the potential long-term antibacterial effect of the doxycycline coating proposed by Xing et al.[96] was tested by analyzing bacteria cultures on doxycycline coated samples that had been immersed in water prior to the *in vitro* study.

Bioluminescence and SEM analysis was also performed for the polyphenol coated discs. However, sedimentation of bacteria from the media was observed to a large extent on these samples, which might have hindered a release of phenolic compounds from the surfaces. To study the effect of released compounds on bacterial viability and growth, the adhesion of planktonic bacteria was minimized by attaching the modified coins upside down to the well lids by means of threaded stainless steel pins (**Figure 14**). This ensured that the phenolic compounds could be released into the bacteria media and that the bioluminescence of the bacteria in contact with the released compounds could be measured. The well lids were furthermore split into three segments, allowing the removal of samples at different time points without having to stop the experiment and without the risk of causing cross-contamination between the wells.

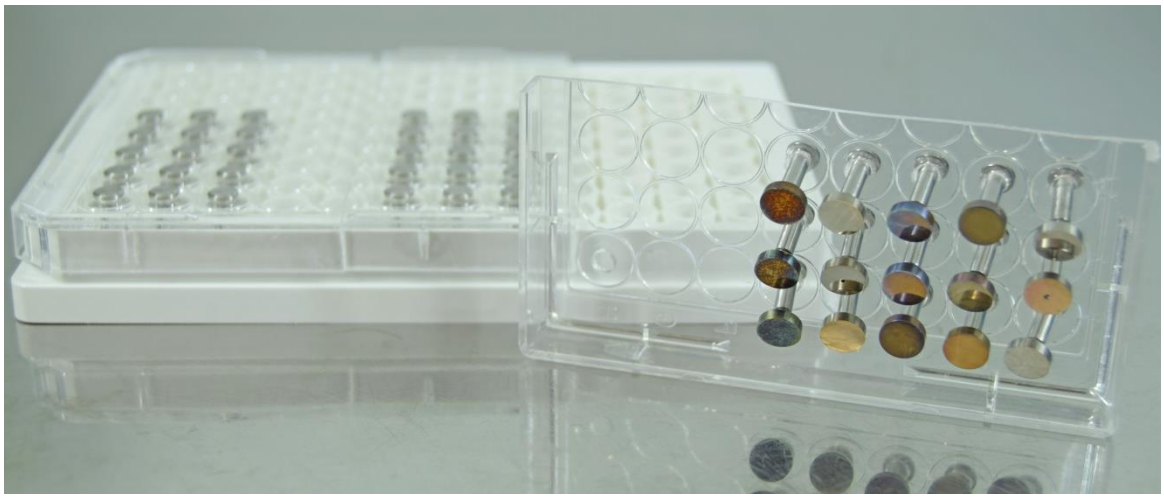

Figure 14. The release of phenolic compounds from the modified surfaces was studied by attaching the discs upside down to the well lid using threaded pins. This was done in order to reduce adhesion of planktonic bacteria to the surfaces. In addition, the lids were cut into three segments to allow removal of samples after different time points.

4 Summary of key findings

4.1 Cathodic polarization with doxycycline

With the process parameters employed in the present study, cathodic polarization was demonstrated not to induce the formation of a hydride layer on mirror-polished titanium surfaces. Instead, the process altered the homogeneous titanium oxide layer present on the control surfaces in an uncontrollable manner, characterized by oxygen diffusion and the formation of oxygen-rich surface pockets. The involvement of titanium hydride in the binding of doxycycline to the surfaces could therefore be excluded. Nonetheless, an organic layer containing doxycycline was found to be adhered to the altered titanium oxide, increasing the surface nano-roughness. While a fraction of this organic layer was released upon simple immersion in water, a firmly adhered layer remained present on the surface.

S. epidermidis cultured in the presence of doxycycline coated surfaces exhibited lower metabolic activity compared to bacteria in the presence of only polarized surfaces, as measured by bioluminescence. While interconnected clusters of bacteria were observed on the polarized samples, doxycycline coated samples showed significantly less bacterial colonization, and the different shape of the bacteria indicated interference of the coating with the bacterial metabolism. However, doxycycline coated samples that had been immersed in water prior to the bacteria assay revealed clearly reduced antibacterial properties. The antibacterial effect of the coatings could thus mainly be attributed to an initial rapid release of active doxycycline molecules which were not strongly attached to the titanium surface.

4.2 Auto-oxidative surface polymerization of phenolic compounds

The auto-oxidative surface polymerization of the phenolic compounds tannic acid and pyrogallol was successfully implemented in a continuous flow QCM-D setup, allowing real time monitoring of the coating deposition on titanium-coated quartz crystals over a time period of 24 h.

