Novel antibacterial strategies to combat biomaterial-associated infection
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General discussion
CHAPTER 10

PATHOGENESIS OF BIOMATERIAL-ASSOCIATED INFECTION

Over the past decades, the use of medical devices such as catheters, artificial heart valves, prosthetic joins and other implants, has grown significantly\(^1\). Despite the continuous improvements in device design, surgical procedures and wound care, biomaterial-associated infection (BAI) is still a major problem in modern medicine. It has been recognized for over half a decade that the presence of a biomaterial in host tissue predisposes for infection\(^2-4\). These infections are most frequently caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*\(^5-7\). BAI generally are difficult to treat, and treatment failure may even lead to removal of the device.

This thesis focuses on the development and characterization of novel antimicrobial agents and delivery systems and tested their effectiveness in the prevention of BAI and other difficult-to-treat biofilm infections, based on a more detailed understanding of the pathogenesis of these infections.

BIOFILM FORMATION

Biofilm formation, most often initiated when bacterial cells attach to the surface of implants during surgery, is considered the major element in the pathogenesis of BAI\(^8-10\). Biofilm-forming bacterial strains are able to cover the surface of the biomaterial, which results in complex communities consisting of bacteria, bacterial products and host proteins\(^10\), highly tolerant to antibiotics\(^11,12\). So, having anti-biofilm activity is an essential trait for novel antimicrobial agents and delivery systems.

The synthetic antimicrobial peptides (AMPs) OP-145 (*chapter 5*), SAAP-145 and SAAP-276 (*chapter 7*), SAAP-148 (*chapter 6*), and TC19 (*chapter 8*), inhibited biofilm formation of a clinical *S. aureus* BAI isolate in a dose-dependent fashion. In the BM2 biofilm medium used for these biofilm prevention assays, the peptides did not display bactericidal activity. This implies that mechanisms other than direct killing, such as inhibition of adherence of the bacteria and inhibition of gene expression for biofilm formation, like shown for LL-37\(^13\), are involved.

The novel synthetic peptides all rapidly permeabilized the membrane of *S. aureus* bacteria (*chapter 6 – 8*), probably implying that the peptides are highly effective against dividing as well as non-dividing biofilm-encased bacteria. This might be an explanation for the reduction of the numbers of *S. aureus* bacteria on the implants owing to the non-bactericidal anti-biofilm activity of OP-145 (*chapter 5*), SAAP-145 and SAAP-276 (*chapter 7*) when injected along subcutaneous implants in mice. Actual *in vivo* biofilm formation at the implant-tissue interface is hard to detect due to technical challenges associated with sectioning of biomaterial-containing histological samples, especially when metals are involved. However,
on titanium subcutaneous implants challenged with *S. epidermidis* we hardly observed any biofilm, but rather colonization of the tissue surrounding the implants (chapter 2).

SAAP-148 also eradicated established biofilms in a dose-dependent way (chapter 6). A subpopulation of these biofilm bacteria might reach a dormant and highly drug-tolerant state, the so-called persisters, which are thought to be responsible for the recurrence of biofilm-related infections14. SAAP-148 even killed persister cells in vitro, making this peptide a promising candidate for further development towards clinical application.

**TISSUE COLONIZATION**

Next to biofilm formation, another important element in the pathogenesis of BAI is bacterial colonization of the tissue around implants, due to dysregulation of the local immune response by the combined presence of bacteria and a foreign body15–18. In chapter 2 we used the mouse subcutaneous BAI model to study the possible routes of infection at the interface between implants and the surrounding tissue. We showed that *S. epidermidis* bacteria applied on the surface of titanium implants, both adhering and as a biofilm, relocate from the material to the surrounding tissue. At 4 days after implantation, the bacteria were present in the tissue surrounding the implants pre-seeded with *S. epidermidis* or carrying a pre-grown *S. epidermidis* biofilm. Similarly, large numbers of *S. aureus* were cultured from the tissues around the (non-coated) titanium implants in chapters 4 and 7, and around silicon elastomer implants in chapter 5, which is accordance with earlier studies18,19. This suggests that it is a more general phenomenon occurring around implants manufactured from biomaterials as diverse as polymer and titanium, and with different bacterial species. In a study by Broekhuizen et al., mice were treated with dexamethasone and BrdU, a nucleotide analogue that is incorporated into bacterial DNA and can be detected immunohistologically, between 14 and 21 days after challenge with *S. epidermidis*20. This study showed regrowth of the bacteria with BrdU incorporated, which had apparently replicated between day 14 and 21, suggesting that tissue rather than the implant provides a hiding place for the bacteria. Moreover, after incubation of peri-catheter tissue biopsies of deceased intensive care unit patients with BrdU, bacteria had incorporated BrdU in situ, proving that bacteria also reside and synthesize nucleic acids within tissue surrounding biomaterials in humans20.

