Novel antibacterial strategies to combat biomaterial-associated infection
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General introduction

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CHAPTER 1

BIOMATERIAL-ASSOCIATED INFECTION

CLINICAL PROBLEM

The use of medical devices, such as catheters, artificial heart valves, prosthetic joints and other implants, has increased dramatically over the past century, and has become a major part of modern medicine and our daily life. With the aging society, the higher demand on medical devices to restore function and quality of life, combined with the ever improving technology within the medical field, the problem of medical biomaterial-associated infection (BAI) is expected to increase.

Transcutaneous devices, such as catheters, and orthopedic devices, such as prosthetic joints, are among the most frequently used devices in human medicine\textsuperscript{1,2}. In practice, catheters suspected for infection are removed and a new catheter is placed at a different location, as using the original location for re-implantation over a guide-wire is strongly discouraged because of the high reinfection risk\textsuperscript{3}. The implantation of prosthetic joints like prosthetic hips, knees, elbows and ankles, is considered a so-called clean procedure\textsuperscript{4}, however, in 0.5-1\% (hip or knee) to over 5\% (elbow or ankle) of cases, infections occur following the primary implant surgery\textsuperscript{5,6}. In general, revision surgery is associated with higher frequencies of infection, due to longer procedures and more extensive tissue damage.

The most common causative microorganisms in BAI are \textit{Staphylococcus aureus}, a major pathogen in wound infections, and \textit{Staphylococcus epidermidis}, the harmless skin commensal\textsuperscript{6–8}. Depending on the type of device and location of application, other coagulase-negative staphylococci, enterococci, streptococci, \textit{Propionibacterium acnes} and yeast can also cause BAI\textsuperscript{9,10}.

In as early as 1957, Elek and Conen studied the minimum infective dose of staphylococci for man in relation to suture infection\textsuperscript{11}. In healthy volunteers, they estimated the minimum pus-forming dose of \textit{S. aureus} – called \textit{Staphylococcus pyogenes} in those days – on intradermal injections in absence of sutures to be 1 – 8 million bacteria, numbers which are improbable in case of a natural infection. However, the presence of a foreign body, a suture in this case, resulted in a dramatic reduction of the minimal inoculum required for pus production: a dose of 300 bacteria led to abscess formation. Higher inoculum doses even resulted in lesions with ‘the size of an orange’, caused fever and took over a week to resolve, in spite of penicillin therapy. This experiment clearly demonstrated the enhancing effect of the presence of a foreign body, but the authors stated that the outcome of the experiment “led to great difficulty in finding further volunteers”. Nowadays, such an experimental set-up would unlikely approved by medical ethical committees, but it gained
crucial insights in the pathogenesis of BAI. Thus, it has been recognized for at least 60 years that the presence of a foreign body predisposes for infection, and this has repeatedly been confirmed in animal studies\(^{12-15}\). In rabbits, for example, only 50 colony forming units (CFU) of \textit{S. aureus} were sufficient for infection in the presence of a cemented hip implant, whereas 10,000 CFU were required in absence of the foreign body\(^{16}\).

### TISSUE COLONIZATION

The combined presence of a foreign body and bacteria leads to dysregulation of the local immune response, facilitating the establishment of an infection around implants, an important element in the pathogenesis of BAI\(^{17-20}\). Already in the 1980s, Zimmerli \textit{et al.} showed reduced neutrophil phagocytic activity in guinea pig tissue cage models infected with \textit{S. aureus}\(^{21}\). When different challenge doses of \textit{S. epidermidis} were injected along subcutaneously implanted catheter segments at the back of mice, the bacteria were more often found in the peri-implant tissue than on the biomaterial itself, and persisted for longer periods in the tissue than on the implant\(^{22}\). Moreover, \textit{S. epidermidis} survives inside macrophages in tissue surrounding implants in mice\(^{20,22}\).

