# CHAPTER VII

General Discussion

Biomaterials research arguably started in the 1950s with the objective of creating synthetic materials with properties that allow replacement of a tissue without deleterious response from the host [1]. Advances in tissue engineering and regenerative medicine allowed the design of biomaterials that replace a tissue but also support and stimulate the regeneration of functional tissue. Examples of these biomaterials are hip prostheses, which are commonly implanted worldwide with good results in restoring function and quality of life [1]. Even so, the risk of bacterial infection remains a serious problem to solve. Biomaterial-associated infection (BAI) is the number one cause of failure of biomaterials implants and devices [2-4]. Often the only solution for eliminating a BAI is to surgical remove the implant and the surrounding, affected tissue [5]. Hence, the aims of this thesis were to (i) develop a model for studying and characterizing the interaction between immune cells, in specific macrophages, and bacteria; (ii) explore strategies that prevent bacterial adhesion while promoting macrophage activity, in specific phagocytosis of bacteria; and (iii) study the outcome of the race for the surface between bacteria, tissue cells and macrophages.

***Interactions between immune cells, bacteria and biomaterials***

Biomaterials commonly induce an inflammatory response from the host upon implantation, mainly due to the tissue injury, but also due to the host reaction to a foreign body [6]. The intensity and duration of this host response is mainly dependent on the site of implantation and the biomaterial characteristics, such as size, shape and physical and chemical properties [7,8]. After implantation a biomaterial rapidly becomes coated with plasma and extracellular matrix proteins that serve as specific receptors for tissue cells and colonizing bacteria [4]. Most BAIs are caused by commensal bacteria [6], which in the absence of a biomaterial hardly

cause infections. The combination of the inflammatory response due to the presence of a biomaterial with a bacterial infection results in a very complex host response. The presence of a biomaterial can trigger an oxidative burst of the immune cells, which may exhaust their metabolic killing mechanisms before phagocytizing all surface adhering bacteria [3,6]. As an infection progresses, the formation of a biofilm may also impair the phagocytic activity [3,6,9].

Several researchers believe that BAI involving *Staphylococcus epidermidis* strains are often low-grade infections, which can remain unnoticed for prolonged periods of time and accordingly stimulate a less aggressive response of phagocytes, opposite to infections involving *Staphylococcus aureus* strains [2,7,10]. In this thesis (Chapter III), we show that this dogma is far from reality, depending on the bacterial strain, the same species can be very or little aggressive to the immune system. For instance, *S. epidermidis* 7391 was one of the studied strains that were recognized and phagocytized faster by all immune cells, while *S. epidermidis* 1457 and *S. aureus* LAC induced a slow reaction from the different immune cells and probably *in vivo* would induce low-grade infections. In addition, murine and human phagocytic cell lines revealed very different behaviors in the presence of bacteria, which highlight the importance for future research studies to identify the differences between immune cells in the animal model versus the human host [11-13]. While some bacteria will not cause infection in mice, they may cause aggressive infections in humans [12,13]. Moreover, in murine models, some vascularized grafts can be well accepted and tissue integrated, whereas such grafts are rapidly rejected in humans [13]. Although there are visible differences between species, murine models are still indispensable for fundamental research and will continue to be the premiere *in vivo* models for human immunology. Different paradigms are well translated between murine models and human hosts, and with the facility with which murine models can be genetically

manipulated, these models will continue to be important tools to provide essential information in future.

***The race for the surface***

The competition for the biomaterial surface *in vivo* will not only depend on the number of bacteria and the immune cells present at the implantation site, but also on the tissue cells involved in the tissue-integration of the biomaterial. Subbiahdoss *et al.* [14] published a method, based on the "race for the surface" model by Gristina [15], to study *in vitro* the outcome of the competition between bacteria and tissue cells for a biomaterial surface. In this thesis, this method was extended to include the influence of macrophages (Chapter V), and revealed that the presence of immune cells prolongs the time that tissue cells survive an attack by aggressive bacterial strains. A high concentration of macrophages would yield clearance of all adhering bacteria from the substratum surface in favor of tissue cell adhesion and spreading. Considering that, *in vivo*, the host can continuously summon immune cells to the affected implantation site, a healthy immune system will be crucial to the success of an implant. However, bacteria also have developed mechanisms to evade the immune response, therewith escaping phagocytosis by macrophages or, when phagocytised, surviving within macrophages [2,16,17]. Antibiotics have been critical to prevent or eliminate bacterial evading the immune system, yet bacteria are remarkably resilient and have developed several ways to resist antibiotics therapies. Unless new non-antibiotic therapies are established, society can be faced, in the near future, with diseases that were treatable but have become untreatable again due to bacterial adaptation.