Bacteria colonizing the surface of a biomaterial not only are a focus of a localized biofilm infection, but can also be the source of tissue colonization. Conversely, bacteria residing in the tissue can be a cause of infection after re-implantation, in experimental infection21 as well as in patients. Tissue-residing bacteria can be hard to eradicate by
antibiotic treatment\textsuperscript{22,23}. For instance, when infected prosthetic joints are removed, patients usually require a prolonged regimen of systemic and local antibiotic treatment in order to reach and kill bacteria present in the tissue prior to re-implantation\textsuperscript{5,24}. Another mechanism of tissue colonization is by introduction of bacteria during surgery. In conclusion, next to the prevention of bacterial colonization of the implant and the subsequent biofilm formation, prevention of bacterial colonization of peri-implant tissue is of vital importance.

Injection of the AMPs OP-145 (\textit{chapter 5}), SAAP-145 and SAAP-276 (\textit{chapter 7}), all inspired by the human cathelicidin LL-37, along subcutaneous implants did not reduce the numbers of \textit{S. aureus} in the surrounding tissue. This might be because the AMPs did not effectively penetrate the tissue, or were not taken up by the host cells and thereby not capable of killing internalized bacteria. When these AMPs were released from Polymer-Lipid Encapsulation Matrix (PLEX) coatings, the numbers of viable \textit{S. aureus} bacteria were reduced in the peri-implant soft tissue in mice (SAAP-145 and SAAP-276) and even in bone in a rabbit humerus intramedullary nail infection model (OP-145). This clearly illustrates the benefit of the PLEX coating technology allowing controlled and prolonged release of the AMPs at the implant-tissue-interface. In \textit{chapter 7}, the SAAP-276-PLEX-coated implants were able to reduce the number of doxycycline-resistant \textit{S. aureus} in the peri-implant tissue, in contrast to the doxycycline-PLEX coated implants which failed to reduce their numbers. This underlines the potency of AMP-PLEX coatings in the fight against BAI caused by multidrug-resistant (MDR) staphylococci. Unexpectedly, the doxycycline-releasing PLEX-coating was still able to reduce the implant colonization by the doxycycline-resistant MDR \textit{S. aureus} significantly, although SAAP-145-PLEX-coated implants were more effective. A possible explanation is the high local concentration of doxycycline released from the PLEX coating at the implant-tissue-interface, exceeding the elevated minimal inhibitory concentration (MIC) of doxycycline for the resistant strain. The observation that release of doxycycline offers partial protection against a doxycycline-resistant \textit{S. aureus} strain was also reported in rabbits\textsuperscript{25}.