### BIOFILMS

Bacterial biofilm formation is considered the major element in the pathogenesis of BAI\(^{1,10,23}\). Biofilm formation is initiated when bacterial cells attach to the surfaces of implants. BAI are often caused by biofilm-forming bacterial strains, able to cover the surface of the biomaterial. Bacteria in biofilms behave differently from planktonic bacteria, particularly in response to antibiotic treatment\(^{24}\). The complex bacterial community of a biofilm is highly tolerant to antibiotics\(^{25}\). This might be due to the complicated structure of the extracellular polymeric matrix of the biofilm, making the antibiotic agents less accessible to the bacteria. As antibiotics often target active cell processes, the slow growth or starved state of the bacteria in a biofilm may also make them more tolerant. A subpopulation of these bacteria, the so-called persisters, reaches a dormant and drug-tolerant state. Such persisters are suggested to be largely responsible for the recalcitrance and recurrence of biofilm-associated infections\(^{26}\). Moreover, biofilm-entrapped bacteria are unreachable to the human immune system. Vancomycin, known to penetrate biofilms and effectively reduce the numbers of bacteria\(^{27}\), is often used in combination with rifampicin to treat BAIs. However, this treatment has a relatively high failure rate\(^{28}\), probably due to the low metabolic activity of bacteria within biofilms\(^{29}\). As mentioned above, peri-implant tissue
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is an important niche for bacteria. Vancomycin has low penetration into eukaryotic cells and therefore does not reach and kill bacteria residing intracellularly. Thus, antimicrobial strategies should be effective against both biofilm-encased bacteria and bacteria present in the peri-implant tissue.

ANTIMICROBIAL RESISTANCE

In addition to the difficulty of treating biofilm-encased bacteria with conventional antibiotic therapy, treating BAI is further hindered by the rising antibiotic resistance among pathogens. The World Health Organization recently endorsed a global action plan to tackle antibiotic resistance. One of the key objectives of this plan is to develop novel antimicrobial drugs. The emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) pathogens, accelerated by the selective pressure exerted by extensive use and misuse of antimicrobials, further underscores the very pressing need for the discovery of novel treatment strategies to replace or complement the conventional antibiotics. Magiorakos et al. defined MDR bacteria as non-susceptible to at least one agent in three or more antimicrobial categories, XDR bacteria as non-susceptible to at least one agent in all but two or fewer antimicrobial categories, meaning that bacterial isolates remain susceptible to only one or two categories, and PDR bacteria as non-susceptible to all agents in all antimicrobial categories. The occurrence of XDR and PDR strains illustrates the clinical challenges that we will be facing in the dark scenario of a possible “post-antibiotic era”. Antimicrobial resistance causing limited treatment options in critically ill patients, stresses the importance of the development of new agents that can be used against drug-resistant bacteria. Clearly, it is vital that novel antimicrobial agents are also effective against drug-resistant Gram-negative bacteria belonging to the so-called ESKAPE panel (Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), which cause the majority of US hospital infections and are associated with high morbidity and mortality.

PREVENTIVE STRATEGIES

Antimicrobial biomaterials have been developed to reduce the risk of infection, deriving their antimicrobial properties from different strategies. Surfaces may be modified to prevent bacterial adherence and biofilm formation, may be directly coated with antimicrobials which are released in a burst-like fashion upon implantation, or with release coatings allowing controlled release of incorporated antibiotics according to a profile considered
optimal to prevent infection of the device or its surrounding tissue\textsuperscript{35}. These approaches all have their benefits and limitations, which need to be taken into account when designing an antimicrobial strategy for a particular device\textsuperscript{36}.

**ANTI-ADHESIVE**

Implant surfaces are ideal substrates for opportunistic bacteria to attach to, colonize, and form biofilms on. Surface properties of the implant, like surface charges, hydrophobicity/hydrophilicity and surface chemistry play a major role in initial bacterial adhesion and proliferation. Already in 1987, Gristina suggested that tissue cell integration and bacterial adhesion compete for a spot on the implant’s surface, the so-called ‘race for the surface’\textsuperscript{37}. In case this race is won by the bacteria, this would result in infection instead of tissue integration. Gristina also realized that colonization of the tissue around implants was another possible mechanism of infection\textsuperscript{37}. Bacterial adhesion and subsequent biofilm formation may be prevented by modifying the physicochemical surface properties of biomaterials. One strategy is to use hydrophilic polymer coatings, e.g. immobilized poly(ethylene glycol) (PEG), as applied on contact lenses, shunts, endotracheal tubes and urinary catheters\textsuperscript{35,38}. Another approach is functionalization of the surface with a dense layer of polymer chains commonly known as polymer brush coatings\textsuperscript{39,40}. Large exclusion volumes of tethered polymer chains result in surfaces difficult to approach by proteins or bacteria. The brush coating may even possess antimicrobially active functional groups.

