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Macrophage-mediated phagocytosis of bacteria adhering on biomaterial surfaces

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Da Silva Domingues, J. (2014). *Macrophage-mediated phagocytosis of bacteria adhering on biomaterial surfaces*. [Thesis fully internal (DIV), University of Groningen]. s.n.

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CHAPTER I

General Introduction:
Bridging the gap between *in vitro* and *in vivo* evaluation of biomaterial-associated infections

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ABSTRACT

Biomaterial-associated infections 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 postoperative 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 biomaterial-associated infections has been intensively studied before *in vivo*, until recently no *in vitro* methodology existed for this purpose. Just very recently 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, we here describe bi-culture (interactions between bacteria and osteoblasts) and tri-culture (interactions between bacteria, osteoblasts and immune cells) experiments that allow better evaluation of multi-functional coatings *in vitro* and therewith bridge the gap between *in vitro* and *in vivo* studies.

GENERAL INTRODUCTION

Biomaterials-associated infections

Biomaterials play an important role in modern medicine in the restoration and maintenance of tissue, organ or body function. A current estimate of the number of total hip replacements in the world is approximately one million a year, while the number of knee replacements is more than 250,000 [1]. One of the major problems emerging from the use of biomaterial implants and medical devices is biomaterial-associated infection (BAI). On average, BAI occurs in approximately 0.5 – 6% of all cases [2,3], strongly depending on the implant site, and more often in cases of trauma or revision surgery [4-6]. Upon adhesion to a biomaterial surface, bacteria start to synthesize a hydrated matrix of extracellular polymeric substances (EPS) to form a biofilm [7]. Biofilm formation on an implant surface is a multi-phase process [8] starting with reversible adhesion of single bacteria, transition to an irreversible state through physicochemical processes and EPS production followed by bacterial growth. BAI is difficult to treat, as the biofilm mode of growth protects the infecting organisms against the host immune system and antibiotic treatment [9]. Bacteria within biofilms generally require 500 – 5,000 times higher doses of antibiotics than planktonic ones suspended in body fluids [8]. In the majority of cases, the final outcome of a BAI is removal of the implant. There are various routes along which microorganisms can gain access to the implant site and cause BAI [10]. The best-documented route is direct contamination of an implant during surgery (perioperative contamination) [10]. BAI can also develop however, due to bacteria gaining access to the implant site immediately post surgery during hospitalization (postoperative contamination) or through microbial spreading via the blood circulation from infections elsewhere in the human body [10-12]. Irrespective of the

route of infection, the fate of a biomaterial implant depends mainly on the outcome of the so-called "race for the surface" between successful tissue integration of the biomaterial implant and bacterial colonization [10].

Routes of infection and infecting organisms

Perioperative contamination implies that an implant becomes contaminated with bacteria before or during implantation into the human body. It is known that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on a wound is about 270 bacteria/cm^2 [13]. Bacterial counts in the operating theatre are generally higher during periods of operating room personnel activity and when more people are present [13]. More recently, through the use of modern, better ventilated operation theatres (20 changes of air per hour) and impermeable patient and personnel clothing, perioperative bacterial contamination may well be less than 270 bacteria/cm^2 falling into the wound area [14].

The second route of infection is postoperative contamination, which may occur during the period of hospitalization immediately post surgery, as caused by direct contamination of open wounds or by the use of invasive devices like infusion tubes, catheters, or drains. Both perioperative and postoperative contamination can cause BAI many years after implantation, because many bacterial strains are known to be able to stay on an implant surface in a low metabolic state for several years post surgery [10]. These bacteria will favor the development of BAI, especially in immunocompromised host [10].

As a third route, BAI can result from hematogenous spreading of bacteria from infections elsewhere in the body to an implanted biomaterial. Hematogenous spreading of bacteria may result from skin infections, surgical or dental interventions, pneumonia, abscesses or bacteremia, which can all cause temporal or chronic

bacteremia resulting in infections [11]. Immune cells such as macrophages may also play a role in transporting bacteria to the implant site, as some strains are capable to survive within macrophages [15,16]. Importantly, BAI due to hematogenous spreading of bacteria to an implant site may occur any time after implantation. Notorious in this respect are abscesses underneath the skin, developing for instance after minor injuries. Dental treatment is also known to be a cause, as even routine inspection of the dentition by the dentist or oral hygienist may give rise to bacteremia [17,18].