***Strategies to prevent bacterial adhesion while promoting the host immune response***

With the knowledge that homogeneous surfaces of commonly used biomaterials, often sustain the same effect on both host cells and bacteria [18], a quest has started for infection-resistant biomaterials for tissue-integratable implants and devices [18,19]. Poly(ethylene) glycol (PEG) has been widely used as an infection-resistant biomaterial coating [20-23], yet there is still the weak adherence of bacteria and possible formation of biofilm. Weak cell-surface interactions were also shown to occur for tissue cells on PEG-coated surfaces, which is an advantage for enhanced macrophage mobility and subsequent phagocytic activity (Chapter II and Chapter VI). On surfaces with PEG-patterned adhesive patches (studied in Chapter VI) there is a great chance that tissue integration can occur smoothly after the fast elimination of pathogens and debris by immune cells. To obtain a better elimination of pathogens, superparamagnetic iron oxide nanoparticles (SPIONs) can be added to the system as shown in Chapter IV. SPIONs can be combined with any biomaterial surface to assist in the elimination of bacteria and replace the current use of antibiotic therapy in the fight against BAI. Nevertheless, there are a few settings that need improvement in the studies shown in this thesis. For healthy tissue integration, there is the need of an optimal number of adhesive sites present on the biomaterial surface, where cells can attach strongly to the biomaterial. In addition, a low concentration of SPIONs should be sufficient to assist macrophages in the elimination of bacteria. Therefore, future research should focus in finding the optimal ratio of adherent and non-adherent patches on a surface, and more importantly finding the minimal concentration of SPIONs needed to aid macrophage-mediated elimination of bacteria without causing any toxic effects on the host cells.

There is an urgent need to obtain non-antibiotic therapies that improve tissue-integration of implants while preventing BAI. To find optimal *in vitro* models that mimic *in vivo* situations can be a decisive requirement to obtain a much-needed connection between *in vitro* data and clinical outcome. The model we propose for future studies (Figure 1) brings a practical and efficient solution to reduce the gap between *in vitro* studies and the *in vivo* situation.

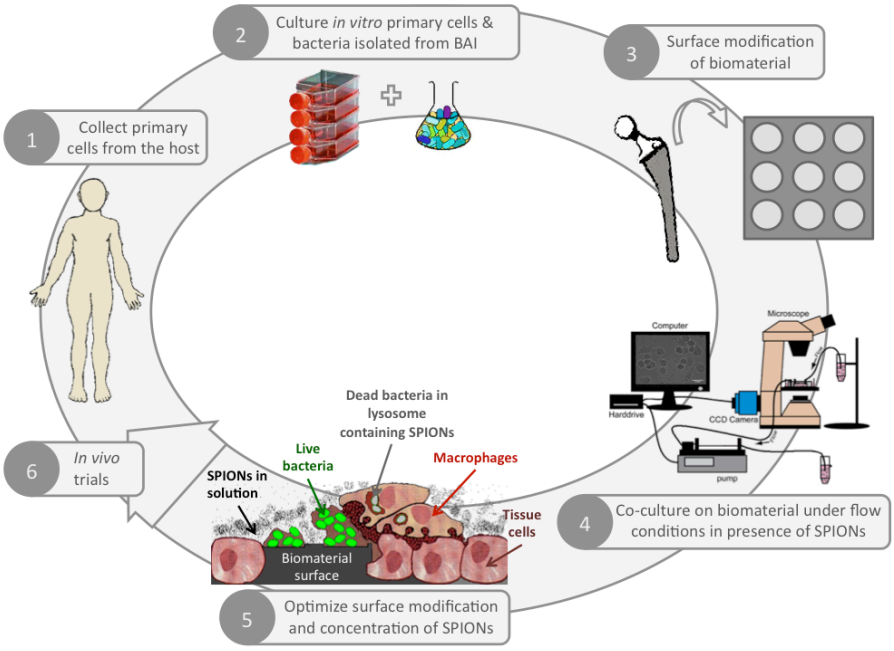


**Figure 1.** Schematic diagram of the optimal *in vitro* model to minimize the gap between *in vitro* and *in vivo* studies of BAI. Primary cells should be collected from the patient (1) and bacteria isolated directly from existing BAI (2). The choice of the biomaterial and surface modification should be specific and appropriate to the clinical situation (3). Co-culture of tissue cells, immune cells and bacteria, previously obtained from the patient, should be done *in vitro* under flow conditions to mimic the *in vivo* situation as closely as possible. Moreover SPIONs should be added in solution as an alternative to the antibiotic therapies (4). After *in vitro* settings, this system can be optimized for the optimal settings of the surface modification and the minimal non-toxic concentration of SPIONs needed to eliminate BAI (5). Finally, after obtaining an optimal *in vitro* outcome, the therapy can be applied directly to the patient, since all variables (tissue cells, immune cells and bacteria) were already specific for the human host (6).

Elements involve first of all the choice of cells. The use of cell lines instead of primary cells in this thesis has been a pragmatic choice, as these cells are immortal(ized), stable, reliable, mature and differentiated (J774) [7,9], although it is recognized that macrophages *in vivo* are in a spectrum of activated phenotypes rather than discrete stable subpopulations [11,24]. By taking human, maybe even patient-specific primary cells the outcome of competition experiments could be closer to the clinical reality. Also the bacteria can be isolated from actual BAI sites.