\section*{INTRACELLULAR SURVIVAL}

In \textit{chapter 2}, we describe that \textit{S. epidermidis} predominantly co-localized with macrophages in the peri-implant tissue of mice, even when the bacteria were present exclusively on the implant surface at the start of the experiment. This interesting observation suggests that the bacteria were either removed from the implant by phagocytosis, or were first detached and subsequently phagocytosed. Both \textit{S. epidermidis} (\textit{chapter 2}) and \textit{S. aureus} (\textit{chapter 4}) were cultured in high numbers from the tissue and co-localized with macrophages in
histology, particularly at 4 days after challenge, suggesting that these macrophages were not effectively killing the bacteria. Most likely, the local host immune response is impaired in presence of an implant, resulting in less or no clearance of bacteria. For instance, neutrophils can have reduced phagocytic and bactericidal capacity in the vicinity of an implant\textsuperscript{26,27}. Moreover, the intracellular killing capacity of macrophages can be reduced due to altered cytokine tissue levels in the presence of a biomaterial\textsuperscript{18,20,27–29}. Bacteria may even form small colony variants, which are more resistant to antimicrobial compounds\textsuperscript{30,31} to adapt to this micro-environment. Chronic osteomyelitis caused by \textit{S. aureus} is challenging to treat and often considered incurable. Next to intracellular infection of osteoblasts and osteocytes\textsuperscript{32}, \textit{S. aureus} residing within canaliculi of live cortical bone is considered a mechanism of chronic infection\textsuperscript{33}. Apparently, when bacteria are initially present near or on the surface of implants this results in ineffective eradication by phagocytes. This might lead to persistence of intercellular bacteria in the peri-implant tissue.

Although the AMPs studied in this thesis reduced the colonization of the peri-implant tissue \textit{in vivo} when released from the PLEX coating (\textit{chapters 5 and 7}), they might still not be able to act on intracellular bacteria. Apparently the rapid initial release of the AMPs killed the vast majority of the infecting bacteria, preventing biofilm formation on the implant surface as well as colonization of the tissue, thereby protecting both these sites against colonization. Treatment of infections featuring intracellular bacteria remains difficult, as observed with the conventional antibiotic vancomycin\textsuperscript{22}, and likely with the novel AMPs as well. A possible way to improve the intracellular entry of AMPs is by adding a specific domain (‘tag’) to the peptides as a signal for uptake by the host cells\textsuperscript{34,35}. However, this is less of a problem when AMPs are used in BAI prevention, as shown with the SAAP-PLEX coatings. By directly killing the bacteria on the implant-tissue interface during surgery, their entry into the tissue will be prevented. In an abraded skin infection model, however, we showed that even in an established infection with biofilms formed on the skin and bacteria possibly residing intracellularly, SAAP-148 was still able to clear the infection effectively. Topical application of a hypromellose gel containing SAAP-148 was highly effective in eradicating MDR \textit{S. aureus} and \textit{Acinetobacter baumannii} in acute and established (24 – 48 h) abraded \textit{ex vivo} human skin and \textit{in vivo} murine skin infection models (\textit{chapter 6}).

Actual intracellular localization and viability/survival of the bacteria is difficult to prove. In general, it is hard to tell the difference between live and dead bacteria in histology, as they can be in a dormant state and therefore not be culturable. A promising technique to allow visualization and localization of individual microbial cells in tissue is fluorescence \textit{in situ}
hybridization (FISH)\textsuperscript{36}. Using FISH, bacteria in peri-implant tissue were observed, as a discrete biofilm at 2 days after injection of \textit{S. epidermidis} along subcutaneous titanium implants in mice (collaboration with A. Moter, Biofilmcenter, German Heart Center, Berlin, Germany). Moreover, strong fluorescence after applying the ribosomal RNA-probe is an indication of RNA synthesis of the bacteria. Using specific probes for the intergenic spacer region (ISR) of the rRNA gene operon between the small (16S) and large (23S) subunits, a region which is excised from the RNA transcript after transcription and is rapidly degraded, only highly active bacteria can be stained, thereby proving the presence of bacteria which were alive at the moment of tissue fixation in an even more sensitive way. The other way around, in case no staining is observed in FISH, this is still no proof that bacteria are dead, as it cannot be excluded that bacteria may be in a dormant state and/or the rRNA content simply below the detection level.

