Biofilms and biocompatibility: discovering alternative targets for preventing biomaterial-associated infections
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Chapter 1

General introduction and aims of thesis
**Chapter 1**

### 1.1 General Introduction

Biomaterial implants play an important role in modern day medicine as they are engineered to either restore function by replacement or direct the course of healing and tissue regeneration after injury or disease. Advances in health care, aging and life-style diseases are coupled with an increasing demand in the usage of medical implants. For example, an estimated 20 million patients undergo repair of an abdominal hernia, which is a protrusion of tissue or part of the intestines through the abdominal wall, due to a rise in risk factors such as abdominal surgery and obesity. Of this, more than 80% are repaired with surgical meshes.

Fully implanted biomaterials are faced with two major hurdles upon introduction into a living host. The first is the lack of tissue integration as a result of the immune response elicited by the host immune defense and the second is bacterial infection during or after surgery. The host response to an implanted biomaterial is a combination of innate immune response towards iatrogenic injury and the presence of the biomaterial. The intensity of this immune reaction, known as the foreign body reaction (FBR), is a primary determinant of implant failure or success.

### 1.2 Host response to biomaterials

The FBR to medical implants, is triggered by surgical damage of (vascularized) living tissue, resulting in the release of plasma proteins such as albumin, fibronectin, fibrinogen and vitronectin. The adsorption of plasma proteins on the surface of an implant occurs within seconds after implantation and leads to the formation of a fibrin-dominated, 2-5 nm thick provisional protein matrix. This provisional matrix enables integrin-mediated interactions with recruited immune cells such as neutrophils and macrophages to participate in subsequent acute and chronic inflammation. Biomaterial characteristics can modulate protein adsorption and thereby affect the course of the foreign body reaction. For example, adsorption of fibronectin and vitronectin is more promoted on charged surfaces than on uncharged surfaces.

Acute inflammation is initiated by the arrival of neutrophils several hours after implantation of the biomaterial. This stage is relatively short and its duration can range from days up to a week depending on the severity of injury caused during implantation. During acute inflammation, neutrophils function mainly by phagocytosis, the production of reactive oxygen species (ROS) to clear cell debris and attack bacteria and produce cytokines to recruit monocytes and macrophages. A persistent presence of neutrophils lasting more than 3 weeks is usually a sign of a bacterial infection. In that case, neutrophils combine phagocytosis and ROS production with the secretion of neutrophil extracellular traps, which in essence consists of a DNA backbone with antimicrobial proteins or peptides. Chronic inflammation begins 2-5 weeks after implantation and spans a period of 2-3 weeks. This phase is characterized by a highly proinflammatory environment with the presence of macrophages. At this stage, recruited monocytes differentiate into macrophages, which in turn secrete chemokines and cytokines to recruit more cells.
macrophages to aid in creating a pro-inflammatory environment, partly aimed at disintegrating the implant\textsuperscript{19}. Macrophages are reported to play diverse roles and are involved in the progression of tissue regeneration or fibrotic encapsulation based on the repertoire of cytokines in their microenvironment as well as physicochemical properties of the biomaterial implant. Macrophages are highly dynamic and exist as M1 and M2 phenotypes or hybrids thereof. M1 phenotype macrophages are known to secrete factors that promote a proinflammatory environment, initiation of angiogenesis and repression of profibrotic behaviour in adjacent fibroblasts-like cells. The pro-healing M2 phenotype has been associated with reduced inflammation, stabilization of newly formed blood vessels and inducing the deposition of collagen by fibroblasts-like cells\textsuperscript{1,20}.

The fusion of macrophages to foreign body giant cells ensues due to macrophage frustrated phagocytosis induced by the presence of nondigestible biomaterials\textsuperscript{15}. Foreign body giant cells promote the production of ROS and degradative enzymes aimed to facilitate the degradation of biomaterial implants. Chronic inflammation is concurrent with the formation of granulation tissue by fibroblast-like cells which eventually becomes a stronger fibrotic capsule, isolating the biomaterial from host tissues\textsuperscript{7,11}.