In this thesis the emphasis was on staphylococcal strains due to their great incidence in BAI, but it is possible that bacteria isolated from the actual BAI possess slightly different characteristics relative to the strains used in the thesis [9,25,26].

## CONCLUSIONS

Even a small number of adhering bacteria can grow into a life-threatening biofilm on a biomaterial implant surface. The current strategies used to prevent BAI are still far from perfect, and therefore, there is an urgent need of improved non-antibiotic therapies. Current research is aimed at finding biomaterials that can help the host cells, to win the race for the surface, and the work in this thesis provides new insights on the materials variables we need to improve. Different strategies to prevent bacterial adhesion while promoting immune cell activity were demonstrated to be promising as future therapies to promote tissue integration while preventing or eliminating BAI. In addition, it is also shown that the numbers of macrophages present in the infection site may decide the outcome of the race for the surface.

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# Summary

Biomaterial-associated infections (BAI) constitute a major clinical problem that is difficult to treat and often necessitates implant replacement. Pathogens can be introduced on an implant surface during surgery or postoperatively and compete with host cells attempting to integrate the implant. The fate of a biomaterial implant has been depicted as a race between bacterial adhesion and biofilm growth on an implant surface *versus* tissue integration. Until today, *in vitro* studies on infection risks of biomaterials or functional coatings for orthopedic and dental implants were performed either for their ability to resist bacterial adhesion or for their ability to support mammalian cell adhesion and proliferation. Even though the concept of the race for the surface in BAI has been intensively studied before *in vivo*, until recently no *in vitro* methodology existed for this purpose. Currently, various groups have proposed co-culture experiments to evaluate the simultaneous response of bacteria and mammalian cells on a surface. As an initial step towards bridging the gap between *in vitro* and *in vivo* evaluations of biomaterials, in **Chapter I** we describe bi-culture, and tri-culture experiments that allow better evaluation of multi-functional coatings *in vitro* and therewith bridge the gap between *in vitro* and *in vivo* studies.

Bacteria in their biofilm mode of growth are relatively insensitive to the host immune system or antibiotic treatment, as also caused by the growing antibiotic-resistance across many current pathogens. Therefore, it is important to understand how immune cells interact with adhering pathogens on different biomaterial surfaces and to find new strategies to cure or prevent bacterial infections. Poly(ethylene)glycol (PEG) coatings have been studied as a strategy to decrease the incidence of BAI. Despite their effectiveness to reduce protein adsorption and a hundred-fold reduction in bacterial adhesion, PEG-based coatings still facilitate weak bacterial adhesion that can form a basis for further biofilms growth. In **Chapter II**, a

methodology is described that enables direct, quantitative and detailed qualitative *in situ* observation of macrophage morphology, migration and phagocytosis of bacteria on biomaterial surfaces. The methodology was used to compare the *in vitro* interaction of macrophages with *Staphylococcus epidermidis* 3399 adhering to commercial, crosslinked PEG-based coatings (OptiChem®), fluorinated ethylene propylene, silicone rubber and glass. Adhesion, phagocytosis and migration were studied real-time in a parallel-plate flow chamber. Macrophages cultured on OptiChem® coatings showed enhanced migration and phagocytosis of bacteria compared to common biomaterials. Bacterial clearance per macrophage on both inert and reactive OptiChem® coatings were about three times higher than on the common biomaterials studied, corresponding with up to 70% reduction in bacterial numbers on OptiChem®, whereas on the biomaterials less than 40% bacterial reduction was obtained. These findings show that bacterial clearance from cross-linked PEG-based coatings by macrophages is more effective than from common biomaterials, possibly resulting from weak adhesion of bacteria on Optichem®. Moreover, macrophages exhibit higher mobility on Optichem® retaining an improved capability to clear bacteria from larger areas than from other common biomaterials, where they appear more immobilized.

The model described in Chapter II led to the design of a simple method to quantify macrophage-mediated phagocytosis of bacteria adhering to a transparent material surface. In **Chapter III**, we describe the rationale behind this method together with its application to six different strains of staphylococci adhering on a glass surface. Phagocytosis of adhering staphylococci by phagocytes was quantified in a parallel-plate flow chamber, and expressed as a phagocytosis rate, accounting for the number of adhering staphylococci initially present and for the duration of phagocytosis. Murine macrophages were more effective in clearing staphylococci

from a surface than human phagocytes, which require differentiation from their monocyte or promyelocytic state during an experiment. Direct visualization of internalization of a GFP-modified *Staphylococcus aureus* strain inside phagocytes confirmed the validity of the method proposed. As a second aim, the differences in phagocytosis rates observed were investigated on a surface thermodynamic basis using measured contact angles of liquids on macroscopic lawns of staphylococci and phagocytes, confirming that phagocytosis of adhering pathogens, can be regarded as a surface phenomenon. In addition, surface thermodynamics revealed that phagocytosis of adhering pathogens is determined by an interplay of physical attraction between pathogens and phagocytes and the influence of chemo-attractants. For future studies, these results will help to place *in vitro* experiments and murine infection models in better perspective with respect to human ones.