\textbf{ALTERNATIVES TO ANTIBIOTICS}

Effective treatment options for infections caused by pathogenic bacteria resistant to multiple antimicrobial agents are becoming limited. The bacteria belonging to the co-called ESKAPE panel (\textit{Enterococcus faecium}, \textit{Staphylococcus aureus}, \textit{Klebsiella pneumoniae}, \textit{A. baumannii}, \textit{Pseudomonas aeruginosa} and \textit{Enterobacter} species) are increasingly prevalent and resistant and thereby a particularly dangerous group of bacteria\textsuperscript{37}. Currently, the majority of hospital infections in the United States is caused by MDR ESKAPE pathogens\textsuperscript{38}. Infections caused by the Gram-negative members of this panel are of even greater concern, because these bacteria are intrinsically resistant to many antibiotics\textsuperscript{39}. In view of this emerging antimicrobial resistance among bacteria, we selected the non-antibiotic antimicrobial agents chlorhexidine (\textit{chapter 4}), synthetic AMPs (\textit{chapter 5 – 8}) and silver (\textit{chapter 9}) as alternatives for antibiotics in our studies.

\textbf{CHLORHEXIDINE}

In \textit{chapter 4} we incorporated chlorhexidine, a frequently used antiseptic agent with broad spectrum activity\textsuperscript{40}, in a controlled release coating for titanium implants. Chlorhexidine is frequently used in hospital settings, for instance for skin disinfection, sterilization of surgical instruments and wound cleaning. This widespread use of chlorhexidine has led to some concerns regarding the emergence of bacterial resistance\textsuperscript{41–43}. However, the reported levels of susceptibility are still considerably lower than the clinically applied dosages\textsuperscript{42}. Therefore, we consider chlorhexidine as potential alternative antimicrobial agent in the fight against BAI caused by MDR pathogens.
ANTIMICROBIAL PEPTIDES
RESISTANCE DEVELOPMENT

In the fight against MDR bacteria, there is a pressing need for novel antimicrobial agents. Therefore, we developed novel synthetic antimicrobial and anti-biofilm peptides (SAAPs). Resistance development against AMPs is unlikely and not common, probably because the general mechanism of action of AMPs does not involve specific target molecules in the bacterial cell. Nonetheless, resistance to AMPs in bacteria can occur and several mechanisms of resistance have been described, including membrane and cell envelope structure alterations increasing positive charge, upregulation of efflux pumps and proteolytic degradation of the peptides\textsuperscript{44,45}. For instance, resistance against LL-37 has been reported to be caused by degradation of the peptide by proteolytic enzymes, up-regulation of efflux pumps as well as bacteria-induced down regulation of LL-37 expression in host cells\textsuperscript{46}. In our studies we therefore investigated possible resistance development of bacteria against our novel peptides, using conventional antibiotics as comparators. In chapter 6 we showed that the LL-37-inspired SAAP-148 is highly effective against a broad range of MDR bacteria, without development resistance against S. aureus and A. baumannii. Furthermore, no resistance development of S. aureus was detected for SAAP-145 and SAAP-276 (chapter 7) and TC19 (chapter 8), peptides highly active against MDR bacteria. In all studies, rifampicin readily induced resistance, as expected for this antibiotic\textsuperscript{47}, once again underlining the importance of combining rifampicin with other antibiotics\textsuperscript{5}. Although no resistance was observed against the novel SAAPs in any of the studies, the general consensus is that, sooner or later, resistance will eventually be developed against any novel antimicrobial agent. Moreover, only a selective panel of bacterial strains has been tested in these studies, mainly focused on the bacterial species encountered with the type of application described.

The urgent need for novel antimicrobials is further underlined by the emergence of Gram-negative bacteria resistant to colistin, which is considered a last-resort antibiotic. SAAP-148 proved to be highly effective against colistin-resistant isolates of Enterobacter cloacae and Escherichia coli (chapter 6).

The pandrug-resistant (PDR) P. aeruginosa was not susceptible to TC19 in the presence of human plasma, whereas it was killed at low micromolar concentrations in absence of plasma (chapter 8). It is known that a plasma-inducible resistance mechanism can make P. aeruginosa strains highly resistant to AMPs. Upon incubation with plasma, N-arabinose is added to the lipopolysaccharide (LPS), making the outer surface more positively charged and thereby repelling cationic AMPs\textsuperscript{44}. However, SAAP-148 did show activity in plasma at low concentrations against the PDR (data not shown) and several extensively drug-resistant (chapter 6) isolates of P. aeruginosa. Thus, the plasma-inducible resistance of P. aeruginosa does not repel all AMPs.
SELECTION

In chapter 5 we reported that OP-145 is highly effective in killing S. aureus in the presence of physiological concentrations of salt and divalent cations, however not in human plasma, possibly due to binding of the peptide to plasma components. We defined stability of the novel antimicrobial activity in situ, like in human plasma, wound fluid or urine, as an important characteristic of a novel antimicrobial agent. Therefore, we selected the novel peptides based on their improved antimicrobial and anti-biofilm activities under physiological conditions (chapters 3, 6 and 8).