**ANTIBIOTICS**

In general, antibiotics are selected based on their activity against biofilms, but not on their ability to kill bacteria in the niches specific for BAI, like in peri-implant tissue and intracellularly in host cells\textsuperscript{35}. Antibiotics often used in the treatment of BAI, such as vancomycin and gentamicin, have low or no penetration into host cells, and are therefore not active against intracellular bacteria. On the other hand, rifampicin (against staphylococci) or fluoroquinolones (against Gram-negative bacilli) do target these intracellularly localized bacteria, but resistance develops rapidly against these antibiotics. The combination of vancomycin and rifampicin is often used to treat BAI, but – as vancomycin does not reach intracellular bacteria – provides a high risk of resistance development towards rifampicin.

Antibiotic-releasing coatings are widely used for medical devices, like in sutures and central venous and urinary tract catheters. However these coatings have two major disadvantages: (i) a patient might be infected with a bacterium resistant to the released
antibiotic, and (ii) due to the local release a gradient of the antibiotic will be present near the
implant thereby increasing the risk to select for resistant bacteria. In view of the increasing
development of resistance, the use of antibiotics for medical device is discouraged by
government regulatory agencies like the American Food and Drug Administration (FDA)\textsuperscript{36,41}.

ANTISEPTICS
As an alternative to antibiotics, commonly used antiseptics and disinfectants might be
used, as they are less prone to induce resistance and in general have a broader spectrum
of activity than antibiotics. These biocides, such as alcohols, aldehydes and biguanides, are
extensively used in hospitals and other health care settings, and also by the general public,
as an essential part of infection control practices\textsuperscript{42}. Probably the most widely used biocide in
antiseptic products (e.g. hand wash and oral products) is chlorhexidine, owing to its broad
spectrum activity, low toxicity and good tolerability of soft tissue. Moreover, resistance
development is extremely rare and chlorhexidine has been shown to prevent infection in
animal models and in patients. It is used topically, for surgical site preparation, and also
intracorporally\textsuperscript{43}, and as dental irrigant fluid\textsuperscript{44}. Chlorhexidine is currently FDA approved for
coatings on intravenous catheters, and these catheters have been shown to be effective at
decreasing catheter-related infection in human trials\textsuperscript{45}.

SILVER
Silver, in particular free silver ions, exhibits broad-spectrum antimicrobial activity\textsuperscript{46,47}
and has therefore been used for a long time in numerous applications, like catheters,
endotracheal tubes, prosthetic heart valves and wound care products\textsuperscript{48-50}, and is generally
regarded as safe\textsuperscript{36}. Although millions of people have been treated with silver-containing
materials, there are no indications for \textit{in vitro} toxicity towards mammalian cells\textsuperscript{51} or local
or systemic toxicological side-effects in silver-coated orthopedic implants in humans\textsuperscript{52}. One
would expect that, if the incidence of complications due to silver would be high, more cases
would be reported. There are, however, concerns about silver nanoparticles (AgNPs), which
are transported and accumulated, and affect cellular processes differently than ionic forms
of silver\textsuperscript{47}, so caution is required when AgNPs are used in free form.

Despite extensive use of silver in medical and non-medical devices, development of
resistance to silver is rare\textsuperscript{53}. Only a few clinical cases of silver resistance have been reported
in strains of \textit{P. aeruginosa}, \textit{Enterobacter cloacae} and \textit{K. pneumoniae}\textsuperscript{54}. The mechanism of
action of silver is associated with multiple targets, including corruption of DNA replication,
cell wall formation, functional protein precursors and the electron transport chain\textsuperscript{54}, making resistance development less likely to occur.