1.3 Biocompatibility versus biotolerability

Encapsulation by, usually acellular, non-vascularized and collagenous tissue, is the common fate of implanted biomaterials. Often the term biocompatibility is linked to biomaterials that accomplish this kind of “separating the problem”, which is frequently the case with non-toxic, non-leaching and sterile materials. Still there is quite some debate about the definition of biocompatibility. Ratner suggested to link the term biocompatibility to materials that allow vascularization and tissue reconstruction rather than shielding off the material from the host environment in which case the term biotolerability is introduced\textsuperscript{21,22}. In the definition of Ratner, biotolerability is “the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reaction” whereas biocompatibility in Ratner’s definition is: “the ability of a material to locally trigger and guide non-fibrotic wound healing, reconstruction and tissue integration”\textsuperscript{21,23}. Non-fibrotic wound healing is for instance extremely relevant for improving glucose monitoring sensors, since fibrotic encapsulation of the transcutaneous sensor causes a distortion of the glucose concentration near the interface of the sensor and delays the glucose signal\textsuperscript{24}. The qualification of biocompatibility in the definition of Ratner is accomplished by materials that control the fate of macrophages that can guide the FBR towards regenerative healing. Such materials are for instance biodegradable materials like decellularized small intestinal submucosa or materials with prefabricated porosities confining macrophages to polarize towards a pro-healing phenotype\textsuperscript{21}. In contrast to biocompatibility, biotolerability seems to be an easily attainable qualification. Materials that pass the in-vitro and in vivo tests (e.g. ISO 10993-5 and -6, respectively) are supposed to elicit a mild FBR. Provided the material is not leaching out toxic substances and is sterile, most, if not all of these materials will lead to biotolerability. However, low degrees of inflammation are only reached if the implantation site is kept fully sterile which is very hard to
achieve as contaminating bacteria may originate from the surgical environment or by hematogenous spreading, even when minimally invasive techniques are applied\textsuperscript{25,26}.

1.4 Implant associated infection

Implant associated infection after implant surgery is a disaster that will nullify the original aim of the surgery. Several factors contribute to the reality that an estimated 40% of all implants, often biotolerable and always implanted in a sterile fashion, can become contaminated with bacteria and raise severe proinflammatory reactions once implanted. Immunosenescence and the presence of a biomaterial implant reduce the efficacy of the host innate immune response to clear invading bacteria. Particularly, the burden of the highly oxidative nature of the inflammatory environment can limit the phagocytosis and bacterial killing by both neutrophils and macrophages\textsuperscript{15}. Also, the presence of an implant predisposes for bacterial infection, since bacteria have a high affinity for the surfaces of both synthetic and biologic implants. It has been shown that the presence of a biomaterial implant in humans or animals, decreases the minimal abscess formation dose by at least 10,000-fold\textsuperscript{27}. Together, these factors give infecting bacteria better opportunity to thrive on the surfaces of medical implants. Treatment of biomaterial-associated infections is mostly coupled with long antibiotic treatments, debridement and in worst case implant removal, which not only cause great discomfort for patients but also comes with high financial costs\textsuperscript{6,27}. Therefore, when it comes to implant associated infections, biofilm prevention is definitely easier than biofilm removal.

1.5 The biofilm life cycle

\textit{Staphylococcus aureus} is an important pathogen that is frequently found in biomaterial-associated infections and the main bacterial species studied in this thesis. \textit{S. aureus} is a Gram-positive opportunistic pathogen which generally forms part of the normal flora on the skin and mucosal surfaces\textsuperscript{28} and a common etiologic factor in biomaterial-associated infections due to its ability to grow into biofilms\textsuperscript{29}. Biofilms are communities of interconnected bacteria that are enclosed in a self-produced matrix of extracellular polymeric substances (EPS), usually consisting of polysaccharides such as poly-N-acetylglucosamine, extracellular DNA (eDNA) and proteins (Fig. 1)\textsuperscript{30,31}. The ability of the EPS matrix to interact with and limit perfusion of antibiotics, combined with the existence of metabolically quiescent bacteria makes biofilms resistant to antimicrobials and the host innate immune response\textsuperscript{32}.

Biofilm formation (Fig. 1) commences with the adhesion of bacteria on a substrate, whether biotic or abiotic. Adhesion occurs in two phases: first, there are reversible Van der Waals and steric-electrostatic interactions between molecules on the bacterial surface and the substrate. This is replaced by irreversible adhesion of the bacteria that is established by hydrogen bonds as well as ionic and dipole-hydrogen interactions\textsuperscript{33}. Microcolony formation and EPS formation occurs after irreversible attachment. Here, the bacteria begin to grow and produce EPS which is controlled via cell-cell signalling, nutrient availability, pH and oxygen concentration\textsuperscript{34}. 
During this phase, deacetylation of poly-N-acetylglucosamine confers a positive charge which promotes interaction with negatively charged components on the bacterial surface. eDNA, crucial for the structural integrity of biofilms, is sourced via lysis of a subpopulation of bacteria or by active vesicular transport. Naturally occurring eDNA on the surface of *S. aureus* has been shown to improve adhesion and also stabilizes biofilm structure by acting as an electronegative string that tether bacteria to each other as well as to EPS polysaccharides and proteins\textsuperscript{35,36}.