One strategy to improve the plasma stability of peptides is the incorporation of D-amino acids, or other unnatural amino acids. In chapter 3 we synthesised a series of modified HHC10 peptides. All HHC10-mimics, including the inverso-HH10 (i.e. different stereo isomer), showed microbicidal activities at low micromolar concentrations against E. coli, S. aureus and S. epidermidis. To be able to immobilize the peptides in a hydrogel, the sequence was elongated with a N-terminal cysteine. The resulting peptide inverso-CysHHC10 showed a slight decrease in activity, but was not significantly degraded when incubated in human serum for 24 h, in contrast to HHC10, which was fully degraded within 4 h.

Interestingly, SAAP-148 was more efficient in killing bacteria under physiological conditions than LL-37 and other (pre-)clinical phase antimicrobial peptides (chapter 6). On the other hand, peptide OP-145, despite its lack of in vitro antimicrobial activity in the presence of plasma, proved to be effective in preventing S. aureus colonization of subcutaneous implants in mice (chapter 5). Thus, in vitro activities under defined conditions do not necessarily predict in vivo potency. It would be of high interest to compare the in vitro results of the (pre-)clinical phase peptides with their final outcome in their clinical application. This will hopefully provide more insight in the predictive value of in vitro assays for the ultimate clinical application.

SILVER

Silver is used in numerous medical applications48–50, has broad-spectrum antimicrobial activity51,52 and is generally regarded as safe (GRAS)53. No toxic side-effects of the use of silver-coated orthopedic implants have been reported54. However, when silver nanoparticles (AgNPs) are used in free form, they may be transported and accumulated, possibly affecting cellular processes52. Therefore, in chapter 9 we stably embedded AgNPs in the titanium oxide layer on additively manufactured titanium implants, only releasing silver ions. Resistance to silver is rare55, and only a few resistant isolates from clinical cases have been reported, including isolates of P. aeruginosa, E. coli, E. cloacae, K. pneumoniae, Proteus mirabilis, and Citrobacter freundii56. As
for AMPs, the mechanism of action of silver ions is associated with multiple targets\textsuperscript{57,58}, making resistance development less likely to occur. Since resistance to silver has only been reported for Gram-negative bacterial strains, we did not test for resistance development of the Gram-positive methicillin-resistant \textit{S. aureus} (MRSA) used in this study. The additively manufactured porous implants showed a local burst release of silver ions within the first 24 h and a constant lower release afterwards. This is a similar profile as we had developed for the chlorhexidine- (chapter 4) and AMP-release coatings (chapters 5 and 7), mainly focusing on prevention of BAI.

**ANTIMICROBIAL STRATEGIES**

As explained above, in addition to biofilm formation on the implant, colonization of peri-implant tissue is an important factor in the pathogenesis of BAI. Therefore, this niche needs to be taken into consideration when designing preventive strategies against BAI. Current strategies mainly focus on the development of three types of antimicrobial surfaces: (i) antifouling surfaces, (ii) contact-killing surfaces, and (iii) surfaces which incorporate and release antimicrobials\textsuperscript{59}. As antifouling will not kill bacteria, these are not included in the scope of this thesis.

**CONTACT KILLING SURFACES**

Contact-killing surfaces will only eradicate bacteria that are in direct contact with the active surface, meaning that clearance of any bacteria further away from the surface will depend on efficient phagocytosis. However, as mentioned before, due to the presence of a biomaterial the local host immune response is dysregulated, and therefore phagocytosed bacteria may not be killed and may even persist intracellularly\textsuperscript{18,28}. An example of a contact-killing surface is the hydrogel network with the covalently attached stabilized inverso-CysHHC\textsuperscript{10} peptide (chapter 3). This coating demonstrated high \textit{in vitro} antimicrobial activity against \textit{S. aureus}, \textit{S. epidermidis} and \textit{E. coli}. Although this coating shows highly promising \textit{in vitro} activities, its capacity to prevent BAI \textit{in vivo}, especially in preventing peri-implant colonization, should be determined.