In general, *Staphylococcus epidermidis* and *Staphylococcus aureus* are the most frequently isolated pathogens from infected biomaterial implants or devices, but also Gram-negative organisms as *Escherichia coli* and *Pseudomonas aeruginosa* are isolated [8,9,19]. Almost 50% of infections associated with catheters, artificial joints, and heart valves are caused by *S. epidermidis* [20]. *S. aureus* is the cause of around 23% of infections associated with prosthetic joints, especially their metal parts [20]. *P. aeruginosa* is the causative organism in around 12% of hospital-acquired urinary tract infections, 10% of bloodstream infections, and 7% of all hip- and knee-joint infections [21]. Another factor that plays an important role in the pathogenesis of BAI is the bacterial virulence [20]. *S. aureus* and *P. aeruginosa* infections usually progress much more aggressively than BAI caused by *S. epidermidis* [20,21]. *S. aureus* appears more frequently in acute infections, within 4 weeks after surgery of complicated total joint arthroplasty compared to *S. epidermidis. S. epidermidis* strains are a common cause of "late" infections [20] and are most commonly implicated in delayed septic loosening of total joint prostheses [22] or even in presumed aseptic loosening [23], indicating their low virulence with only minor clinical symptoms of infection. *Pseudomonas* is also more virulent than *S. epidermidis*, which is ascribed to the more aggressive endotoxins in its EPS. The low virulence of *S. epidermidis* strains

compared to *S. aureus* or *P. aeruginosa* is due to the lack of additional genes responsible for producing tissue-damaging toxins [22-24]. In *S. epidermidis* infections, biofilm formation is considered the only virulence factor as the organism lacks the genes to produce toxins and tissue-damaging exoenzymes that are produced by for instance *S. aureus,* and therefore infections are usually subacute or chronic [24-27].

Bacterial and mammalian cell adhesion to biomaterials: a race for the surface

The introduction of bacteria by any of the above routes is the initial step in the development of BAI, although often preceded by adsorption of macro-molecular components from body fluids, such as tear fluid, saliva, serum or plasma to yield the so-called conditioning film on the biomaterial surface [11]. Bacterial adhesion can be mediated by high affinity, localized interactions between cell surface structures and specific molecular groups on a substratum surface, or overall interaction forces, such as Lifshitz-Van der Waals, electrostatic, and acid-base interactions. Conditioning films can greatly affect the interaction forces in bacterial adhesion. Many adsorbed proteins reduce bacterial adhesion, but others, like fibronectin and fibrinogen, have been shown to promote adhesion of certain *S. epidermidis* and *S. aureus* strains [8].

The best protection against BAI is rapid and complete tissue integration of an implant surface, although not all implants and devices require or allow tissue integration. Mammalian cells can adhere to both bare implant surfaces as well as to implant surfaces with adsorbed conditioning films through integrins and other receptors present upon the cell surface. Therefore, it is important to control protein adsorption to biomaterial surfaces in order to stimulate tissue integration [28], especially since mammalian cells have high affinity to the arginine-glycine-aspartic acid (RGD) motive, which is present in a number of different extracellular matrix

proteins, such as fibronectin, laminin, collagen and vitronectin [29]. Unfortunately, surface chemistries that promote tissue integration very often also mediate bacterial adhesion, like conditioning films comprising fibronectin or fibrinogen [8].

The realization that it may well be impossible to design a surface modification that discourages bacterial adhesion and at the same time encourages tissue integration is catching on. Polymer brush coatings for instance, consisting of a layer of adsorbed, highly hydrophilic poly(ethylene glycol) (PEG) in a brush configuration, resist not only protein adsorption and bacterial adhesion, but also adhesion and spreading of mammalian cells [30,31], which are imperative for successful tissue integration. This realization has led to the development of multifunctional coatings. Multifunctional coatings can be based on a non-adhesive PEG base, decorated with RGD-sequences to promote mammalian cell adhesion and spreading, but due to a low decoration density do not increase bacterial adhesion [30,32]. The use of such bifunctional coatings enables direct control over the outcome of the race for the surface, an expression coined in 1987 by the orthopedic surgeon Anthony Gristina to describe the fate of a biomaterial implant [9].