Apart from creating surfaces that allow macrophages to be mobile, these phagocytic cells can also be supported in their deactivation of bacteria by the use of superparamagnetic iron-oxide nanoparticles (SPIONs) as described in **Chapter IV**. *S. aureus* biofilms were exposed to macrophages together with SPIONs and microscopically examined. SPIONs were internalized by macrophages, yielding increased generation of reactive oxygen species and less survival of staphylococci (17 – 21%) compared with the presence of macrophages (50 – 74%) or SPIONs (80 – 87%) alone. Herewith, we present a novel, original, non-antibiotic-based approach to increase macrophage efficacy to remove staphylococci from infectious biofilms.

Macrophages do not act alone when a biomaterial is implanted in the body. Tissue cells will try to colonize the surface. If bacteria are present a competition will start. In an *in vitro* model, this competition is mimicked for different bacterial strains and human U2OS osteoblast-like cells colonizing a poly(methylmethacrylate) surface. The role of macrophages in this competition was investigated, as described in

**Chapter V**. Bacteria were seeded on the surface at a shear rate of 11 $s^{-1}$ prior to adhesion of U2OS cells and macrophages. Next, bacteria, U2OS cells and macrophages were allowed to grow simultaneously under low shear conditions (0.14 $s^{-1}$). The outcome of the competition between bacteria and U2OS cells for the surface critically depended on bacterial virulence. In absence of macrophages, highly virulent *S. aureus* or *Pseudomonas aeruginosa* stimulated U2OS cell death within 18 h of simultaneous growth on a surface. Moreover, these strains also caused cell death despite phagocytosis of adhering bacteria in presence of murine macrophages. Thus U2OS cells are bound to lose the race for a biomaterial surface against *S. aureus* or *P. aeruginosa*, even in the presence of macrophages. In contrast, low-virulent *S. epidermidis* did not cause U2OS cell death even after 48 h, regardless of the absence or presence of macrophages. Clinically, *S. aureus* and *P. aeruginosa* are known to yield acute and severe biomaterial-associated infections in contrast to *S. epidermidis*, mostly known to cause more low-grade infection. Thus it can be concluded that the model described possesses features concurring with clinical observations and therewith has potential for further studies on the simultaneous competition for an implant surface between tissue cells and pathogenic bacteria in presence of immune system components.

The theme of mobile macrophages was further explored by confining bacteria on smartly patterned PEG-coatings, with adhesive patches as described in **Chapter VI**. Adhesive patch diameters and inter-patch distances were varied to yield different adhesive area fractions of glass in a PEG-matrix. Patterns with adhesive patch diameters in the order of 2 – 5 μm separated by distances greater than the patch diameter promoted osteoblast adhesion, spreading and growth and simultaneously reduced staphylococcal colonization. Moreover, macrophages were more efficient in eradicating adhering staphylococci from patterned surfaces than from the PEG-

matrix or glass surfaces, because staphylococcal growth was confined to the adhesive patches. This new feature of confined bacterial growth in adhesive patches aided macrophages in their search for adhering bacteria.

From the experiments described in this thesis an outlook on future treatment of biomaterial-associated biofilms is given in the general discussion (**Chapter VII**).

# Sumário

Infecções associadas a biomateriais (BAI) constituem um grave problema clínico, difícil de tratar, e que frequentemente conduz à substituição do implante. Durante ou após cirurgia, agentes patogénicos podem ser introduzidos na superfície do implante e competir com a integração do implante nas células do corpo hospedeiro. O destino de um biomaterial implantado tem sido descrito como uma competição entre a adesão de bactérias e a formação de biofilme na superfície do implante *versus* a integração do biomaterial com o tecido do hospedeiro. Até hoje, vários estudos *in vitro* abordaram os riscos de infecções de biomateriais ou biomateriais com modificações funcionais, para implantes ortopédicos ou dentários, mas apenas com a intenção de promover resistência à adesão de bactérias ou de melhorar a adesão e proliferação de células de animais. Apesar do conceito "competição pela superfície" em relação a BAI já ter sido extensamente estudado *in vivo*, até recentemente não existia uma metodologia *in vitro* para este propósito. Actualmente, vários grupos de investigação propuseram experiências de co-cultura para avaliar a resposta de bactérias e células animais em simultâneo numa superfície. Como um primeiro passo para diminuir a lacuna entre as avaliações *in vitro* e *in vivo* de biomateriais, no **Capítulo I** descrevemos experiências com bi-culturas e tri-culturas que permitem avaliar modificações de superfície multi-funcionais *in vitro* e assim criar uma melhor correspondência entre os estudos *in vitro* e *in vivo*.

