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Bridging the gap between in vitro and in vivo evaluation of biomaterials-associated infections

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Contents

CHAPTER 1

General Introduction

Biomaterial-Associated Infections

Biomaterials play an important role in modern medicine in the restoration of tissue, organ or body function. For example, a current estimate of the number of total hip replacements in the world amounts approximately one million per year and knee replacements more than 250,000. More than 10% of all hospitalized patients are using urinary catheters, biomaterial implants or other medical devices. The use of biomaterial implants and medical devices is mainly restricted by complications due to biomaterial-associated infections (BAI). On average, BAI occurs in approximately 0.5 – 6% [1,2] of all cases, strongly depending on the implant site, and more often in cases of trauma or revision surgery [3-5]. The presence of a biomaterial in the body significantly compromises the host to cope with invading microorganisms. There are various routes along which microorganisms can enter the body and develop a BAI in the case of permanent implants [6]. The best-documented route is direct contamination of an implant during surgery (peri-operative contamination). BAI can also be initiated immediately post-surgery during hospitalization (post-operative contamination) or microbial spreading through blood from infections elsewhere in the human body [6-8]. These different routes of infection will be briefly discussed.

Routes of Infection

Peri-operative contamination means that an implant becomes contaminated with bacteria before implantation into the human body or during the implantation process. During the implantation of a biomaterial implant, microorganisms from the skin can reach the implant surface. 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 cm^{-2}. The

bacterial counts are generally higher during periods of activity and when more people are present in the operating room [9]. More recent, through the use of modern, better ventilated operating theatres (20 changes of air per hour) and impermeable patient and personnel clothing, peri-operative bacterial contamination levels may well be less [10].

The second route of infection is post-operative contamination which may occur during the period of hospitalization immediately post-surgery, caused by direct contamination of open wounds or by the use of invasive devices like infusion tubes, drains and catheters. Both peri-operative and post-operative infections can cause BAI many years after implantation, because bacteria on a biomaterial implant surface are known to be able to stay on the implant in a low metabolic state for several years [6].

As a third route, BAI can result from hemotogenous spreading of bacteria from infections elsewhere in the body, which upon adhering to a biomaterial implant surface form a biofilm. This includes skin infections, surgical or dental interventions, pneumonia, abscesses or bacteriuria, which can cause temporal or chronic bacteremia resulting in BAI [7]. It is also shown that macrophages play a major role in transporting bacteria to the implant surface, as some strains are capable to survive within macrophages [11,12].

Microbial Adhesion to Biomaterials

The introduction of bacteria by any of the above routes to the biomaterial surface is the initial step in the development of BAI. Microorganisms can reach the biomaterial surface as early as during implantation and interact with bare substratum surfaces, not even covered with a conditioning film, i.e. a film of adsorbed serum or plasma

proteins [8]. In general, microbial adhesion is mediated by the specific interactions between cell surface structures and specific molecular groups on the substratum surface, or by non-specific interaction forces, such as Lifshitz-Van der Waals, electrostatic charge, acid-base interactions and Brownian motion forces. Irrespective of the presence or absence of a conditioning film, the physico-chemical surface properties of the biomaterial and microorganisms play an important role in the adhesion process. Conditioning films on biomaterial surfaces and on bacterial cell surfaces play important roles too by changing the physico-chemical surface properties. Most proteins in conditioning films are capable of reducing microbial adhesion, but fibronectin and fibrinogen have been shown to promote the adhesion of certain *Staphylococcus epidermidis* and *Staphylococcus aureus* strains [13]. Microbial adhesion mechanisms in post-operative infections or hematogeneous infections are unclear. Biomaterial implants are usually not completely integrated with host tissue, especially when they consist of metal parts which are not easily colonized by host tissue cells. An uncovered metal surface can be a site for microbial colonization. Moreover, it is possible that by repeated hinging of orthopedic implants, cell/tissue damage can occur, providing adhesive sites for microorganisms [6].

Once microorganisms adhere to the biomaterial surface, the second step is the subsequent growth of the adhering bacteria, accompanied by production of Extracellular Polymeric Substances (EPS), leading to biofilm formation, which protects the infecting organisms against host immune system and antibiotic treatment [14,15]. Eradication of a biofilm is difficult because the populating bacteria are protected from the immune system and antibiotics. Bacteria within biofilms generally require 500-5000 times higher doses of antibiotics than planktonic ones suspended in

body fluids [16]. In the majority of cases, the final outcome of a BAI is removal of the implant.

Pathogens Causing BAI

In general, *S. epidermidis* and *S. aureus* are the most frequently isolated pathogens from infected biomaterial surfaces. Additionally isolated organisms include *Escherichia coli* and *Pseudomonas aeruginosa* [6,15,17]. Almost 50% of infections associated with catheters, artificial joints and heart valves are caused by *S. epidermidis* [18]. *S. aureus* is the cause of around 23% of infections associated with prosthetic joints [18]. *P. aeruginosa* is the causative organism of around 12% of all hospital acquired urinary track infections, 10% of bloodstream infections and 7% of hip-joint infections [19]. *S. epidermidis* are the common cause of late infections [18].

Another factor that plays an important role in the pathogenesis of BAI is the bacterial virulence [18]. *S. aureus* and *P. aeruginosa* infections usually progress much more aggressively than BAI caused by *S. epidermidis* [18,19]. *S. aureus* appears more frequently in acute infections within 4 weeks after surgery, compared to *S. epidermidis*. *S. epidermidis* is most commonly implicated in delayed septic loosening of total joint prostheses [20] or even in presumed a-septic loosening [21], indicating its low virulence with only minor clinical symptoms of infection. Pseudomonas is also much more virulent than *S. epidermidis,* which is ascribed to the more aggressive endotoxins in the slime. 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 [18,19,22]. In *S. epidermidis* infections, biofilm

formation is considered the only virulence factor and therefore infections are usually sub-acute or chronic [23-25].

Mammalian Cell Adhesion to Biomaterials

Mammalian cell adhesion to a biomaterial implant surface is crucial for the successful integration of the implant within the host tissue. *In vivo*, when a biomaterial is implanted, it becomes immediately coated with proteins (conditioning film) that are adsorbed from the local body fluids. Depending on the body site, the surrounding fluid can be saliva, urine, tissue fluid, serum or blood and the conditioning film mostly consists of adsorbed proteins. The physico-chemical properties of the biomaterial surface (chemical composition, hydrophobicity and surface charge) control the nature of the adsorbed protein layer [26,27]. Cell adhesion to adsorbed proteins is mediated through integrins and other receptors present within the cell membrane. Upon adhesion to the protein film, a cascade of intracellular signaling events is triggered. Therefore, controlling protein adsorption on the biomaterial surface may be critical to control and direct cell responses to biomaterials. Unfortunately, proteins like fibronectin and fibrinogen in conditioning films have been shown to promote the adhesion of certain *S. epidermidis* and *S. aureus* strains [13].

The Race for the Surface

In 1987, the orthopedic surgeon Anthony Gristina coined the term "race for the surface" to describe the fate of a biomaterial implant in relation with the development of BAI [6], as illustrated in Fig. 1. The fate of a biomaterial implant was depicted as a

race between microbial adhesion and biofilm growth on an implant surface *versus* tissue integration. 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 and tissue cell functions are hampered by bacterial virulence factors [6]. Irrespective of the route of infection, the fate of biomaterial implants depends mainly on the outcome of the so-called 'race for the surface'.
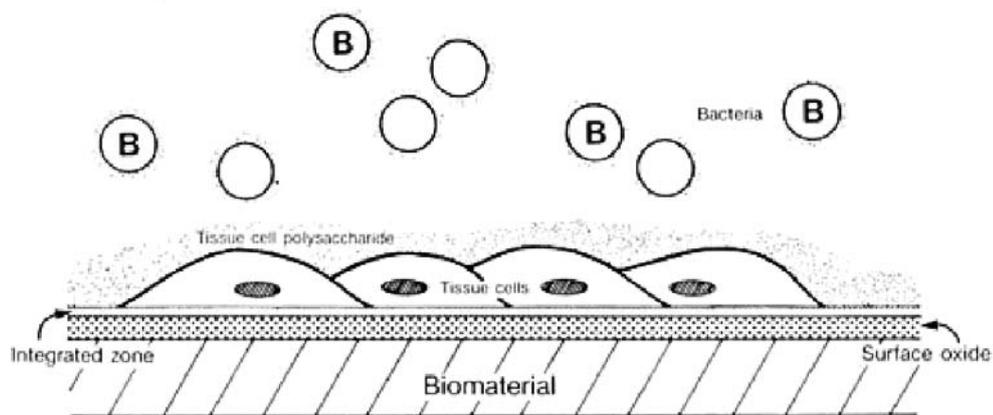
Fig. 1. Schematic diagram representing the race between bacteria and tissue cells for the biomaterial surface [6].