During these early stages of *S. aureus* biofilm formation, a transient period of heightened nuclease production occurs which reportedly results in a first round of bacterial dispersal\textsuperscript{37}. Gradients in environmental conditions such as O\textsubscript{2} and nutrients, can give rise to distinct subpopulations characterized by four different metabolic states: bacteria that grow aerobically or anaerobically, dormant cells or dead cells\textsuperscript{32}. Bacteria within the top layers of the biofilm are mostly aerobic metabolically active while fermentative or slow growing and persister cells are sheltered at the hypoxic interior of the biofilm\textsuperscript{38}. As the biofilm matures, bacteria continue to grow and produce EPS. Once matured biofilms are established, the *agr* quorum sensing system enables biofilm dispersal and colonization of novel sites\textsuperscript{39,40}. Bacteria prefer the biofilm mode of growth over the planktonic state as the EPS protects resident bacteria from the host immune defense and also by conferring a high tolerance to antibiotics as it can interact and limit perfusion of antibiotics. The presence of metabolically quiescent and persister cells further render antibiotics inefficient in killing resident bacteria in biofilms\textsuperscript{32}. 

**Figure 1.** Developmental stages of a biofilm

1.6 *Neutrophil extracellular traps and micrococcal nuclease*

Acute inflammation is dominated by neutrophils. In addition to phagocytosis
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and macrophage recruitment, neutrophils also excrete Neutrophil Extracellular Traps (NETs) through a process known as NETosis. NETs contain DNA tagged with antimicrobial peptides such as citrullinated histones, myeloperoxidases, neutrophil elastase and cathepsin. NETs were first observed in connection with bacterial infections, but recently have been found in reaction towards sterile implants as well, suggesting that NETs form part of the FBR. As a particular virulence factor, *S. aureus* bacteria are able to excrete nucleases which can pacificate the antimicrobial properties of NETs and therewith, directly impact the FBR. Micrococcal nucleases have the ability to cleave both DNA and RNA and have been reported to promote biofilm dispersal by cleaving eDNA in the biofilm matrix. Moreover, nuclease activity facilitates the escape of *S. aureus* from the effects of NETs. *S. aureus* is known to independently express two forms of nucleases, the extracellular Nuc1 and the membrane bound Nuc2, encoded by the *nuc1* and *nuc2* genes respectively. The nuclease enzyme has both endo and exo 5'phosphodiesterase activity against DNA and RNA to give 3-mono- and di-nucleotides but have a higher affinity for DNA.

The staphylococcal nuclease is a globular protein of 146 residues, the tertiary structure of which consists of 5 highly twisted β-strands and 3 α-helices. Ca²⁺ ions are a prerequisite for the enzyme's activity. Nuclease production is regulated by the saePQRS regulatory system which also regulates target genes such as α-hemolysin and leukocidins. H₂O₂ and α-defensins produced by human neutrophils are known to activate the saePQRS system. However, in mice, other activators may be present as murine neutrophils are capable of activating the saePQRS system although unable to produce α-defensins.
2 Aims of this Thesis

Despite the benefits of implantable medical devices for patients, the presence of a biomaterial in tissues provokes the foreign body response which contributes to the innate immune defense being dysfunctional in clearing invading bacteria. Therefore, the increased application of biomaterials to support or restore biological function fuels the surge in biomaterial-associated infections, making it a major contributor to bacterial infections, predicted to be the leading cause of deaths by the year 2050 as a result of antibiotic resistance. At the moment, antibiotic treatment of implant infections is costly due to prolonged hospital stays with often unsatisfactory outcomes. This is because the biofilm mode of growth and tissues surrounding the implant can protect bacteria from antibiotics. Hence, there is a need for novel complementary solutions to prevent or destabilize biofilm formation as well as support the host immune response in fighting implant-related infections.

This thesis investigated whether the influence of micrococcal nuclease activity on biofilm formation can be modulated by biomaterial hydrophobicity in vitro (Fig. 2, Chapter 2). We subsequently aimed in Chapter 3 to assess the role of micrococcal nuclease activity on biofilm formation and infection progression in vivo (Fig. 2, Chapter 3). In addition, we investigated the prospects of utilizing polymer coatings as antibacterial remedies by prevention bacterial adhesion or by modulating the foreign body response (Fig. 2, Chapters 5 and 6).

Figure 2. An overview of the scope of this thesis. Blue dots indicate bacteria, arrows indicate the studied interactions between the various aspects addressed in this thesis.
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References


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