Bactérias em modo de formação de biofilme são relativamente insensíveis ao sistema imunitário do hospedeiro ou a tratamento com antibióticos, o que também resulta do aumento da resistência a antibióticos de vários agentes patogénicos actuais. Consequentemente, é importante compreender como as células do sistema imunitário interagem com microorganismos aderidos em differentes superfícies de biomateriais, e encontrar novas estratégias para prevenir ou eliminar infecções bacterianas. Modificações de superfície com polietilenoglicol (PEG) têm sido

estudadas como uma estratégia para diminuir a incidência de BAI. Apesar desta modificação ser eficaz na redução da adsorção de proteínas e resultar numa redução de cem vezes na adesão bacteriana, estas modificações baseadas em PEG continuam a permitir uma fraca adesão bacteriana, o que pode formar a base para o crescimento de um biofilme. No **Capítulo II**, é descrito um método que permite a observação *in situ*, directa, quantitativa e detalhada da morfologia, migração e fagocitose bacteriana por macrófagos, em superfícies de biomateriais. Este método foi utilizado *in vitro* para comparar a interacção de macrófagos com *Staphylococcus epidermidis* 3399 aderindo a superfícies modificadas com PEG polimerizado (OptiChem®), propileno de etileno fluorinado, borracha de silicone e vidro. Os fenómenos de adesão, fagocitose e migração foram estudados em tempo real numa célula de fluxo laminar. Macrófagos cultivados em superfícies de OptiChem® revelaram uma maior migração e fagocitose de bactérias do que em biomateriais comuns. A remoção de bactérias pelos macrófagos, em superfícies com OptiChem®, tanto inerte como reactivo, revelou-se três vezes mais elevada do que nos biomateriais comuns estudados. Esta redução em superfícies com OptiChem®, correspondeu a uma redução de 70 % do número de bactérias, enquanto nos biomateriais comuns a redução do número de bactérias foi menor do que 40%. Estes resultados provam que a remoção de bactérias por macrófagos em superfícies modificadas com PEG polimerizado é mais eficaz do que em superfícies de biomateriais comuns, possivelmente devido à fraca adesão de bactérias em Optichem®. Além disso, em superfícies modificadas com Optichem®, os macrófagos exibiram elevada mobilidade e capacidade para eliminar bactérias de maiores áreas do que em outros biomateriais comuns, onde apareceram menos móveis.

O modelo descrito no Capítulo II conduziu ao design de um método simples para quantificar a fagocitose de bactérias, aderidas na superfície de um material

transparente, por macrófagos. No **Capítulo III** descrevemos a lógica subjacente a este método, assim como a sua aplicação a seis estirpes diferentes de staphylococci aderidos numa superfície de vidro. A fagocitose de bactérias aderidas foi quantificada numa célula de fluxo laminar, e descrita como a velocidade de fagocitose, tendo em consideração o número de staphylococci presentes inicialmente e a duração da fagocitose. A linha celular de macrófagos de rato revelou-se mais eficiente na eliminação de bactérias duma superfície do que as linhas celulares de fagócitos humanos, que necessitavam de ser diferenciadas do estado de monócitos ou promielocítico durante uma experiência. A validade do método proposto foi confirmada pela visualização directa de uma estirpe de *Staphylococcus aureus*, modificada para expressar proteínas fluorescentes verdes (GFP), dentro dos fagócitos. Como objectivo secundário, foram investigadas as diferenças entre as velocidades de fagocitose com base em termodinâmica de superfície, através da medição de ângulos de contacto de diferentes líquidos em camadas macroscópicas de bactérias ou fagócitos. Estas diferenças confirmaram que a fagocitose de microorganismos aderidos pode ser considerada um fenómeno de superfície. A termodinâmica de superfície revelou também que a fagocitose de microorganismos aderidos é determinada por uma combinação da atracção física entre microorganismos e fagócitos bem como, pela influência de quimio-tácticos. Estes resultados contribuirão para uma melhor comparação entre experiências *in vitro* e os modelos de infecção animal, fornecendo uma melhor perspectiva em relação aos humanos.

Além da criação de superfícies que permitem maior mobilidade dos macrófagos, estas células imunitárias também podem ser ajudadas a desactivar bactérias através do uso de nanopartículas superparamagnéticas de óxido de ferro (SPIONs), como descrito no **Capítulo IV**. Biofilmes de *S. aureus* foram expostos a uma combinação de macrófagos e SPIONs e examinados microscópicamente. Os SPIONs foram

internalizados pelos macrófagos resultando numa maior produção de espécies reactivas de oxigénio e menor quantidade de staphylococci sobreviventes (17 – 21%) comparando com a presença de apenas macrófagos (50 – 74%) ou SPIONs (80 – 87%). Como resultado, apresentamos uma terapia nova, original e sem antibióticos, para aumentar a eficácia com que os macrófagos removem staphylococci de biofilmes infecciosos.