**RELEASE SYSTEMS**

The peri-implant tissue is considered as an important niche for bacteria. Therefore, antimicrobial-releasing surfaces or coatings from which the antimicrobial agent also reaches this niche are preferred to prevent BAI. The main drawback of antimicrobial-eluting coatings is that at a certain point in time they will release sub-inhibitory concentrations of the antimicrobial agent (e.g. antibiotic) that promote the selection of resistant strains. Antibiotic-releasing coatings are widely used for different medical applications, such as central venous
catheters and urinary tract catheters. However, coatings releasing antibiotics for orthopaedic devices remain mainly experimental\textsuperscript{60–64}. The first commercially available gentamicin-releasing intramedullary tibia nail has recently shown promising results in the first prospective study\textsuperscript{65}. Obviously, coatings releasing antimicrobial agents that are less likely to induce resistance, such as chlorhexidine, AMPs or silver ions, would be preferred in view of reducing rise of resistance development, and compatibility with use of antibiotics for prophylaxis or treatment. To prevent the spread of bacteria from the implant surface to the surrounding tissue, and to eradicate bacteria contaminating tissue during surgery, a rapid initial release of antimicrobials is required. If this release is delayed, bacteria may ‘escape’ into host cells before effective levels of the antimicrobial agent have been established. Subsequently, prolonged local release of the antimicrobial agent at sufficiently high concentrations will be required to eradicate any residual bacteria\textsuperscript{66,67}. Such a release profile has been established for the antimicrobial agents released from the coatings described in this thesis (chapter 4, 5 and 7).

**CHLORHEXIDINE**

The epoxy-based coatings containing 10 wt\% chlorhexidine displayed an initial burst release within the first 24 h and the residual release occurring within 4 days (chapter 4). This coating proved to be highly efficacious in preventing \textit{S. aureus} infection in the mouse subcutaneous implant infection model. At 1 day after challenge only a relatively low level of neutrophil influx in the tissue surrounding the implants releasing chlorhexidine was observed, likely due to the efficacy of the coating to kill \textit{S. aureus} rapidly, and thereby reducing neutrophil attraction. This supports the notion that an initial high level release, ensuring rapid killing, is a beneficial property of a release coating.

**ANTIMICROBIAL PEPTIDES**

The PLEX-OP-145 coating developed in chapter 5 is characterized by a high initial release rate of OP-145, followed by a continuous slow release, and provided effective \textit{in vitro} antimicrobial activity against \textit{S. aureus}. The effectiveness of the coating was tested in a rabbit intramedullary nail infection model. At 28 days after implantation and infection, the humeri of rabbits having received the PLEX-OP-145-coated implants were more frequently culture-negative than those of rabbits having received an uncoated implant. Although the numbers of \textit{S. aureus} bacteria cultured from the implant, bone and soft-tissue were considerably lower in rabbits with the PLEX-OP-145-coated implant than from rabbits with uncoated implants, this difference was not significant. One reason for this might be the low number of animals per experimental group, but another explanation could be that the peptides are less
active in a complex environment, as described above. Therefore, one might expect the novel LL-37-inspired SAAP-145 and SAAP-276 released from a PLEX coating (see chapter 7) to even perform better in the rabbit model. Although in several of the rabbits having received the OP-145-PLEX-coated implants the numbers of bacteria retrieved were still relatively high, these rabbits showed much better values for the clinical parameters, such as weight and level of white blood cells, than the rabbits having received a non-coated implant. As these parameters are highly relevant for the clinical outcome of the rabbits, the coating seems to be very effective.