Bridging the Gap Between *In Vitro* and *In Vivo* Studies

In vivo, processes occurring at the biomaterial site are complex, involving multiple cell types depending on the site, cytokines, chemokines, excretion of bacterial and cell substances. Normalization of macrophage response on biomaterials allows appropriate antibacterial activities, healing and integration. During infection, macrophages play a key role in the elimination of bacterial colonization [17].

In the path towards reducing the risk of BAI, biomaterials research has been focused on the development of biomaterials or coatings that can prevent bacterial adhesion and stimulate mammalian cell growth. Till today, biomaterials or functional

coatings were evaluated *in vitro* either for their ability to resist bacterial adhesion or for their ability to support mammalian cell adhesion and proliferation based on the concept of the race for the surface [28-33]. Shi *et al*. [28] showed that a surface composed of chitosans and RGD-containing peptides discouraged bacterial adhesion but enhanced cell attachment and alkaline phosphatase activity. Dexter *et al*. [30] 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*. [33] 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.

However, none of the above studies have made an attempt to address the simultaneous effects of the presence of bacteria and mammalian cells on a biomaterial surface, which according to the concept of the 'race for the biomaterial surface', is crucial for the ultimate fate of a biomaterial implant. The reason for this omission is, that hitherto no methodology exists to this end. A proper method to study the race for the surface on an experimental basis, would constitute a valuable bridge between *in vivo* and *in vitro* studies.

Aim of this Thesis

The aim of this thesis is to develop a method that could bridge the gap between *in vitro* and *in vivo* studies on BAI (Fig. 2).
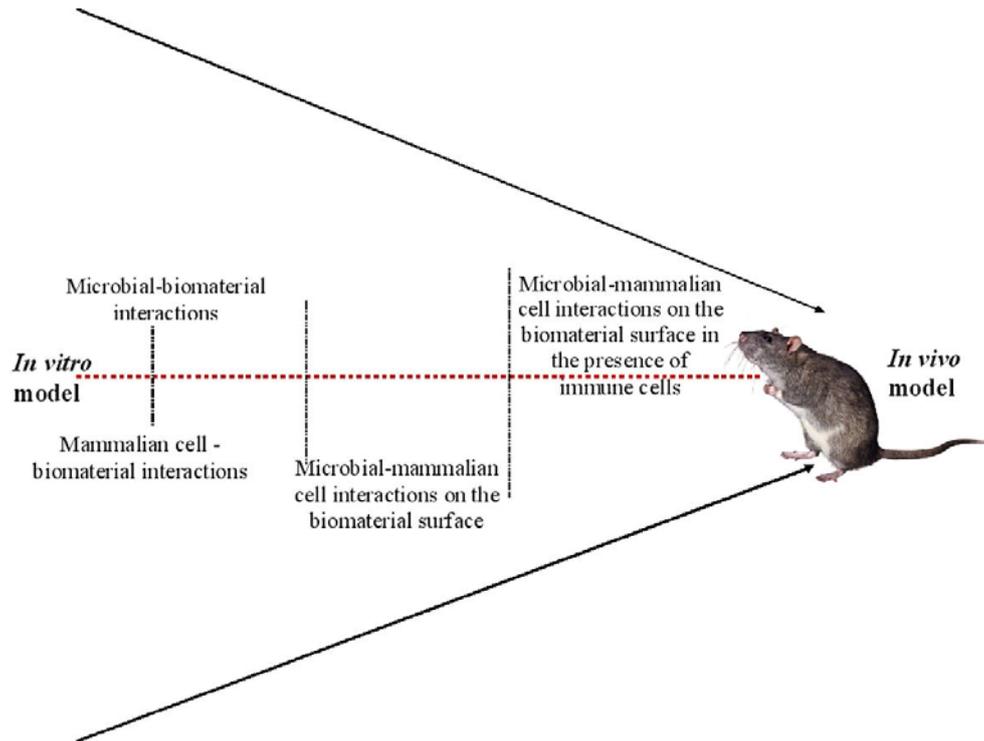
Fig. 2. Schematic diagram representing the bridge connecting the gap between traditional *in vitro* and *in vivo* studies on BAI.

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Microbial Biofilm Growth *versus* Tissue Integration: "The Race for the Surface" Experimentally Studied

Guruprakash Subbiahdoss, Roel Kuijer, Dirk W. Grijpma, Henny C. van der Mei, Henk J. Busscher

Introduction

Biomaterials play a major role in modern medicine for the restoration of function, frequently used examples being prosthetic joints or heart valves. Biomaterials-associated infections (BAI) pose a serious complication, which is of growing concern due to the increasing use of biomaterial implants and devices. On average, BAI occurs in approximately $0.5 - 6\%$ [1,2] of all cases, strongly depending on the implant site, and more often in cases of trauma or revision surgery [3-5]. BAI is difficult to treat, as the biofilm mode of growth protects the infecting organisms against the host immune system and antibiotic treatment [6,7]. In most cases, the final outcome of a BAI is removal of the implant. There are various routes along which a BAI can develop. The best-documented route is direct contamination of the implant during surgery (peri-operative contamination) or contamination during hospitalization [8-10]. Since microorganisms can remain dormant for several years on a biomaterial surface [9,11] inside the human body or in adjacent tissue [9], BAI can become clinically manifest years after insertion of an implant. Moreover, late BAI can develop by microbial spreading through blood from infections elsewhere in the human body, but evidence for haematogenous spreading is mainly anecdotal.

In 1987, the orthopedic surgeon Anthony G. Gristina coined the term "race for the surface" to describe the fate of biomaterial implants in relation with the development of BAI [9]. The fate of a biomaterial implant was pictured as a race between microbial adhesion and biofilm growth on an implant surface *versus* tissue integration. 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 rapidly covered by a biofilm and tissue cell functions are hampered by bacterial virulence factors and toxins. To the aid of the

implant, its surface may generate an inflammatory reaction at the tissue interface, resulting in the activation of the immune system which may hamper bacterial colonization [9,12]. Unfortunately, microorganisms are frequently introduced on an implant surface during surgery and *in vivo*, microorganisms start the race for the surface before tissue integration can occur.

The concept of the race for the surface has been embraced by many researchers in the field, but hitherto there has been no *in vitro* experimental methodology forwarded to study the actual race. New biomaterials or functional coatings are either evaluated for their ability to resist bacterial adhesion and biofilm formation [13-16] or for their ability to support tissue cell adhesion and proliferation [13,16-18]. The aim of this study is to describe an *in vitro* experimental methodology to investigate the race for the surface between bacteria and tissue cells in a single experiment.

Materials and Methods

U2OS cell culturing and harvesting. U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagle's Medium (DMEM) -low glucose supplemented with 10% fetal calf serum (FBS), 0.2 mM of ascorbic acid-2-phosphate (AA2P) denoted in the paper as DMEM+FBS. U2OS cells were maintained at 37°C in a humidified 5% CO_2 atmosphere, and cells were passaged at $70 - 90\%$ confluency using trypsin/EDTA.

Bacterial growth conditions and harvesting. *Staphylococcus epidermidis* ATCC 35983, originally isolated from human blood of a patient with an infected intravascular catheter, was used throughout this study. First, the strain was streaked on

a blood agar plate from a frozen stock and grown overnight at 37°C. The plate was then kept at 4°C. For each experiment, a colony was inoculated in 10 ml of tryptone soy broth (TSB; OXOID, Basingstoke, United Kingdom) and cultured for 24 h. This culture was used to inoculate a second culture in TSB, which was grown for 17 h prior to harvesting. Bacteria were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were sonicated on ice (3 x 10 s) in sterile PBS (10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) in order to break bacterial aggregates. This suspension was further diluted in sterile PBS to a concentration of 3×10^5 bacteria per ml.