No caso da implantação de um biomaterial, os macrófagos não agem sozinhos, as células presentes no local do implante também tentarão colonizar a superfície. Na presença de bactérias, inicia-se uma competição pela superfície. Esta competição foi mimetizada para diferentes estirpes de bactéria e células humanas tipo osteoblastos (U2OS) colonizando uma superfície de poli(metacrilato de metilo), num modelo *in vitro*. No **Capítulo V**, este modelo foi extendido para investigar o papel dos macrófagos nesta competição. Antes da adesão das células U2OS e macrófagos, as bactéria foram aderidas na superfície a uma velocidade de fluxo de 11 $s^{-1}$. De seguida, as células U2OS e os macrófagos foram inseridos, e a cultura continuada na presença de meio a circular em baixa velocidade (0.14 $s^{-1}$). Foi verificado que o resultado desta competição pela superfície, entre bactérias e células U2OS, depende da virulência da estirpe de bactéria estudada. Na ausência de macrófagos, as estirpes muito virulentas, como *S. aureus* ou *Pseudomonas aeruginosa*, estimularam a morte das células U2OS em apenas 18 h de crescimento em simultâneo na superfície. Além disso, estas estirpes na presença de macrófagos de rato também causaram morte celular de U2OS, apesar da fagocitose de bactérias aderidas. Assim, as células U2OS estão destinadas a perder a competição pela superfície de um biomaterial contra *S. aureus* or *P. aeruginosa*, mesmo que na presença de macrófagos. Em contraste, estirpes pouco virulentas, como *S. epidermidis*, não conduziram à morte das células U2OS mesmo após 48 h de crescimento, independentemente da presença ou ausência de

macrófagos. Em termos clínicos, *S. aureus* e *P. aeruginosa* são conhecidas como estirpes que causam infecções associadas a biomateriais agudas e severas, ao contrário de *S. epidermidis*, que são geralmente consideradas infecções de baixo risco. Em conclusão, o modelo descrito possui características coerentes com observações clínicas e tem assim potencial para ser usado em futuros estudos sobre a competição pela superfície de um implante entre tecidos celulares e bactérias patogénicas na presença de componentes do sistema imunitário. O tema da mobilidade de macrófagos foi novamente explorado no **Capítulo VI**, ao limitar a adesão de bactérias em superfícies com modificações de PEG com um smart design de áreas adesivas. O diâmetro das áreas adesivas e a distância entre estas áreas foram variados para obter diferentes fracções de adesão em superfícies de vidro com uma matrix de PEG. As superfícies com áreas adesivas de diâmetros na ordem dos 2 – 5 μm, e separadas por distâncias maiores do que o diâmetro da área adesiva, promoveram a adesão e proliferação de osteoblastos e simultâneamente reduziram a colonização por staphylococci. Os macrófagos foram mais eficientes em eliminar bactérias aderidas nas superfícies com diferentes designs de PEG, devido à limitação da área de adesão das bactérias, do que em superfícies completamente modificadas com PEG ou de vidro. A limitação do crescimento de bactérias em áreas adesivas ajudou os macrófagos na procura de bactérias aderidas.

Através das experiências descritas nesta tese, a discussão geral (**Capítulo VII**) fornece uma visão do que será o futuro no tratamento de biofilmes associados a biomateriais.

# Samenvatting

Biomateriaal-geassocieerde infecties (BAI) vormen een belangrijk klinisch probleem dat moeilijk te behandelen is en waardoor implantaatvervanging vaak noodzakelijk is. Zowel tijdens een operatie als postoperatief kunnen pathogene bacteriën op het oppervlak van een implantaat geïntroduceerd worden en de concurrentie aangaan met gastheercellen die proberen het implantaat te integreren. Het uiteindelijke lot van een geïmplanteerd biomateriaal wordt beschreven als een wedstrijd tussen bacterie-adhesie en biofilmgroei op het oppervlak van een implantaat versus weefselintegratie ("race for the surface"). Tot op heden zijn *in vitro* studies naar infectierisico's van biomaterialen of functionele coatings voor orthopedische en tandheelkundige implantaten gericht op òf het tegengaan van bacterie-adhesie aan een implantaat, òf de bevordering van adhesie en proliferatie van weefselcellen. Hoewel het "race for the surface"-concept uitgebreid onderzocht is *in vivo*, tot voor kort bestond er geen *in vitro*-methode voor dit doel. Op dit moment hebben verscheidene onderzoeksgroepen methoden ontworpen waarmee de simultane respons van bacteriën en weefselcellen op een oppervlak onderzocht kan worden. Als eerste stap om het hiaat tussen *in vitro* en *in vivo* onderzoek van biomaterialen te overbruggen, beschrijven we in **Hoofdstuk I** kweekexperimenten waarbij twee, en drie entiteiten gelijktijdig gekweekt worden met als doel de evaluatiemogelijkheden van multifunctionele coatings *in vitro* te verbeteren en daarmee het kennishiaat tussen *in vitro* en *in vivo* onderzoeken te overbruggen.