**ANTIMICROBIAL PEPTIDES**

As discussed earlier, due to the major problems arising from resistance to conventional antibiotics, there is a strong need for antimicrobials not associated with resistance development. Antimicrobial peptides (AMPs) are innate defence molecules of animals, plants and microorganisms, commonly with a broad spectrum of antimicrobial activity and low risk of resistance development\textsuperscript{55,56}. Antimicrobial peptides are mostly amphipathic, cationic peptides that display antimicrobial activity against a wide variety of pathogens, including bacteria, fungi and viruses. In addition, many of them have immunomodulatory and wound healing activities\textsuperscript{57}. The low risk of resistance development is due to the fact that AMPs interact with microbial membranes, mostly resulting in membrane depolarisation, destabilisation and/or disruption leading to rapid cell death, or passing of the membrane to reach intracellular targets\textsuperscript{58}. Naturally occurring human antimicrobial peptides are considered as excellent templates for the development of novel synthetic antimicrobials. Indeed, native AMPs have been used as design templates for a large variety of synthetic AMPs, some of which have now reached the stage of phase 2 and 3 clinical trials\textsuperscript{59,60}.

Several AMPs also have the capacity to prevent biofilm formation. Mansour et al. demonstrated that a synthetic peptide (named 1018) inhibited biofilm formation by \textit{S. aureus} and various other species by blocking (p)ppGpp, an important signal molecule in biofilm development, at concentrations that did not affect bacterial growth\textsuperscript{61}. A peptide derived from CRAMP, the mouse homologue of the human defence peptide LL-37 (cathelicidin), inhibited biofilm formation of the yeast \textit{Candida albicans}, and also prevented biofilm formation by different bacterial species\textsuperscript{62}. Many more examples of AMPs with anti-biofilm activity have recently been listed in the specialised biofilm-active antimicrobial peptides (BaAMPs) database\textsuperscript{63}.

Recently, one series of novel synthetic AMPs were developed based on thrombocidin-1 (TC-1), the major antimicrobial protein of human blood platelets\textsuperscript{64,65}, and another series inspired by LL-37, the principal human AMP produced by mucosal epithelial cells and multiple immune cells. LL-37 is an $\alpha$-helical peptide with a broad range of activities, including antimicrobial activity against Gram-positive and Gram-negative bacteria\textsuperscript{66}. The LL-37-inspired peptide OP-145 (formerly designated as P60.4Ac\textsuperscript{67}) has reached the stage of clinical development, and showed safety and efficacy in treatment of recurrent otitis media patients\textsuperscript{68}. 

\textit{General introduction}
For biomaterials, the predominant AMP-related antimicrobial strategies are coating by tethering peptides to the surface, or to apply the peptides in controlled release coatings. Immobilisation of AMPs on surfaces has been performed with a variety of peptides, and with many different chemistries. An excellent overview of immobilisation strategies has recently been published by Silva et al. For peptides to be effective after immobilisation, they should retain the structural characteristics important for their antimicrobial activity. Other decisive factors for success are length, flexibility, and kind of spacer connecting the peptide to the surface, the AMP surface density and the orientation of the immobilised peptides. Although peptides are considered to be active through insertion into the microbial membranes, even short surface-attached peptides, which are unlikely to have a free interaction with the membrane, have antimicrobial activity. This activity is thought to be due to destabilisation of the membrane by displacement of positively charged counterions, changing bacterial surface electrostatics and activating autolytic enzymes or disrupting the ionic balance.

It should be noted that surface attachment of peptides may have certain disadvantages. Chemical procedures of tethering AMPs to surfaces may cause strong decrease in their antimicrobial activity, or even their inactivation, depending on the combination of peptides and immobilization technology. Apart from this reduction of activity due to the tethering process, proteins, blood platelets and dead bacteria may block the antimicrobial groups on the surface. Moreover, since the antimicrobial activity is restricted to the surface of the implant, there is a lack of antimicrobial impact on bacteria in the tissue surrounding the implant.