Development of modified culture medium. In order to grow both *S. epidermidis* and U2OS cells simultaneously, a suitable medium had to be developed. To this end, bacterial medium (TSB) and tissue growth medium (DMEM+FBS) were combined in different ratios and growth rates of both *S. epidermidis* and U2OS cells were determined.

To determine U2OS cell growth, 1 ml U2OS cell suspension, containing 600,000 cells, was mixed in combined media with different amounts of TSB and seeded into T25 cell culture flasks. After incubation at 37°C in a humidified 5% CO_2 atmosphere for 48 h, cells were detached using trypsin-EDTA solution (Invitrogen, Breda, The Netherlands) and counted in a Bürker-Türk counting chamber. During incubation, cell adhesion, spreading and morphology were assessed every 24 h using phase-contrast light microscopy.

S. epidermidis ATCC 35983 was inoculated from agar plates in 10 ml of the combined media consisting of different amounts of TSB and (DMEM+FBS) for 24 h.

This culture was used to inoculate a second culture in combined media, which was grown overnight. Bacteria were counted using a Bürker-Türk counting chamber.

The medium composition showing optimal *S. epidermidis* and U2OS cell growth was chosen for further studies and will be denoted as "modified culture medium" in the remainder of this study.

Substratum. For ease of use (optimal transparency), glass was used as a substratum surface. Microscope glass slides were cleaned in a 2% RBS 35 detergent solution (Omniclean, Breda, The Netherlands) under sonication and thoroughly rinsed in demineralized water, methanol, water again and finally washed with sterile ultrapure water. This cleansing yielded full spreading of water, immediately after cleaning.

The race for the surface under static conditions. Glass slides were exposed in Petri dishes to different concentrations of *S. epidermidis* ATCC 35983 and incubated at 37°C for 30 min. Subsequently, the bacterial suspensions were removed by rinsing with PBS. Images of adhering bacteria were obtained using a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope (Leica Microsystems Ltd, Germany) with a x30 objective and bacterial adhesion was expressed as the number of bacteria adhering cm^{-2}. Subsequently, U2OS cells suspended in modified culture medium were seeded on bacterial-coated glass plates to a density of 20,000 cells cm^{-2}. *S. epidermidis* and U2OS cells were maintained at 37°C in a humidified 5% CO_2 for 48 h. Images were obtained using Leica DMIL microscope (Leica Microsystems Ltd, Germany) at x10 magnification after 48 h and analyzed using Scion image software.

The race for the surface under flow conditions. The parallel plate flow chamber and image analysis system have been described in detail previously [19]. The flow

chamber used was equipped with heating elements and kept at 37°C throughout the experiment. Bacterial and cellular deposition were observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Leica DM2000 (Leica Microsystems Ltd, Germany) with a x30 objective for bacteria and x10 objective for tissue cells.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, and before the addition of the bacterial suspension, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, the bacterial suspension in PBS was perfused through the chamber at the same shear rate and images were obtained as a function of time. As soon as the desired density of adhering bacteria (10^2 cm^{-2} or 10^5 cm^{-2}), was reached, flow was switched to sterile PBS in order to remove the bacterial suspension from the tubes and chamber. Subsequently, a U2OS cells suspension in modified culture medium was allowed to enter the flow chamber. Once the entire volume of buffer inside the chamber was replaced by cell suspension, flow was stopped for 1.5 h in order to allow cells to adhere and spread on the substratum. Finally, modified culture medium supplemented with 2% HEPES was perfused through the system at a low shear rate of 0.14 s^{-1} for 48 h without recirculation, and images were obtained continuously and analyzed real-time.

After 48 h of medium flow, the samples from the flow chamber were prepared for immunocytochemical staining to assess U2OS cell morphology and spreading. For fixation, glass slides with *S. epidermidis* and U2OS cells were placed in a Petri dish with 30 ml of 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5 min, the fixation solution was replaced by 30 ml of fresh CS for another 5 min. Subsequently

cells were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS and stained for

30 min with 5 ml PBS containing 49 µl DAPI and 2 µg ml^{-1} of TRITC-Phalloidin.

The cells on the glass slide were washed 4 times in PBS and examined with confocal

laser scanning microscopy (Leica DMRXE with confocal TCS SP2 unit). The number

of adhering cells per unit area and the average area per spread cell were determined

using Scion image software. For each density of adhering bacteria, 6 images (900 x

700 µm) per sample were randomly chosen and analyzed.

In order to assess the viability of U2OS cells adhering to the glass slide after

48 h of flow, vitality staining solution (2 µM calcein-AM and 3.4 µM ethidium

homodimer-1 (Molecular Probes Inc.) in PBS) was directly added to the adhering

U2OS cells, after which slides were left for 15 min in the dark at room temperature

with a coverslip on top.

Statistics. Experiments for each density of adhering bacteria both under static and

flow conditions were carried out in triplicate. Data are represented as a mean with

standard deviation. Statistical ANOVA analysis was performed, followed by a Tukey

HSD post-hoc test and a *P*-value of <0.05 was considered significant.

Results

Development of a modified culture medium. U2OS cells were cultured in different

ratios of DMEM+FBS and TSB media, but did not show any growth in media

containing more than 30% TSB (see Fig. 1). No changes in cell morphology were

observed, when U2OS cells were cultured in media containing 2% and 4% TSB

compared to cells grown in 100% DMEM+FBS. For higher percentages of TSB,

change in U2OS cell morphology and subsequent cell death were observed.

Analogously, the growth of *S. epidermidis* ATCC 35983 dropped considerably when the TSB content was less than 20% and close to zero in 100% DMEM+FBS medium (see also Fig. 1). From the above results, while considering U2OS cell morphology as the most important factor, it was decided to employ a modified medium consisting of 98% DMEM+FBS and 2% TSB for further experiments. In this modified growth medium U2OS cells had an advantage in growth over the bacteria, but both showed significant growth (see also Fig.1).
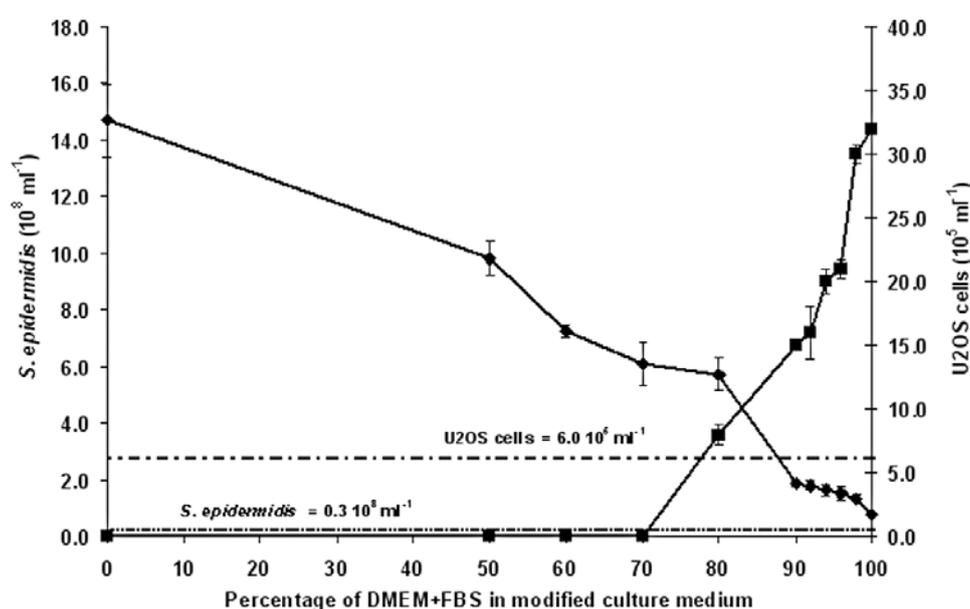
Fig. 1. Growth of U2OS cells (■) and *S. epidermidis* ATCC 35983 (♦) as a function of the percentage of DMEM+FBS in combination with TSB medium. Horizontal lines indicate the number of U2OS cells (dash-dotted) and bacteria (dotted) prior to growth. Growth was registered after 48 h.