The fate of a biomaterial implant was depicted as a race between bacterial adhesion and biofilm growth on an implant surface *versus* tissue integration (Figure 1). If the race is won by tissue cells, then the surface is covered by tissue and less vulnerable to bacterial colonization. On the other hand, if the race is won by bacteria, the implant surface will become colonized by bacteria.

Although the concept of the race for the surface stems from 1987, it was only recently that various groups have proposed co-culture experiments to evaluate the simultaneous response of bacteria and mammalian cells on a surface. Co-culture experiments allow the *in vitro* evaluation of multifunctional coatings and therewith bridge the gap between *in vitro* and *in vivo* studies.

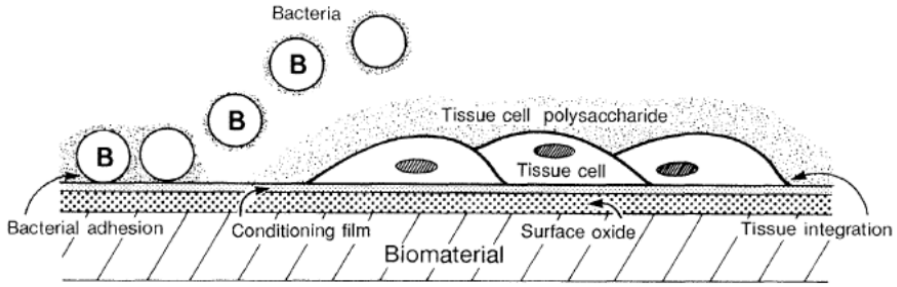

Figure 1. The race between bacteria and tissue cells for the biomaterial surface [9].

Bridging the gap between in vitro and in vivo studies

In the path towards the reduction of infection risk, biomaterials research has been focused on the development of biomaterials or coatings that can prevent bacterial adhesion and stimulate mammalian cell growth. 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 [32-37]. Shi *et al.* [32] showed that a surface composed of chitosans and RGD-containing peptides discouraged bacterial adhesion but enhanced osteoblast attachment and subsequent differentiation as indicated by increased alkaline phosphatase activity. Dexter *et al.* [33] in a study suggested that an optimal concentration of seeded 3T3 fibroblasts and conditions to stimulate cell adhesion without stimulating bacterial adhesion could probably reduce infection. Ploux *et al.* [37] showed an opposite adhesion behavior of bacteria compared to human osteoprogenitor cells on the nano-patterned surfaces prepared by pulsed plasma polymerization and UV-irradiation. In none of these *in vitro* studies, the effects of the bacterial presence on mammalian cell attachment to a biomaterial surface, cell spreading, and growth were studied, which could completely change the fate of the biomaterial implant according to the concept

of the "race for the biomaterial surface". Even though the concept of the race for the surface in biomaterial-associated infections has been intensively studied before *in vivo*, until recently no *in vitro* methodology existed for this purpose.

Bacterial-mammalian cell interactions

As a first step towards bridging the gap between *in vitro* and *in vivo* studies, an *in vitro* model was developed to investigate bacterial biofilm formation and mammalian cell growth in a single experiment based on the perioperative infection route [38]. In this study, bacteria were allowed to adhere to a biomaterial surface prior to mammalian cell adhesion and spreading to mimic the clinical situation where an implant becomes contaminated prior to implantation (perioperative contamination). The outcome of the competition between *S. epidermidis* ATCC 35983 and U2OS osteoblast-like cells appeared to be dependent on the number of bacteria present prior to mammalian cell seeding and the absence or presence of fluid flow. Mammalian cells lost the competition in the absence of flow probably due to the accumulation of bacterial toxins, but were able to grow when culture medium was continuously refreshed by flowing medium in a flow chamber [38]. A further study on the race for the surface on different biomaterials demonstrated that mammalian cell interactions with biomaterials were hampered by bacterial biofilm formation on some of the most commonly used biomaterial surfaces [39]. Yet, PMMA showed better mammalian cell adhesion and spreading in the presence of adhering *S. epidermidis* ATCC 35983 than other biomaterials [39]. A study on comparison of different bacterial species involved in BAI on the competition between bacteria and mammalian cells showed that mammalian cells are bound to lose the competition for the surface in the presence of adhering, highly virulent *S. aureus* and *P. aeruginosa* [40]. Buchholz and co-workers [41] showed that in a group of 64 BAI