Bacteriën die in een biofilm groeien zijn relatief ongevoelig voor het immuunsysteem van de gastheer of voor behandeling met antibiotica, mede veroorzaakt door de groeiende antibioticaresistentie van pathogenen. Daarom is het belangrijk om te begrijpen hoe de interactie is tussen immuuncellen en gehechte pathogenen op verschillende biomateriaal-oppervlakken en om nieuwe strategieën te

vinden om bacteriële infecties te behandelen of te voorkomen. Er is onderzoek gedaan naar poly(ethylene)glycol (PEG) coatings als strategie om de incidentie van BAI te verlagen. Ondanks effectieve reductie van eiwit-adsorptie en een factor 100 vermindering van bacterie-adhesie door PEG-gebaseerde coatings, faciliteren dergelijke coatings bacterie-adhesie enigszins, waardoor een basis gelegd kan worden voor verdere biofilmgroei. In **Hoofdstuk II** wordt een methode beschreven waarmee directe kwantitatieve en gedetailleerde kwalitatieve *in situ* observatie van macrofaagmorfologie, -migratie en -fagocytose van bacteriën op biomateriaaloppervlakken mogelijk wordt. Deze methode is gebruikt om de in *vitro* interactie tussen macrofagen en *Staphylococcus epidermidis* 3399 te vergelijken, wanneer deze gehecht zijn aan commercieel verkrijgbare, gecrosslinkte PEG-gebaseerde coatings (OptiChem®), gefluoridiseerd ethyleen propyleen, siliconenrubber en glas. De adhesie, fagocytose en migratie werden real time bestudeerd in een parallelle plaat doorstroom kamer. Macrofagen die gekweekt werden op OptiChem® coatings lieten een versterkte migratie en fagocytose van bacteriën zien ten opzichte van gangbare biomaterialen. Op zowel inerte als op OptiChem® coatings was de bacteriële klaring per macrofaag ongeveer drie keer hoger dan op de andere gangbare biomaterialen die bestudeerd werden. Analoog aan deze bevindingen werd op OptiChem® tot 70% reductie in bacterie-aantallen behaald, terwijl de reductie op de biomaterialen minder dan 40% bedroeg. Deze bevindingen tonen aan dat macrofagen bacteriën effectiever verwijderen van gecrosslinkte PEG-gebaseerde coatings dan van gangbare biomaterialen, mogelijk als gevolg van zwakke adhesie van bacteriën aan OptiChem®. Bovendien zijn macrofagen meer mobiel op OptiChem® waardoor zij beter in staat zijn om bacteriën van grotere oppervlakken te verwijderen dan van andere gangbare biomaterialen, waarop de macrofagen meer geïmmobiliseerd lijken te zijn.

Het model dat beschreven werd in Hoofdstuk II heeft geleid tot het ontwerp van een simpele methode om de macrofaag-gemedieerde fagocytose van bacteriën, die gehecht zijn aan een transparant materiaaloppervlak, te kwantificeren. In **Hoofdstuk III** beschrijven we de principes achter deze methode en diens toepassing op zes verschillende stafylokokken stammen gehecht aan glas. De fagocytose van gehechte stafylokokken door fagocyten werd gekwantificeerd in een parallelle plaat doorstroom kamer, en uitgedrukt als een fagocyteer-snelheid, waarbij gecorrigeerd werd voor het aantal gehechte, initieel aanwezige stafylokokken en voor de duur van de fagocytose. Van een muis afkomstige macrofagen waren effectiever in het verwijderen van stafylokokken van een oppervlak dan van een mens afkomstige fagocyten, die eerst moeten differentiëren vanuit het stadium van monocyt of promyelocyt gedurende een experiment. Directe visualisatie van het opnemen van een GFP-gemodificeerde *Staphylococcus aureus*-stam in fagocyten bevestigde de validiteit van de voorgestelde methode. Als tweede doel werden de geobserveerde verschillen in fagocyteer-snelheid verder onderzocht op basis van oppervlakte-thermodynamica door middel van het meten van randhoeken van verschillende vloeistoffen op macroscopische lagen van stafylokokken en fagocyten, waardoor bevestigd werd dat de fagocytose van gehechte pathogenen beschouwd kan worden als een oppervlaktefenomeen. Daarnaast liet oppervlakte thermodynamica zien dat fagocytose van hechtende pathogenen wordt bepaald door een interactie van fysische aantrekkingskracht tussen pathogenen en fagocyten en door de invloed van chemo-attractiva. Deze resultaten kunnen in toekomstig onderzoek bijdragen aan het beter in perspectief plaatsen van *in vitro* experimenten en infectiemodellen in muizen in vergelijking met infectie-experimenten in mensen.