Adhesion of U2OS cells in the presence of *S. epidermidis* - static versus flow conditions. The response of U2OS cells to the presence of adhering staphylococci under static conditions *versus* low fluid flow is shown in Fig. 2. In the absence of staphylococcal adhesion, slightly more ($p < 0.05$) U2OS cells adhere after 48 h under static conditions than under flow. Under static conditions and in the presence of adhering staphylococci however, U2OS cell adhesion was greatly reduced as

compared to the control, i.e. in the absence of adhering bacteria ($p < 0.05$). Under medium flow conditions, the number of adhering U2OS cells was significantly reduced for both staphylococcal concentrations as compared to the control ($p < 0.05$).
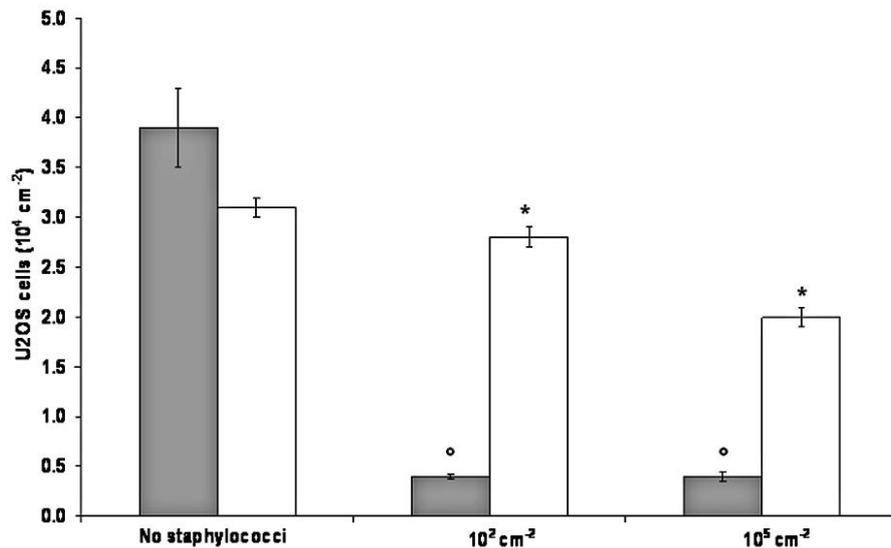
Fig. 2. Number of U2OS cells seeded on a glass substratum after 48 h under static conditions (■) and under medium flow (□) and in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates, with separate bacterial and cell cultures. *,° Significantly different ($p < 0.05$) from the control (absence of staphylococci).

Under static conditions and irrespective of the number of *S. epidermidis* initially present, floating granular particles were observed and interpreted as cell debris, indicating U2OS cell death. In the presence of *S. epidermidis*, U2OS cells showed better spreading and survival rate under flow as compared to static conditions. This is due to the continuous flow, which likely removes the majority of bacterial endotoxins produced. Bacterial endotoxin-induced cell death was confirmed by culturing U2OS cells in growth medium consisting of 50% fresh modified growth medium and 50% supernatant from a 48 h mixed U2OS cell and bacterial culture. No growth and subsequent U2OS cell death was observed in this mix of fresh and spent medium,

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confirming that bacterial endotoxins are responsible for cell death during the race for the surface under static conditions. Fluorescent dead-live stain, comprising of calcein-AM and ethidium homodimer-1, also confirmed that adhering U2OS cells were alive in the presence of *S. epidermidis* after 48 h under flow, as shown in Fig. 3.
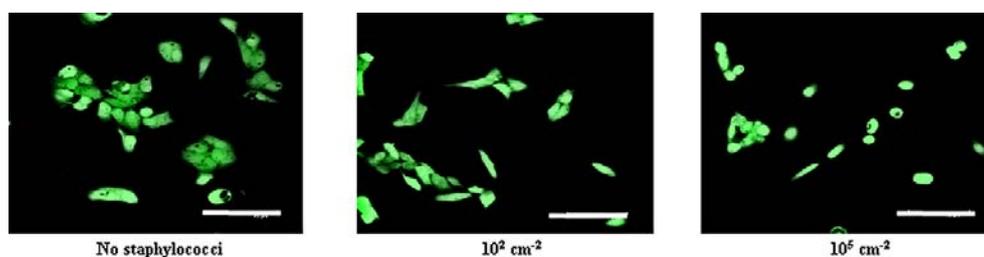
Fig. 3. CSLM live-dead images of U2OS cells after 48 h on glass under medium flow in the absence and presence of adhering staphylococci. Bar denotes 75 μm.

Influence of the number of adhering *S. epidermidis* on adhesion and spreading of U2OS cells under medium flow. Adhesion and spreading of U2OS cells after 1.5 and 48 h of growth in the presence of different number of adhering *S. epidermidis* are shown in Fig. 4. After 48 h the number of adhering bacteria had also increased due to growth, which made it difficult to analyze the cell number and spreading. For this reason, the adhering U2OS cells were stained and analyzed by CLSM (see Fig. 5).
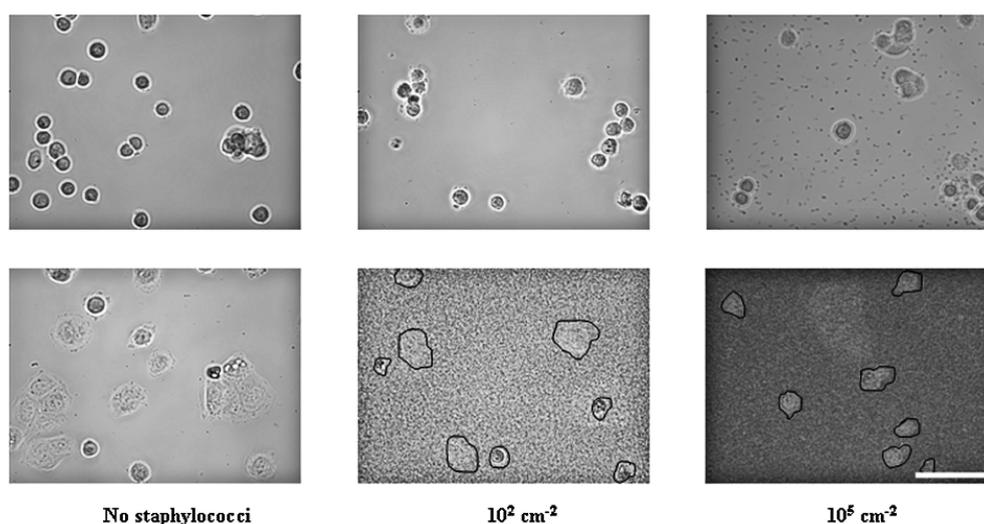
Fig. 4. U2OS cells adhesion and spreading after 1.5 h (top series) and 48 h (bottom series) under medium flow on a glass substratum in the absence and presence of adhering staphylococci. Bar denotes 75 μm. U2OS cells are differentiated by a contour line from *S. epidermidis* biofilm.

U2OS cells showed highest spreading in the absence of staphylococci. The extent of spreading was reduced with increasing density of adhering staphylococci and in the presence of adhering staphylococci many non-spread U2OS cells were observed. Quantitative analysis of cell spreading in the absence and presence of staphylococci is shown in Fig. 6. After the initial adhesion of U2OS cells for 1.5 h, the average area of U2OS cells on all surfaces was between 250 μm^2 and 380 μm^2 per cell. After 48 h of flow, significant differences were observed in the average area per cell as a result of varying densities of adhering *S. epidermidis*. In the absence of *S. epidermidis*, the average area of a spread cell was 960 μm^2 after 48 h, but in the presence of 10^5 staphylococci cm^{-2} spreading decreased to approximately 390 μm^2 per cell.