patients with an infection by a low-virulent organism 54 (84%) were free from infection 2 years later. Alternatively, when *S. aureus* was the causative organism, recurrent infection occurred in 28% of all patients [41], and almost 50% of the patients with a Pseudomonas infection experienced a relapse. The low virulence of *S. epidermidis* strains compared to *S. aureus* or *P. aeruginosa* is supposed to be due to the lack of additional genes responsible for producing tissue-damaging toxins [21,22,24]. In *S. epidermidis* infections, biofilm formation is considered the only virulence factor and therefore infections are usually subacute or chronic [25-27]. Bennion *et al.* [42] experimentally showed that with bacterial strains such as *S. aureus*, contamination of an implant is inoculum dependent. When the critical inoculum size is reached, local host defenses are overwhelmed and an infection is established [43].

In the concept of the race for the surface, tissue integration is an important protective factor against bacterial contamination of an implant surface [6]. Based on postoperative contamination, the effects of different degrees of mammalian cell coverage on the balance between bacterial biofilm formation and mammalian cell growth were investigated [44]. Mammalian cell growth was severely impaired when bacteria were introduced on surfaces with a low initial mammalian cell density (2.5×10^4 cells/cm^2) but in the presence of higher initial cell densities (17×10^4 cells/cm^2), contaminating staphylococci did not affect cell growth (Figure 2) [44].

Bacterial-macrophages interactions

In a healthy host, the host immune system comes to the aid of tissue cells [45]. Macrophages are one of the most predominant immune cells that arrive within minutes to hours at an implant site and can remain at a biomaterial surface for several weeks to orchestrate the inflammatory process and foreign body reactions

[45]. During infection, macrophages detect bacteria via cell surface receptors that bind to bacterial ligands and opsonines [46-48]. Subsequently, macrophages ingest pathogens and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to destroy phagocytozed microorganisms and recruit other cells from the adaptive immune system [48]. However, it has been shown that the presence of a foreign body may impair the host immune system and consequently low numbers of adhering bacteria can already be sufficient to cause a BAI [49].