Based on their high effectivity against (MDR) \textit{S. aureus} clinical strains and their anti-biofilm activity, we selected the novel SAAP-145 and SAAP-276 for incorporation in the PLEX coating (chapter 7). These PLEX coatings were modelled according to the OP-145-PLEX coating, and showed a similar release profile \textit{in vitro}, namely an initial burst release followed by a constant low level of peptide release. At 1 day post infection, the SAAP-145- and SAAP-276-PLEX-coated implants were effective against (MDR) \textit{S. aureus} on the implants and in the peri-implant tissue. Efficacy at later time points remains to be established in such models as the rabbit intramedullary nail infection model mentioned above.

\section*{3D-PRINTED IMPLANTS WITH IMMOBILIZED SILVER NANOPARTICLES}

3D-printed of medical devices is a major breakthrough, especially in the field of orthopedics, in part owing to the possibilities to produce these implants with high porosity and thereby increasing the surface area. These aspects make this technique attractive for personalized solutions in clinical application. However, as with conventional implants, the additively 3D-printed are susceptible to infection. In chapter 9 we produced porous implant using selective laser melting. These implants were biofunctionalized through plasma electrolytic oxidation treatment in the presence of AgNPs, resulting in an oxide layer with AgNPs incorporated. These implants released silver ions over time, showing \textit{in vitro} bactericidal activity against MRSA, including prevention of biofilm formation. The porous implants had a more than 3-fold increase in surface area, when compared to solid implants. The benefits of an increased surface area might be the increased release of active molecules, more than 4-fold higher release of silver ions in this case, and providing pore space for bone regeneration and osseointegration. However, these pores could reduce the implant stability and might be a perfect hiding place for bacteria to avoid phagocytosis by host immune cells. Again, an initial high release of antimicrobial molecules is key to prevent BAI at later time points. In accordance, the implants we developed released high doses of silver ions within the first day, followed by much lower doses for at least one month. The silver ions-
releasing porous implants prevented bacterial survival in an ex vivo mouse femur implant infection model. In the ex vivo experiments however, an active immune system that likely is instrumental in even further reducing the number of bacteria is lacking. Therefore, the implants should be further tested in in vivo implant infection models. The use of an ex vivo model to determine and optimize the antimicrobial properties of the novel implants does allow the final number of animals needed for in vivo studies to be strongly reduced.

CONCLUSIONS AND FUTURE PERSPECTIVE
Prevention of BAI is a challenging problem, in particular due to the increased risk of resistance development associated with current antibiotic-based strategies. In this thesis we showed the evidence of biofilms as a source for peri-implant tissue colonization, clearly showing the importance of preventive measures to be able to act against both implant and tissue colonization. Subsequently, we described different strategies to prevent BAI and other difficult-to-treat biofilm infections. The hydrogel coating with covalently attached stabilized AMPs shows high in vitro activity, but it might very well be that these contact-killing coatings will not be sufficient for final application as a single measure to keep both the implant and its surrounding tissue infection free. Therefore, future research should also focus on the development of combination devices with both anti-fouling or contact-killing capacities – to protect the implant – and controlled release of an antimicrobial agent to protect the surrounding tissue. The release of silver ions from 3D-printed implants is a promising technique to prevent bacteria to colonize either of these niches. Furthermore, the porous structure even creates possibilities for the addition of antimicrobial agents, like antimicrobial peptides. In this view, a coating releasing one of the LL-37-inspired synthetic peptides will have great benefits if applied in a clinical setting. Since all compounds of the PLEX coating have already been approved for human use, the translation to man may be relatively rapid.

In general, all synthetic AMPs investigated in this thesis have great potential for clinical application. OP-145 is able to prevent S. aureus osteomyelitis in rabbits, even though it has reduced in vitro activity in plasma. The newly developed synthetic peptides, i.e. SAAP-145, SAAP-148, SAAP-276 and TC19, proved to have broad spectrum activity including activity against highly dangerous multi-drug resistant pathogens, to prevent biofilm formation, and to have in vivo activity, either in preventing biomaterial-associated or wounded skin infections. From this set, SAAP-148 is probably the most promising candidate for further development into a clinical application to treat e.g. skin wounds infected with highly antimicrobial resistant bacteria, as well as possible applications to prevent BAI.
REFERENCES