No staphylococci 10^2 cm^{-2} 10^5 cm^{-2}
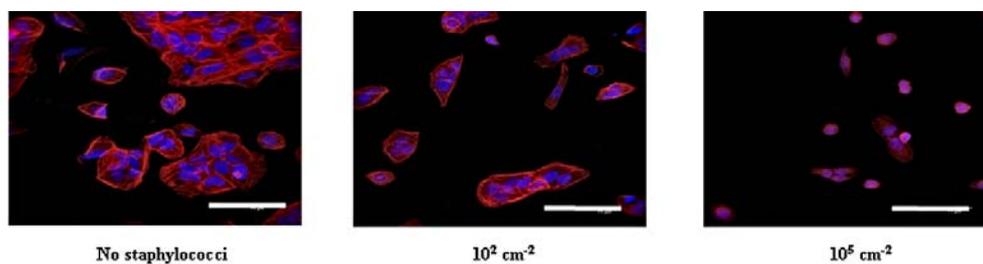
Fig. 5. CLSM images of U2OS cells after 48 h on glass under medium flow in the absence and presence of adhering staphylococci. U2OS cells were stained with 5 ml PBS containing 49 μl DAPI and 2μg ml^{-1} of TRITC-Phalloidin. Bar denotes 75 μm.
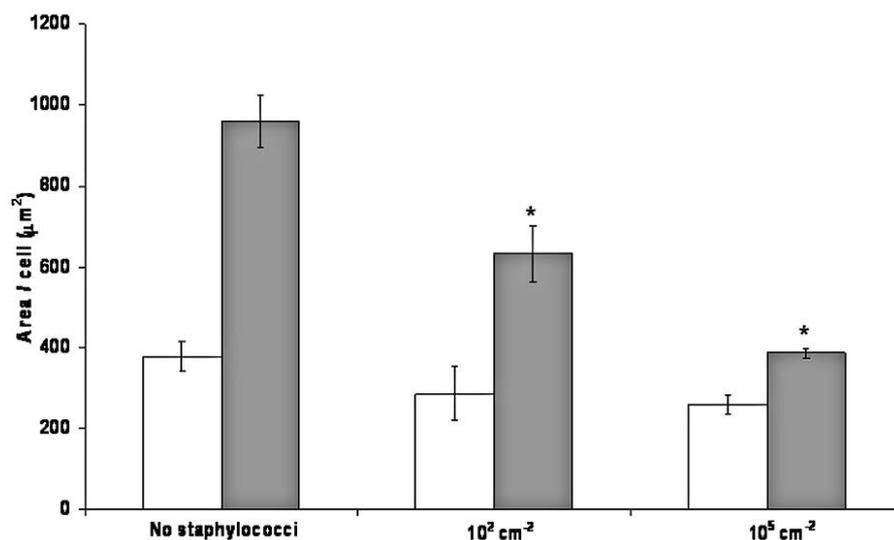
Fig. 6. Average area per spread U2OS cell after 1.5 h (□) and 48 h (■) of growth on glass under medium flow in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates with separate bacterial and cell cultures. * Significantly different (p<0.05) from the control (absence of staphylococci).

Discussion

This paper presents the first experimental set-up to study the race between bacteria and tissue cells for a biomaterial surface *in vitro*. The need for such a system is enormous at current, as many coatings that are propagated to attract low numbers of adhering bacteria, such as polymer brush coatings [15,20], may also be expected to support poor adhesion and spreading of tissue cells, which is currently stimulating the development of bi-functional coatings that support cell spreading and repel microorganisms at the same time. This development necessitates the use of a methodology, as described in this manuscript. Under static culturing conditions, U2OS cells did not have a chance to win the race for the surface and detached from the surface in the presence of adhering staphylococci. Under medium flow, however, U2OS cells remained adhering in the presence of adhering staphylococci and spread

more when the density of adhering staphylococci was lower. Therewith we have presented a method for the evaluation of biomaterial coatings that encompasses tissue cell adhesion and spreading as well as bacterial growth.

The effects of two different densities of adhering bacteria were evaluated in this study. Bacteria were allowed to adhere prior to cell adhesion and spreading, which is a fine-tuning of the model toward peri-operative bacterial contamination of implant surfaces. It has been documented that during an operation procedure of 1 h [21], the total number of bacteria-carrying particles falling on a wound is about 270 cm^{-2}, while the risk of infection depends on the number of viable bacteria present in the wound area at the time of wound closure. Bacterial counts are generally higher during periods of activity or during increased numbers of personnel in the operation theatre [21]. The presence of a biomaterial implant in the body usually stimulates infection by a smaller inoculum of bacteria than in non-biomaterial surgery [22]. Hence, the densities of adhering bacteria prior to cell adhesion employed in this study between 10^2 and 10^5 bacteria cm^{-2} may be considered relevant for minimum and maximum contaminations occurring clinically.

U2OS cell response was studied under static conditions and flow. *In vivo*, fluid is continuously flowing through the network of fine channels of osteocytes to facilitate the diffusion of nutrients and waste products from the bone surface to deeply buried osteocytes and vice versa [23]. Using computer simulation, Klein-Nulend and co-workers [24] showed that flow rates are low in the region of bone immediately ahead of the basic multicellular units. They also showed that cells die in this stagnant area, possibly because of lack of nutrients. Similarly in our study under static conditions, cell death was observed irrespective of different densities of adhering bacteria. Under flow, detachment of cells was not observed and cells were alive due to

the continuous incoming of fresh medium and removal of endotoxins. Hence, conducting the race for a biomaterial surface between bacteria and tissue cells *in vitro* under low flow may be considered clinically relevant, although exact flow rates as occurring *in vivo* are unknown.

Several researchers predict the outcome of the race for the surface by studying the bacteria-surface interactions and tissue cell-surface interactions separately, but this is not how we interpret Gristina's meaning of "the race for the surface", neither do we think this is the right way to study the possible fate of a biomaterial implant *in vitro*. Many biomaterial coatings have been identified as non-adhesiveness to bacteria or support tissue integration separately [6,13-15,17,18], but the combination of both bacteria and tissue cells on the same biomaterials surface has never been studied. Shi *et al*. [16] for instance, promoted a surface composed of chitosans and Arginine-Glycine-Aspartic acid (RGD) peptide sequences. In separate experiments, it was shown that these combined surfaces discouraged bacterial adhesion, and enhanced cell attachment and Alkaline Phosphatase (ALP) activity. However, effects of bacterial presence, including the influence of bacterial activities and toxins [12] on the tissue cell attachment were not studied, which could completely change the fate of a biomaterial implant according to the concept of "race for the biomaterial surface". The methodology forwarded here allows simultaneous growth of bacteria and tissue cells on the same biomaterial surface and will be useful for the evaluation of new functional and biomimetic surfaces.

Conclusion

A novel *in vitro* methodology to study the race between bacteria and tissue cells for a biomaterial surface has been developed. Although due to the cell type chosen and use

of a staphylococcal strain, the methodology as described here may seem geared toward orthopedic applications, we emphasize that its principles are equally applicable to other implant systems, such as surgical meshes, or vascular grafts. Both the absence and presence of flow, as well as the number of adhering bacteria appeared to determine whether tissue cells were able to grow on a biomaterial surface. The methodology forwarded is expected to become indispensable for *in vitro* evaluation of bi-functionalized, biomimetic biomaterial coatings currently being developed in different groups worldwide.

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Microbial Biofilm Growth *versus* Tissue Integration on Biomaterials with Different Wettabilities and a Polymer-Brush Coating

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Henk J. Busscher, Roel Kuijer

Introduction

Biomaterial implants are indispensable in human function restoration after damage to the human body beyond natural repair. The number one cause of failure of biomaterial implants is infection, partly as a cause of unsuccessful tissue integration. Microorganisms involved in biomaterial-associated infection (BAI) are resistant to antibiotics due to their biofilm mode of growth, and infected implants often have to be removed before the infection can be fully eradicated from surrounding tissue and a new implant can be inserted. On average, BAI occurs in approximately $0.5 - 6\%$ of all primary implant patients [1]. BAI has an at least two- to three-fold higher incidence in revision surgery. Primary implants can become contaminated with microorganisms during implant surgery (peri-operative contamination) or hospitalization [2], as the onset of BAI. Whether or not microbial contamination eventually results in BAI, depends on the outcome of the so-called 'race for the surface' between successful tissue integration of the biomaterial implant and biofilm growth [2]. If this race is won by tissue cells, then the biomaterial surface is covered by a cellular layer and less vulnerable to biofilm formation. On the other hand, if the race is won by bacteria, the implant surface will become colonized by bacteria and tissue cell functions are hampered by bacterial virulence factors and toxins [2,3]. Since microorganisms are frequently introduced on an implant surface during surgery, microorganisms have a head start in this race for the surface.