TA coating deposition only occurred within the first 5 h and could be divided into three main phases. Initially, a compact and rigid layer adsorbed to the crystal surface, followed by the deposition of an increasingly viscoelastic layer. After approximately 5 h, precipitation of large polyphenol particles was observed in the coating solutions, accompanied by discontinuation of coating formation. In contrast, PG adsorption to titanium surfaces was seen during the entire 24 h of observation time and was of biphasic nature. The first phase was characterized by deposition of a thin and viscoelastic layer. After 2-3 h, the coating kinetics changed and further mass adsorption occurred with a slower increase in layer viscoelasticity. Ellipsometric assessment of the coating thickness confirmed the coating kinetics observed by QCM-D.

The phenolic coatings were found to be of the same chemical nature as the phenolic particles formed in the coating solutions during the process. Various interactions between the phenolic molecules seemed to be involved in the auto-oxidative coating formation, including both physical and chemical interactions. Moreover, a potential contribution of metal ion coordination to the coating formation was hypothesized. However, due to the complexity of the oxidative systems, the exact molecular mechanisms remain unclear.

While thin PG coatings deposited for 2 h on titanium surfaces showed no release of phenolic compounds, PG coatings deposited for 24 h and TA coatings deposited for 2 and 24 h exhibited mass release from the surfaces. *In vitro* assessment of the biological performance revealed no toxic effects of the coatings on human osteoblasts, although delay in osteoblast maturation was observed for coatings that released compounds from the surfaces. These coatings also induced downregulation of IL6, which may be a sign for the anti-inflammatory potential of the coatings. Thin PG coatings seemed to promote osteoblast maturation and caused increased calcium deposition. The phenolic coatings could not prevent the colonization and biofilm formation of *S. epidermidis* on the surfaces. However, the released phenolic compounds had a clear effect on the planktonic bacteria, as evidenced by a significant reduction in optical density and number of colony-forming units. Bioluminescence measurements did not imply a clear effect of the coatings on the metabolic activity of the bacteria. Due to the unknown composition and unknown concentrations of the released phenolic compounds, no conclusive statement about the mechanisms involved in the interaction of the coatings with osteoblasts and bacteria could be made.

5 Discussion

Since the pioneering work of Brånemark, dental implants made from titanium have demonstrated a great story of success, and the high survival rates imply that currently available implants with their moderately rough surfaces represent an advanced solution for accomplishing osseointegration. Indeed, the traditional strategy of altering the topographical design of the surface to enhance osseointegration has reached a high degree of sophistication.[78, 172] However, there is a diversity of challenging clinical scenarios, mainly with respect to situations where bone sites for implant placement are compromised. The lack of sufficient bone quantity or quality to achieve implant stability represents one of the major drawbacks in modern implant therapy.[6, 80] Moreover, bacterial colonization and subsequent biofilm formation remain problematic for establishing and maintaining osseointegration.[40] Surface functionalization using biologically active substances to influence the microenvironment around the dental implant with the aim to enhance interfacial healing processes and to prevent bacterial colonization represents a promising route to achieve improved implantation outcome, particularly in the compromised patient. This perception is reflected in the vast amount of ongoing research in this field,[79-80, 173-174] and was the underlying motivation for the present thesis.

As already suggested by Gristina, the best strategy for preventing bacterial colonization may be to create an implant surface that is adhesive for appropriate tissue cells and thus aids the cells to occupy the implant surface.[39] Having healthy host tissue with a functioning host defense system in direct contact with the implant surface is not only of importance for the long-term stability of the implant, but also for the prevention of late bacterial colonization, which may for instance result in peri-implantitis. However, establishing early host tissue integration may be challenging in the presence of bacteria. The first 2-6 h after implant placement have been reported to be decisive for the prevention of bacterial adhesion, as the implant is particularly susceptible to surface colonization during this period, but introduced pathogens are still in an inactive state and do not increase in number.[175-179] Therefore, the question arises whether cell-adhesive surfaces are able to integrate sufficient host tissue within this time window before bacteria enter the exponential growth phase. If this is not the case, even an initially small amount of present bacteria can win the race for the surface, since bacteria can replicate in as fast as 30 min, while cell division for eukaryotes typically takes 24 h.[180] For this reason, the approach in the present work was to functionalize implant materials in a way to address both sides of the race for the surface, i.e. promote host tissue integration and fight bacterial colonization simultaneously.