Behalve het creëren van oppervlakken die macrofagen meer mobiel maken, kunnen deze fagocytische cellen ook ondersteund worden bij het deactiveren van

bacteriën door de toepassing van superparamagnetische ijzeroxide nanodeeltjes (SPIONs), zoals beschreven wordt in **Hoofdstuk IV**. Biofilms van *S. aureus* werden blootgesteld aan macrofagen en SPIONs en microscopisch onderzocht. SPIONs werden opgenomen door macrofagen, resulterend in verhoogde aanmaak van reactieve zuurstofsoorten en lagere overleving van stafylokokken (17 – 21%) in vergelijking met de aanwezigheid van macrofagen (50 – 74%) of SPIONs (80 – 87%) alleen. Hiermee presenteren wij een vernieuwende, originele, niet op antibiotica gebaseerde methode om de efficiëntie waarmee macrofagen stafylokokken uit een infectieuze biofilm verwijderd worden, te verhogen.

Macrofagen zijn niet de enige cellen die in actie komen wanneer een biomateriaal in het lichaam geïmplanteerd wordt. Weefselcellen zullen proberen het oppervlak te koloniseren. Wanneer bacteriën aanwezig zijn, zal er concurrentie ontstaan. In een *in vitro*-model wordt deze wedloop nagebootst voor verschillende bacteriestammen en menselijke U2OS osteoblast-achtige cellen, die een poly(methylmethacrylate) oppervlak koloniseren. De rol van macrofagen in deze wedloop werd onderzocht, zoals beschreven in **Hoofdstuk V**. Bacteriën werden gezaaid op het oppervlak onder een afschuifsnelheid van 11 $s^{-1}$ voorafgaand aan adhesie van U2OS cellen en macrofagen. Vervolgens werden bacteriën, U2OS-cellen en macrofagen tegelijk geïncubeerd onder lage afschuifsnelheid (0.14 $s^{-1}$). De uitkomst van de wedloop tussen bacteriën en U2OS-cellen om te hechten aan het oppervlak was voor een groot deel afhankelijk van de virulentie van de bacteriën. Wanneer macrofagen afwezig waren veroorzaakten sterk virulente *S. aureus* of *Pseudomonas aeruginosa* verhoogde celdood van U2OS cellen binnen 18 uur na gelijktijdige incubatie. Daarnaast veroorzaakten deze stammen ook celdood ondanks fagocytose van gehechte bacteriën in de aanwezigheid van uit een muis afkomstige macrofagen. U2OS cellen zijn dus gedoemd om de strijd tegen *S. aureus* of *P.*

*aeruginosa* om een biomateriaaloppervlak te verliezen, zelfs in aanwezigheid van macrofagen. Laag-virulente *S. epidermidis* daarentegen veroorzaakte geen celdood bij U2OS-cellen, zelfs niet na 48 uur, ongeacht of er macrofagen aanwezig waren of niet. Vanuit de kliniek is bekend dat *S. aureus* en *P. aeruginosa* acute en ernstige BAI op kunnen leveren, in tegenstelling tot *S. epidermidis*, die meer laaggradige infecties veroorzaakt. Geconcludeerd kan worden dat het beschreven model eigenschappen bezit die overeenkomen met klinische observaties en daarom potentieel heeft voor verder gebruik in het onderzoek naar de gelijktijdige strijd om het oppervlak tussen weefselcellen en pathogene bacteriën in aanwezigheid van componenten van het immuunsysteem.

Het thema mobiliteit van macrofagen werd verder uitgewerkt door bacteriën te plaatsen op PEG-coatings waarin volgens een bepaald patroon gebiedjes aangebracht zijn waarin de bacteriën zich aan kunnen hechten, zoals beschreven in **Hoofdstuk VI**. Gevarieerd werden de diameters en onderlinge afstand tussen de adhesie-gebiedjes om verschillende adhesie-oppervlakken van glas te verkrijgen in een PEG-matrix. Patronen waarbij de adhesie-gebiedjes een diameter hadden in de orde van grootte van 2 – 5 μm en met een onderlinge afstand die groter was dan de diameter van het adhesie-gebiedje stimuleerden aanhechting, spreiding en groei van osteoblasten en tegelijkertijd verminderden dergelijke patronen kolonisatie door stafylokokken. Daarnaast waren macrofagen meer effectief bij het verwijderen van gehechte stafylokokken van oppervlakken met een patroon dan van een volledige PEG-matrix of van glas, omdat de groei van de stafylokokken slechts beperkt was tot de adhesie-gebiedjes. Deze nieuwe methode, waarbij de groei van bacteriën tot een bepaald gebied beperkt wordt, ondersteunde macrofagen bij hun zoektocht naar gehechte bacteriën.

Een visie op de behandeling van biomateriaal-geassocieerde biofilms in de toekomst op basis van de experimenten in dit proefschrift beschreven, wordt gegeven in de algemene discussie (**Hoofdstuk VII**).

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*Charles Darwin*

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