A better understanding of the combined interaction of tissue cells and bacteria on biomaterial surfaces is required in order to develop new biomaterials or functional coatings that resist bacterial adhesion and biofilm formation [4-8] and simultaneously support tissue cell adhesion and proliferation [5-9]. Since the development of biomaterials and functional coatings, bacterial adhesion and biofilm formation are

studied independently from tissue cell adhesion and proliferation, and the combined outcome of these two interactions, i.e. the race for the surface, remains unknown. Recently, an *in vitro* experimental methodology to investigate the race between bacteria and tissue cells in a single experiment has been forwarded [10]. The outcome of the race for the surface between staphylococci and tissue cells appeared dependent on the number of bacteria present prior to cell seeding and the absence or presence of fluid flow. Cells lost the race for the surface in the absence of flow due to accumulation of bacterial endotoxins, but were able to grow in the presence of flow due to continuous incoming of fresh medium and removal of endotoxins by the flow [10].

Many biomaterials that are often used in the clinic have not yet been investigated with respect to the influence of their surface properties on the outcome of the race for the surface between bacteria and tissue cells. Surface wettability is one of the important properties influencing bacterial or cellular interactions with biomaterials. Gottenbos *et al.* [11]. showed relatively similar bacterial adhesion between materials with different wettabilities. Schakenraad *et al.* [12]. reported that tissue cells spread best on wettable and poorly on less wettable surfaces. Therefore the aim of this paper is to determine the influence of wettability on the outcome of the race for the surface on different biomaterial surfaces. In addition, a surface coated with a hydrophilic polymer-brush is included, since these have been shown to discourage microbial adhesion and biofilm formation [13].

Materials and Methods

Biomaterial surfaces. Polyethylene (PE) (Goodfellow, Cambridge, UK), Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) (Fluorplast, Raamsdonkveer,

The Netherlands), poly (methyl methacrylate) (PMMA) (Vink Kunststoffen, Didam, The Netherlands), Polystyrene (PS) (Colltec, Groningen, The Netherlands), High-Throughput microArraying, multifunctional slide (HTA) (Greiner Bio One, Alphen aan den Rijn, The Netherlands) and glass coated with a hydrophilic polyethylene oxide (PEO) layer were used. All samples except hydrophilic PEO-coating were rinsed thoroughly with ethanol (Merck, Darmstadt, Germany) and washed with sterile ultrapure water before use.

Hydrophilic PEO-coated glass (polymer-brush coating) was prepared by first cleaning microscope glass slides in a 2% RBS35 detergent solution (Omniclean, Breda, The Netherlands) under sonication and thorough rinsing in demineralized water, methanol, water again and finally washed with sterile ultrapure water. Glass surfaces were made hydrophobic by application of a dimethyldichlorosilane coating (DDS, Merck, Germany), yielding a water contact angle of 107 ± 2 degrees. Exposure to a solution of 0.5 g l^{-1} Pluronic F-127 solution ($PEO_{99}PPO_{65}PEO_{99}$, molecular weight 12600; Sigma-Aldrich, USA) in phosphate buffered saline (PBS: 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) for 20 min created a hydrophilic polymer-brush coating, that has appeared stable and effective against bacterial adhesion for at least 48 h [13].

Biomaterial surface characterization. The wettability of the surfaces was determined by water contact angle measurements at room temperature with an image analyzing system, using the sessile drop technique. Each value was obtained by averaging five droplets on one sample.

The elemental surface composition of the biomaterial surfaces was measured using X-ray Photoelectron Spectroscopy (XPS). The S-probe spectrometer (Surface

Science Instruments. Mountain View, CA, USA) was equipped with an aluminium anode (10 kV, 22 mA) and a quartz monochromator. The direction of photoelectron collection angle was 55 degrees with the sample surface and the electron flood gun was set at 10 eV. Broad spectrum survey scans (binding energy range of 1 to 1100 eV) were made at low resolution (pass energy, 150 eV). The area under each peak was used to calculate peak intensities, yielding elemental surface concentrations for carbon, oxygen, nitrogen, fluorine and silicon, after correction with sensitivity factors provided by the manufacturer. Elemental surface compositions were expressed in atom percentages of carbon, oxygen, silicon, fluorine and/or nitrogen. Two separate measurements were taken on different spots of each biomaterial.

U2OS cell culturing and harvesting. U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagle's Medium (DMEM) -low glucose supplemented with 10% fetal calf serum (FBS), 0.2 mM of ascorbic acid-2-phosphate (AA2P) and denoted in the paper as DMEM+FBS. U2OS cells were maintained at 37°C in a humidified 5% CO_2 atmosphere, and cells were passaged at 70 − 90% confluency using trypsin/EDTA.

Bacterial growth conditions and harvesting. *Staphylococcus epidermidis* ATCC 35983, originally isolated from human blood of a patient with an infected intravascular catheter, was used throughout this study. First, the strain was streaked on a blood agar plate from a frozen stock and grown overnight at 37°C. The plate was then kept at 4°C. For each experiment, a colony was inoculated in 10 ml of tryptone soy broth (TSB; OXOID, Basingstoke, United Kingdom) and cultured for 24 h. This culture was used to inoculate a second culture in TSB, which was grown for 17 h prior

to harvesting. Bacteria were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were sonicated on ice (3 x 10 s) in sterile PBS (10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) in order to break bacterial aggregates. This suspension was further diluted in sterile PBS to a concentration of 3 x 10^6 bacteria per ml.

The race for the surface. The race for the surface was studied on the bottom plate of a parallel plate flow chamber (175 x 17 x 0.75 mm^3) prepared from the biomaterials or coating under investigation, as described in detail before [10]. The flow chamber was equipped with heating elements and kept at 37°C throughout the experiments. Bacterial and U2OS deposition were observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Leica DM2000 (Leica Microsystems Ltd, Germany) with a 30x objective for bacteria and 10x objective for tissue cells.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, and before the addition of the bacterial suspension, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, the bacterial suspension in PBS was perfused through the chamber at the same shear rate and phase-contrast images were obtained and image-analyzed as a function of time. As soon as the desired density of adhering bacteria (10^3 cm^{-2} or 10^5 cm^{-2}), was reached, flow was switched to sterile PBS to remove the bacterial suspension from the tubes and chamber. Subsequently, a U2OS cell suspension in modified culture medium, consisting of 98% DMEM+FBS and 2% TSB and suitable for the simultaneous growth of U2OS cells and *S. epidermidis* [10], was allowed to enter the flow chamber.

Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped for 1.5 h in order to allow tissue cells to adhere and spread on the substratum. Subsequently, phase contrast images (6 images, 900 x 700 μm each) were taken and the number of adhering cells per unit area and area per spread cell were determined using Scion image software. Finally, modified culture medium supplemented with 2% HEPES was perfused through the system at a low shear rate of 0.14 s^{-1} for 48 h without recirculation, and phase-contrast images were obtained continuously.

Immuno-cytochemical staining. After 48 h of flow, the biomaterial surfaces or coating were prepared for immuno-cytochemical staining to assess the tissue cell morphology and spreading. For fixation, surfaces with adhering bacteria and tissue cells were placed in a Petri dish with 30 ml of 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5 min, the fixation solution was replaced by 30 ml of fresh CS for another 5 min. Subsequently tissue cells were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS and stained for 30 min with 5 ml PBS containing 49 μl DAPI and 2 μg ml^{-1} of TRITC-Phalloidin. The cells on the surfaces were washed 4 times in PBS and examined with confocal laser scanning microscopy (CLSM, Leica DMRXE with confocal TCS SP2 unit). The number of adhering tissue cells per unit area and the average area per spread cell were determined using Scion image software. For each density of adhering bacteria, 6 images (900 x 700 μm) per sample were randomly chosen and analyzed.