In this thesis, the broad-spectrum antibiotic doxycycline was chosen as a candidate molecule for such a multifunctional surface modification, since beside its known antibacterial activity,[95] it has also been suggested to promote bone formation.[94, 181-182] Moreover, a beneficial effect of locally released doxycycline has been reported for the treatment of peri-implantitis.[183] The doxycycline coated titanium surfaces produced in this work exhibited antibacterial properties, as demonstrated by the reduced bacterial

surface colonization and decreased planktonic bacterial growth of *S. epidermidis* in **paper I**.

However, there are general concerns over the potential role of antibiotic-modified biomaterials in contributing to the development and spreading of multiresistant bacteria strains.[184] Similar to many other antibiotics, doxycycline interacts with bacteria by penetrating the bacterial cell and inhibiting protein biosynthesis.[95, 185] Therefore, the molecules should be released from the implant surface in order to have an effect. The release of molecules from the functionalized surfaces in **paper I** was demonstrated by simply immersing the modified surfaces in water. Having control over the release kinetics is crucial with regard to antibiotics, as the concentration of released antibiotics needs to be over a certain threshold to effectively inhibit bacterial growth, the so-called minimum inhibitory concentration (MIC).[186] Ideally, implant surfaces functionalized with antibiotics should ensure that the concentration of the antibiotic in the peri-implant tissue is several times higher than the *in vitro* MIC over a sufficient time to at least cover the decisive time window after implant placement.[184] However, longer effective release periods might be desirable in order to prevent late bacterial colonization. In the presence of periodontal disease for example, the critical exposure period of the periodontal pocket to the antibacterial drug has been reported to be between 7 and 10 days.[187-188] Therefore, the needed exposure time certainly depends on the preconditions found at the implantation site and on the time period needed for peri-implant wound healing. If only an initial burst release of the antibiotic takes place, the concentrations might rapidly fall below the effective concentration and become subinhibitory. Such subinhibitory concentrations further increase the risk of selecting resistant bacteria strains,[189] and have for some antibiotics even been demonstrated to have the contrary effect of promoting bacterial biofilm formation.[190-193]

The results in **paper I** revealed typical signs for such a burst release of doxycycline from the functionalized surfaces. After the immersion of the modified surfaces, and thus the removal of the initially released compounds, the antibacterial activity of the coated titanium surfaces showed a clear reduction. Even though a certain effect on the bacteria was still seen after the immersion, bioluminescence monitoring revealed increased bacterial growth compared to the surfaces before immersion. This suggests that after the initial burst release, the concentrations of doxycycline were lower than the inhibitory concentration and therefore allowed bacteria to survive and grow. These findings are in agreement with those by Xing et al., who reported a release from doxycycline coated abutment surfaces only up to 6 h.[96] Thus, there may be a risk to promote and spread bacterial resistance, since resistance to tetracyclines has already been observed.[185, 194]

The functionalization process used for incorporating antibiotics onto implant surfaces plays an important role in defining the release kinetics of the antibiotic. In general, there are three methods to attach biomolecules to the implant surface: physical adsorption, physical entrapment, and covalent immobilization.[195] Physical adsorption has the disadvantage of involving relatively weak interactions between the substrate and the biomolecule, therefore often resulting in a burst release within a relatively short initial

time period, and no sustained release over longer periods. As an example, vancomycin adsorbed to calcium phosphate coatings only showed effective release within the first 1-24 h.[196] Physical entrapment can for instance be achieved by coating the implant surface with biodegradable or non-biodegradable drug-loaded polymers.[197-201] However, such methods may be accompanied by limited chemical stability, responses of the host immune system to the composition of the coatings, or unfavorable release kinetics.[202] A further physical entrapment technique is the incorporation of antibiotics in nanotubes grown by means of an anodization process.[202-203] However, rapid release could also be observed for such systems, for instance within 50-150 min in the study by Popat et al.[202] In order to obtain a more stable and controllable attachment of the biomolecule, which exhibits antibacterial activity for the longer term, the covalent immobilization of antibiotics on titanium-based surfaces has also been explored and has to a large extent focused on the attachment of the antibiotic vancomycin.[204-207]

Furthermore, electrochemical processes have attracted attention for the stable immobilization of biomolecules on titanium surfaces. The incorporation of single stranded nucleic acids as an anchor within an anodically grown titanium oxide layer has been reported.[208-209] This anchor strand was then used to covalently attach biomolecules such as RGD-containing peptides or bone morphogenic proteins to the modified titanium oxide surfaces.[208, 210] In the present thesis, cathodic polarization was employed in order to functionalize titanium surfaces with the antibiotic doxycycline. With respect to the results of previous studies,[92-94, 96] it was hypothesized that this method could be applied to establish a stable surface layer of the antibiotic through direct chemical linking of the molecules to a titanium hydride layer created during the polarization process without the use of a linker system, accomplishing a long-term effect on the peri-implant microenvironment. Based on the results of **paper I**, however, it can be concluded that no hydride layer was created with the used process parameters in the polarization setup. The process induced uncontrollable changes to the oxide layer on the surfaces, and doxycycline was found to be adhered to this oxide layer probably via physical interactions. This finding is consistent with the observed burst release and short-term antibacterial effect of the doxycycline coating in the conducted *in vitro* bacterial study.