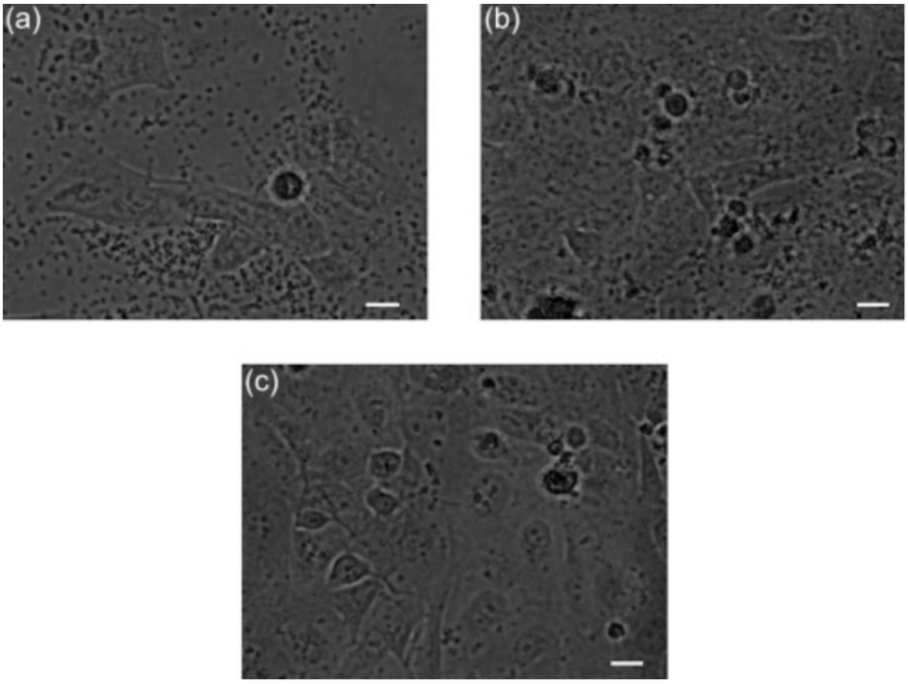

Figure 2. Phase-contrast images of U2OS cells seeded to a density of (a) 2.5×10^4 cells/cm^2, (b) 8.2×10^4 cells/cm^2 and (c) 17×10^4 cells/cm^2 after 24 h of simultaneous growth in the presence of adhering *S. epidermidis* ATCC 35983 on PMMA. Scale bars 10 μm [44].

In general, immune cells migrate, engulf, and kill invading microorganisms [50-52]. A previous study on the simultaneous interaction between macrophages and colonizing *S. epidermidis* showed that macrophage behavior is surface dependent [53]. Macrophage migration towards bacteria and phagocytosis was enhanced on highly hydrated, cross-linked PEG-based polymer coatings compared to uncoated substrata due to the weak adhesion of macrophages and bacteria to the PEG coating [53].

Kubica *et al.* [54] showed that intracellular *S. aureus* can survive within human macrophages for several days until bacteria escaped the intracellular confinement, proliferated in the conditioned medium, and killed the cells. Garzoni *et al.* [55] indicated that some coagulase-negative staphylococci could promote infection by intracellular colonization.

Bacterial-mammalian cells-macrophages interactions

The pathogenesis of BAI is complex and depends on factors such as bacterial virulence, physicochemical properties of the biomaterial surface, and alterations in the host defense [49]. Several studies have demonstrated that immune cells lose their ability to kill bacteria in the presence of a biomaterial [52,56-58]. Neutrophils exhibited decreased bactericidal activity and reduced superoxide production in the presence of extracellular slime producing *S. epidermidis* [59-63], while phagocytized *S. aureus* suppressed the production of superoxide inside macrophages [58]. In a murine model, high numbers of *S. epidermidis* could not only persist within macrophages in pericatheter tissue without showing any signs of inflammation [49], but were also able to proliferate. Macrophages in the periphery of a biomaterial *in vivo* showed deficient intracellular killing of pathogens, resulting in a compromised local host defense [49]. *In vivo,* bacteria may well survive inside macrophages for

prolonged periods of time. These bacteria will favor the development of BAI, especially when the physical condition of a patient disturbs the balance between bacteria and the host response [49].

In addition to bi-culture models, a tri-culture model was developed to further bridge the gap between *in vitro* and *in vivo* evaluation of biomaterials [40]. In tri-cultures, bi-cultures of mammalian cells and bacteria are supplemented with macrophages. Macrophages can prolong the time that U2OS cells can survive an attack by adhering *S. aureus* and *P. aeruginosa* on PMMA surface. However, adhering *S. aureus* and *P. aeruginosa* still cause death of all adhering U2OS cells and macrophages within 10 – 14 h of growth.

In summary, it is important to be aware of conditions distinguishing *in vitro* and *in vivo* methods. *In vitro* models can be used to study the short-term mechanisms by which bacteria, mammalian and immune cells and the biomaterial interact, whereas *in vivo* models include long-term treatment effects and biological integration. Despite these limitations, the *in vitro* models described open possibilities to address many of these processes step by step and to unravel underlying mechanisms in the field of biomaterial-associated infections.

AIMS OF THE THESIS

Biomaterial-associated infections are a common complication associated with the use of biomaterial implants and devices. In a healthy individual, the immune cells are usually very efficient in removing planktonic pathogens; however they are not able to eliminate biofilms, especially not when on a biomaterial surface, while antibiotics, when effective at all against biofilms, are rapidly losing their efficacy. Alternative ways to prevent and cure infections associated with the use of biomaterial implants and devices while promoting tissue integration need to be explored. Therefore the aims of this PhD thesis were to

(i) develop a model for studying and characterizing the interaction between immune cells, in specific macrophages, and bacteria;

(ii) to 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.

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