Statistics. Experiments for each density of adhering bacteria on different surfaces were carried out in triplicate. Data are represented as a mean with standard deviation. Statistical ANOVA analysis was performed followed by a Tukey's HSD post-hoc test and a *P*-value of <0.05 was considered significant.

Results

Biomaterials wettability and surface composition. The water contact angles and elemental surface compositions of the biomaterials and polymer-brush coating evaluated are summarized in Table 1. The biomaterial surfaces extend over a wettability range from 36 to 103 degrees, and are composed for the major part of carbon and oxygen, with the exception of FEP, containing fluorine and HTA, containing nitrogen. The polymer-brush coating has a wettability of 41 degrees, and shows some silicon, originating from the glass underneath the thin polymer-brushes consisting of carbon and oxygen.

Table 1. Water contact angle and elemental surface compositions of biomaterials and PEO-coating evaluated. ± indicates the standard deviation over three independently prepared end measured samples.

Biomaterial	θ (degrees)	%C	%O	%F	%N	%Si
HTA	36 ± 3	80.1 ± 2.0	16.9 ± 0.6	-	3.1 ± 1.2	-
PMMA	73 ± 3	73.2 ± 0.2	25.9 ± 1.4	-	-	-
PS	80 ± 2	94.8 ± 2.2	5.2 ± 2.2	-	-	-
PE	95 ± 2	97.2 ± 1.2	2.8 ± 1.2	-	-	-
FEP	103 ± 1	27.6 ± 0.3	0.6 ± 0.3	72.1 ± 0.8	-	-
Polymer-brush coating [1)	41 ± 5	59.5 ± 0.8	33.6 ± 0.3	-	-	6.9 ± 0.9

1) data taken from Roosjen *et al*. [14].

U2OS cell adhesion and spreading in the absence and presence of adhering *S. epidermidis*. Immediately after seeding, U2OS cell adhesion and spreading was observed in the absence and presence of adhering staphylococci on all biomaterial surfaces evaluated, but not on the polymer-brush coating. At 1.5 h, there was no significant difference between the number of adhering U2OS cells on the biomaterials (25000 cells cm^{-2} on average), with the exception of FEP where far less adhering tissue cells were found (approximately 12000 cells cm^{-2}). On the polymer-brush coating, however, the number of U2OS cells on the surface was 25000 cells cm^{-2} on average at 1.5 h, but these tissue cells adhered loosely and were removed at the applied shear rate of 0.14 s^{-1}. Phase-contrast images of U2OS cell adhesion, spreading and simultaneous biofilm formation of *S. epidermidis* after 24 h of growth on the biomaterials evaluated and the polymer-brush coatings are shown in Fig. 1. On the biomaterials, adhering staphylococci had grown into a biofilm, whereas at the same time U2OS cell adhesion and spreading were observed. On the polymer-brush coating, the adhering U2OS cells that could withstand the applied shear, did not spread and remained rounded even after 24 h (see Fig. 1).
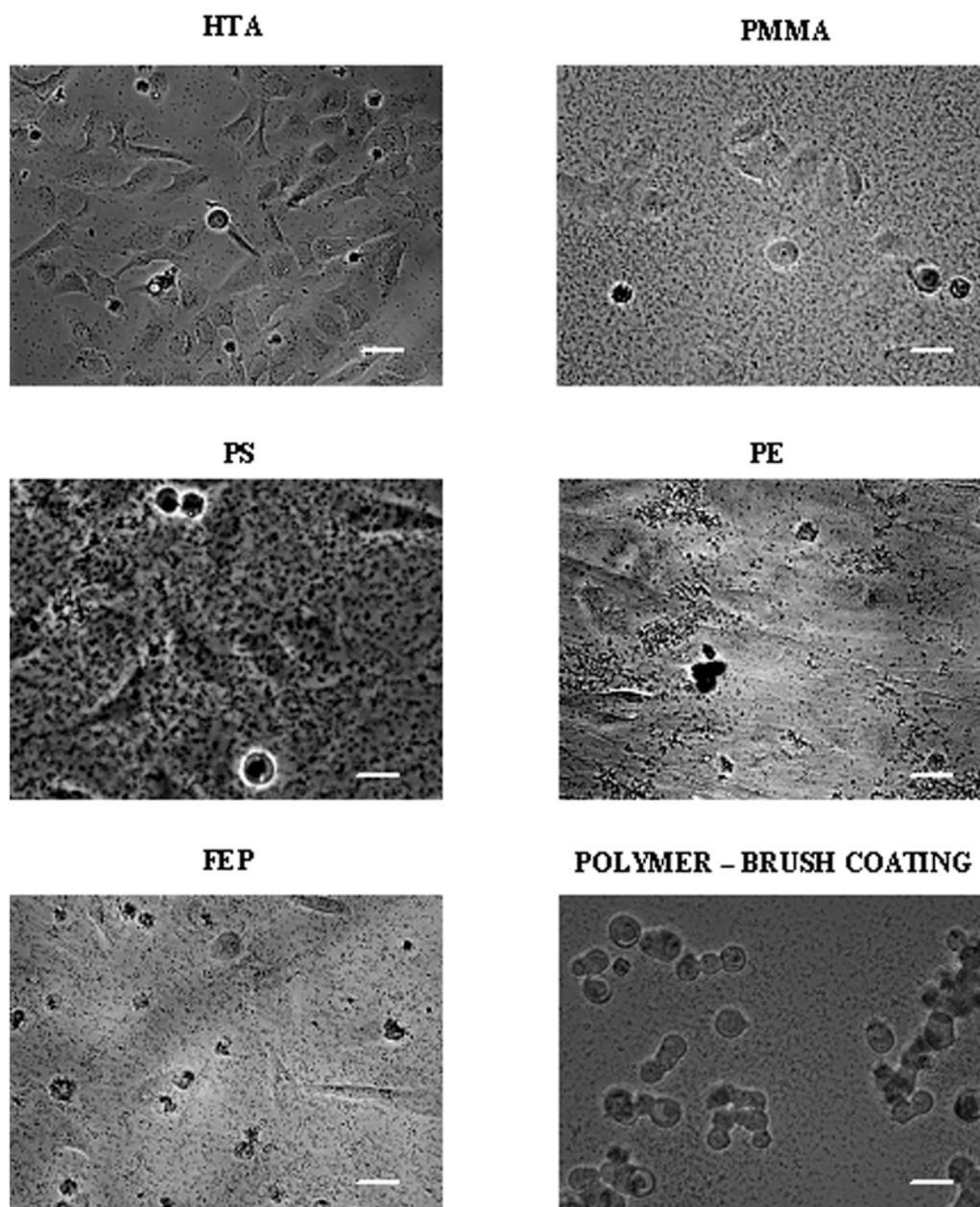
Fig. 1. U2OS cell spreading and staphylococcal biofilm formation (initial number of staphylococci present is 10^3 cm^{-2}) after 24 h of growth on biomaterials evaluated (HTA = High-Throughput microArraying, multifunctional slide, PMMA = Poly (methyl methacrylate), PS = Polystyrene, PE = Polyethylene, FEP = Poly (tetrafluoroethylene-co-hexafluoropropylene)) and a polymer-brush coating. Bar denotes 10 μm.