With respect to the criteria defined in the design of research (see **Figure 3**), it can be stated that even though the doxycycline coatings have been shown not to be cytotoxic and to have positive effects on bone formation,[94] only a limited initial antibacterial effect could be seen. In addition, the low controllability of the process outcomes made clear that cathodic polarization is not an appropriate functionalization method to ensure a long-term influence of doxycycline coatings on the tissue surrounding a dental implant.

For this reason, cathodic polarization with doxycycline was discarded as a functionalization method in this work and a different path was pursued. Inspired by the recent developments in the field of nature-inspired coating systems, the second part of the present thesis focused on exploring the potential application of auto-oxidative polyphenol coatings for the functionalization of dental implant surfaces. Polyphenols represent an interesting group of compounds that have attracted much attention due to their association

with a variety of beneficial effects on human health.[211-217] For example, the polyphenol tannic acid has been reported to prevent bacterial surface colonization without inhibiting the growth of planktonic bacteria.[218-221] Such mechanisms, which only interfere with bacterial biofilm formation but do not kill bacteria, would be favorable because they are less prone to the development of bacterial resistance.[220] On the other hand, the ability of tannic acid and the simple phenolic compound pyrogallol to inhibit bacterial growth has also been shown, and the minimum inhibitory concentrations against a wide range of different bacterial strains were determined.[222] Moreover, Sileika et al. reported that coatings produced by auto-oxidative surface polymerization of tannic acid and pyrogallol demonstrated strong contact-based antibacterial properties, while not being toxic to fibroblastic cells.[106] Thus, it was speculated that such coatings could be suitable for dental implant applications.

So far, the effect of auto-oxidative tannic acid and pyrogallol coatings on cells involved in bone formation has not been reported. As one of the goals of this thesis was to create bone promoting implant surfaces, the biological performance of the phenolic coatings was tested using primary human osteoblasts. Similar to the doxycycline coatings, none of the phenolic coatings were toxic to human osteoblasts. The effects the coatings had on osteoblasts seemed to be related to the release of phenolic compounds from the surfaces. For thin pyrogallol coatings deposited for 2 h, no release was detected, and these coatings seemed to promote the maturation of osteoblasts. However, when these coatings were deposited for 24 h, mass release from the surfaces occurred, causing a delay in osteoblast maturation. A similar but not as pronounced delay was seen for tannic acid coatings deposited for 2 and 24 h, both releasing compounds from the surfaces. Moreover, significant downregulation of IL6 expression was observed for the coatings that exhibited release, suggesting a potential effect of such coatings on inflammatory conditions around the implant. This effect should be further assessed, for example by mimicking an inflammatory situation *in vitro* as it has been done for quercitrin-coated surfaces.[223]

While none of the phenolic coatings in this work could prevent bacterial surface colonization and biofilm formation of *S. epidermidis*, tannic acid and pyrogallol coatings deposited for 24 h had a significant effect on planktonic bacteria. Since this effect was also clearly related to the release of phenolic compounds from the surfaces, the same question as discussed for the doxycycline coated surfaces arises regarding the concentration of the released compounds. The fact that significant effects on planktonic bacteria was only observed for tannic acid coatings deposited for 24 h but not for 2 h coatings, both of which showed release, indicates that the effect is dependent on the amount and thus the concentration of the released compounds. The finding that the coatings could not inhibit biofilm formation, unlike previously reported for tannic acid in suspension,[220-221] could therefore be a sign that the concentrations of the released compounds were not appropriate to cause an effect on biofilm formation. Since there was no controlled release, there is also the potential risk of inducing bacterial resistance, similar to the release of doxycycline. However, unlike for doxycycline, where the interaction of the molecules with bacteria is known, the mechanisms behind the effect of

the released phenolic compounds in not clear. Knowing the underlying mechanisms of bacterial interaction with the compounds is crucial in order to optimize the system and to evaluate the risk of resistance.