Incorporation of AMPs in controlled release coatings has not yet been extensively developed, although a number of AMPs have already reached clinical phase 2 or 3 testing, such as OP-145, IB-367 (Iseganan) and Omiganan. Application of AMPs in antimicrobial surface coatings is, however, a subject of increasing interest. Self-organized and vertically oriented titanium oxide nanotubes loaded with the broad spectrum AMP HHC36 showed in vitro bactericidal activity against S. aureus in liquid surrounding the nanotubular surface and reduced bacterial colonization on the surface approximately 200-fold. In vitro release of Tet213, also a broad spectrum AMP, from microporous calcium phosphate coatings applied on titanium showed bactericidal activity against S. aureus and P. aeruginosa. Furthermore, controlled release of Tet213 linked to collagen IV inhibited early S. aureus biofilm formation in vitro. However, these types of coatings have not yet been tested in vivo.
In addition to direct antimicrobial activity, AMPs can prevent excessive activation of pro-inflammatory responses due to bacterial endotoxins such as lipopolysaccharide (LPS) of Gram-negative bacteria, and peptidoglycan (PG) and lipoteichoic acid (LTA) of Gram-positive bacteria. In this way, AMPs combine the desired characteristics of both direct antimicrobial agents and immunomodulators. The immunomodulatory activity may be used to increase efficacy of clearance of bacterial biofilm infection, and might help to prevent derangement of immune responses which increase susceptibility to infection. Last but not least, several AMPs have osteogenic potential. In a trabecular bone growth in vivo study, cylindrical titanium implants with a coating containing the peptide HHC36 offered in vitro antimicrobial property, and in vivo osteoconductivity in rabbits. Similarly, fusion peptide P15-CSP showed anti-biofilm activity and pro-osteogenic activity, and LL-37 promoted bone regeneration in a rat calvarial bone defect model and accelerated bone repair in NOD/SCID mice by human mono-osteophils.

OUTLINE OF THIS THESIS
The general aim of this thesis is to develop and characterize novel antimicrobial agents and delivery systems and to test their effectiveness in the prevention of BAI and other difficult-to-treat biofilm infections, based on detailed understanding of the pathogenesis of these infections. In chapter 2 we investigate whether surface-localized S. epidermidis – either adherent to or in a biofilm on the implant – can be the source for colonization of the peri-implant tissue. Novel antimicrobial strategies should focus on preventing both biofilm formation and tissue colonization. Chapter 3 describes the in vitro potency of covalently attached stabilized antimicrobial peptides, based on the previously published peptide HHC10, to a hydrogel with a single-step immobilization/polymerization strategy for the development of soft antimicrobial coatings. Soft coatings, however, might be less suitable for metallic orthopedic devices, such as non-cemented implants, because these are often inserted involving high mechanical stress. To avoid abrasion during the implantation procedures, we therefore developed a mechanically stable epoxy-based coating releasing chlorhexidine in chapter 4, and determine its efficacy to prevent biomaterial-associated S. aureus infection in mice.

S. aureus and S. epidermidis have been recognized as the most frequent cause of BAIs. These bacteria form biofilms, and when encased in such biofilms they are much less susceptible to conventional antibiotics, resulting in hard-to-treat chronic infections. The
emergence of (multi)drug-resistant staphylococci further complicates the treatment of BAI by conventional antibiotics. Since AMPs are considered promising alternative antimicrobial agents we investigated their potential in chapters 5 – 8. Chapter 5 describes the development of an antimicrobial coating for bone implants that allows the prolonged controlled release of OP-145, a previously developed synthetic AMP, designed based on the sequence of LL-37. The ability of this OP-145-releasing coating to prevent bacterial colonization of titanium implants is assessed \textit{in vitro}, and ultimately prevention of implant-associated osteomyelitis is assessed in a rabbit intramedullary nail \textit{S. aureus} infection model. In chapter 6 we develop a panel of LL-37-inspired synthetic antimicrobial and anti-biofilm peptides (SAAPs), highly effective against drug-resistant bacteria, biofilms and persister cells, without inducing resistance. The efficacy and safety of one of these peptides, SAAP-148, for topical application is assessed in \textit{ex vivo} and \textit{in vivo} wound infection models. In chapter 7 we select two other peptides equally potent to SAAP-148 from the LL-37-inspired peptide set, SAAP-145 and SAAP-276, for further development in controlled release coatings for prevention of BAI caused by drug-resistant \textit{S. aureus}. A similar approach is used in chapter 8 to develop the thrombocidin-1 inspired synthetic peptide TC19 for prevention of skin infections of biofilm-forming drug-resistant \textit{S. aureus} and \textit{A. baumannii}.

3D-printing has recently gained tremendous attention in the medical device field, especially in orthopaedics. However, just like any conventional medical device, implants synthesized in this way are susceptible to bacterial colonization and infection. Chapter 9 describes the development of 3D-printing porous implants with immobilized silver nanoparticles with strong antimicrobial activity against drug-resistant \textit{S. aureus} in an \textit{ex vivo} femur implant infection model. Finally, the main results and implications of the research reported in this thesis are discussed in chapter 10.
REFERENCES