This leads us to one of the main limitations concerning the interpretation of the *in vitro* results obtained in **paper III**. As demonstrated in **paper II**, a variety of interactions of both physical and chemical nature were suggested to be involved in the auto-oxidative formation of tannic acid and pyrogallol coatings. The exact composition of the released compounds is unknown, and is likely to comprise a mix of differently sized and reacted polyphenol aggregates. Therefore, it is difficult to relate the detected biological effects of the released compounds to the effects of the unreacted precursor molecules reported in literature, especially because the structure of polyphenols has been described to influence the biological activity.[222, 224] However, the observed effect is likely to be connected to the presence of pyrogallol groups for both the tannic acid and the pyrogallol system, as this group has been related to high antibacterial activity.[222] Further investigations on the nature of the released compounds are necessary in order to gain insights into the structure-activity relationship of the presented coatings.

Since the inherent properties of the assessed phenolic coatings could not evoke as clear effects as desired for a functionalized dental implant surface, further modifications of the coating systems are required. The results obtained in **paper II** of this thesis provide a basis for understanding the mechanisms behind the coating deposition of auto-oxidative tannic acid and pyrogallol coatings. In this respect, the coatings could be used as a linker system to attach further biomolecules to the titanium surfaces. This could either be achieved in a two-step process, in which the polyphenol coatings are deposited first and subsequently functionalized with other molecules, or in an integrated process, in which additional molecules become included in the coating during the coating process. The fact that the coatings can be produced close to physiological pH and at room temperature is advantageous when dealing with sensitive molecules. Moreover, as hypothesized in **paper II**, metal ions could play a role in the coating formation. The formation of polyphenol-metal networks has previously been investigated for creating diverse building blocks,[225-227] and such interactions between phenolic compounds and metal ions may be useful for tuning the stability and release kinetics of the coatings. The implemented QCM-D setup in **paper II** provides a powerful tool for future studies on the formation, stability, or release of such coating systems.

The polyphenol coatings used in the present thesis feature one particularly interesting characteristic: similar to polydopamine coatings, they can be used as a universal coating system and be deposited on virtually any kind of material[99, 106-107] without the need for conductivity as in the case of cathodic polarization. This material-independence opens a wide range of new possibilities for future applications. Despite the success of titanium as a dental implant material, the use of ceramic materials is becoming more popular.[228-229] The reason for this is mainly the better esthetic outcome compared to titanium implants, as ceramic materials are white and thus the grey shimmer of metallic implants can be avoided when mucosa and bone around the implant are thin.[229] Furthermore, concerns

regarding the release of metal ions in the surrounding tissue have been raised.[230-231] Optimized polyphenol coatings could therefore also be applied to functionalize such ceramic surfaces. Moreover, the functionalization method is not limited to dental implants, and could for example also be transferred to other biomedical applications where antibacterial properties are needed, such as orthopedic implants (for instance hip or knee implants), or for catheter applications.

Furthermore, while implants with a rough surfaces enhance early osseointegration,[232] rough surfaces are also associated with the promotion of bacterial adhesion.[233-234] In addition, the treatment of severe peri-implantitis has been reported to achieve higher success rates when the implant surface was only turned and not modified.[235] The phenolic coating systems could thus also help to improve the early performance of smooth implant surfaces. A particularly interesting application could be the functionalization of smooth dental abutment surfaces to enhance the soft tissue seal around the implant, which could help in preventing bacterial invasion.

6 Conclusion and future perspectives

Two different surface modification techniques were tested in this thesis with regard to their applicability to functionalize titanium dental implant surfaces for enhanced bone formation and reduced infection risk.

Cathodic polarization was found to induce changes to the oxide layer of the titanium surfaces which appeared to be rather random and uncontrollable. This lack of process control was reflected in the uncontrolled release behavior of doxycycline from the surfaces. Different process parameters such as longer polarization times or higher current densities may be necessary to create the previously reported titanium hydride layer, but would most likely conflict with the stability of the antibiotic. A thorough electrochemical investigation would be required to understand the exact interactions of the doxycycline molecules with the polarized surfaces.

In contrast, auto-oxidative surface polymerization of tannic acid and pyrogallol appeared to be the more promising route for functionalizing surfaces. The coatings revealed to be readily applicable and first insights in the mechanisms behind the coating formation were gained. The assessed properties of such coatings suggested a high potential for biomedical applications, although further studies are required to completely understand the relationship between coating structure and biological activity. Similar to doxycycline coatings, the biologic effect of the phenolic coatings was largely dependent on the release of compounds from the surfaces, which occurred in an uncontrolled way. Therefore, future studies should focus on unraveling the nature of the phenolic compounds released from the surfaces and on obtaining control over the release kinetics. With this knowledge, the coating systems could be optimized and further functionalized to enhance the biological performance.

7 References

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