After 48 h of growth, the number of adhering bacteria had increased to the extent that it impeded quantification of cellular adhesion and spreading and hence adhering U2OS cells were immunocyto-stained for CLSM analysis (see Fig. 2) to

derive the number and spread area of U2OS cells in the absence and presence of staphylococci. In Fig. 3 it can be seen, that the % increase in number of adhering U2OS cells was significantly reduced due to the presence of adhering staphylococci on all biomaterials surfaces as compared to the control, i.e. in the absence of adhering bacteria ($p<0.05$). The number of adhering U2OS cells on hydrophobic FEP showed a significant reduction ($p<0.01$) in % increase with respect to the initial number of *S. epidermidis* present.
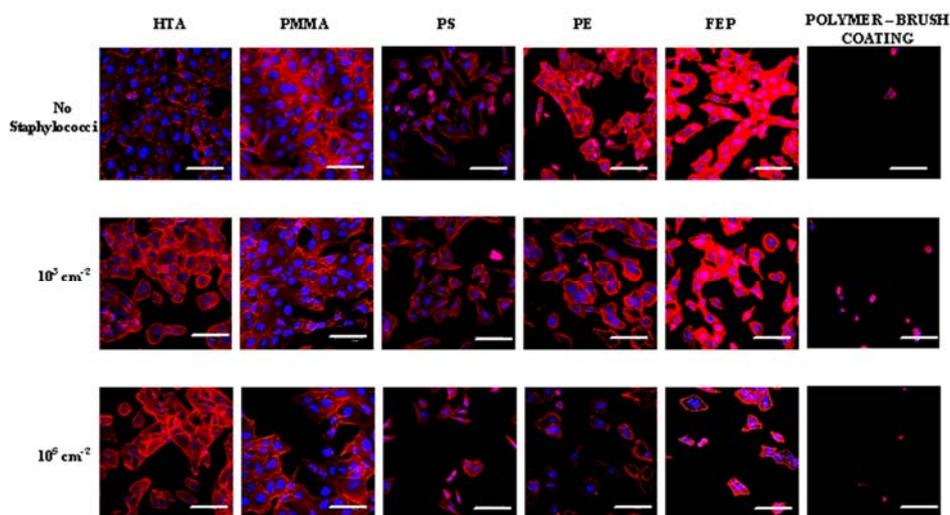
Fig. 2. CLSM images of immunocyto-stained U2OS cells after 48 h of growth on the biomaterials evaluated (HTA = High-Throughput microArraying, multifunctional slide, PMMA = Poly (methyl methacrylate), PS = Polystyrene, PE = Polyethylene, FEP = Poly (tetrafluoroethylene-co-hexafluoropropylene)) and a polymer-brush coating in the absence (no staphylococci) and presence of adhering staphylococci (initial number of adhering staphylococci amounted either 10^3 cm^{-2} or 10^5 cm^{-2}). Bar denotes 75 μm.
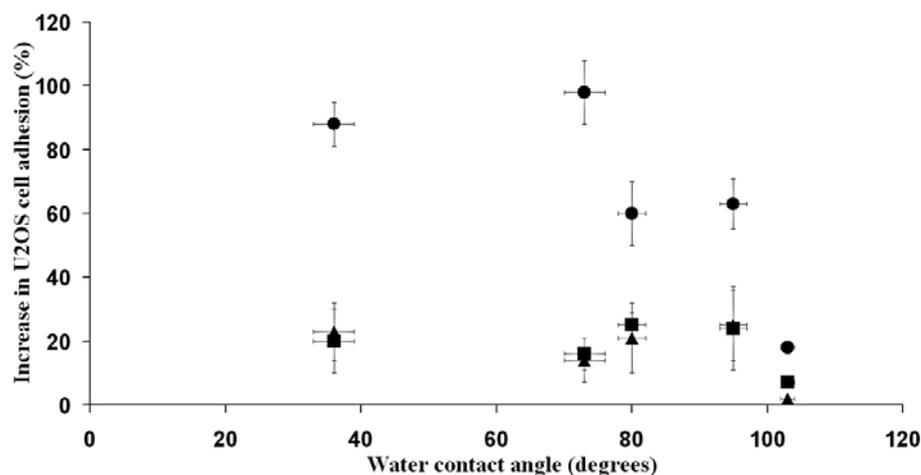
Fig. 3. Percentage increase in the number of adhering U2OS cells after 48 h of growth with respect to their initial adhesion at 1.5 h as a function of the water contact angles of the biomaterials evaluated in the absence (•) and presence (■ – 10^3 cm^{-2}, ▲ – 10^5 cm^{-2}) of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates, with separately cultured bacteria and tissue cells.

U2OS cells showed maximum spreading in the absence of staphylococci on all biomaterials evaluated irrespective of their wettabilities, as compared to the presence of staphylococci. U2OS cells spread best on hydrophilic surfaces (HTA and PMMA) and showed the least spreading on the hydrophobic FEP surface. It is interesting to see that the adhering tissue cells, spread equally well in the presence of staphylococci as in their absence (see Fig. 4). Note that the total surface coverage of the U2OS cells is different in the presence of staphylococci, which is caused by a lower number of the adhering bacteria (see Fig. 2). Tissue cell spreading only appeared hampered due to the presence of adhering staphylococci on the most hydrophobic surface, FEP. On the polymer-brush coating, no increase in the number of adhering U2OS cells was observed, neither did the adhering cells spread within 48 h, irrespective of the absence or presence of staphylococci.
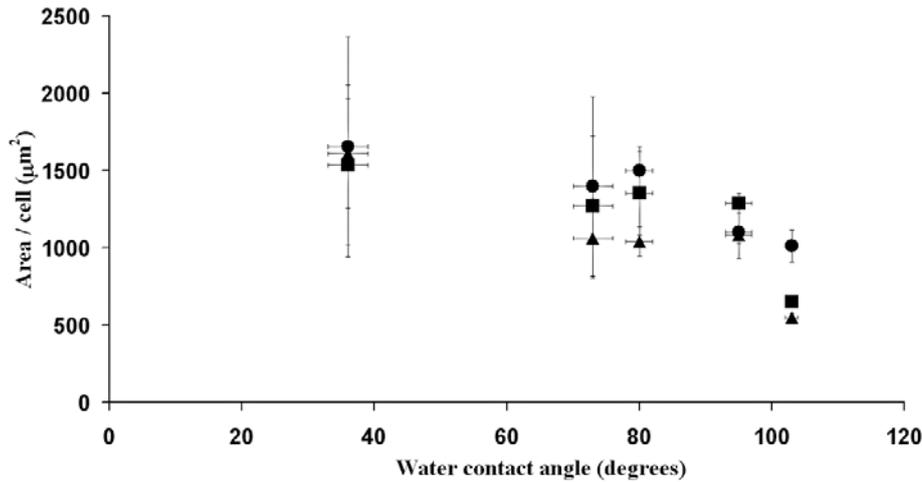
Fig. 4. Average area per spread U2OS cell after 48 h of growth as a function of the water contact angles of the biomaterial evaluated in the absence (●) and presence (■ – 10^3 cm^{-2}, ▲ – 10^5 cm^{-2}) of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates, with separately cultured bacteria and tissue cells.

Discussion

This paper presents the first experimental study on the race between bacteria and tissue cells on biomaterials with different wettabilities as well as on polymer – brush coated glass. Two different densities of bacteria (10^3 and 10^5 bacteria cm^{-2}) were allowed to adhere prior to cell seeding, adhesion and spreading, which mimicks a situation of peri-operative bacterial contamination of implant surfaces. In the past, it has been documented that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on the wound is approximately 270 per cm^2. The bacterial counts are generally higher during the periods of activity and correlate positively with the number of personnel present in the operation theatre [15]. In more recent, conventionally ventilated operation theatre (20 changes of air per hour) and the use of impermeable patient and personnel clothing [16], bacterial contamination will be far less, which makes, the bacterial adhesion densities chosen in our experiments a worst case scenario.

Amongst other material properties, surface wettability plays a major role in bacterial or cellular interaction with biomaterials. Wettability of biomaterial surfaces has been related to bacterial adhesion, cell adhesion and spreading. Gottenbos *et al.* [11]. showed that adhesion of staphylococci is relatively similar on different biomaterials, such as PMMA, PE and FEP, irrespective of differences in wettability. Likewise, these biomaterials in our study showed similar biofilm formation irrespective of their differences in wettability. The adhesion of staphylococci on the polymer – brush coating was slow and not as strong as on the biomaterials evaluated. A recent study by Nejadnik *et al.* [13]. showed that the polymer – brush coatings reduced adhesion of staphylococci considerably but the few adhered bacteria still formed a biofilm when allowed to grow. This biofilm formed on the polymer – brush coating detached when exposed to high fluid shear [13].

In case of tissue cells, many papers [12,17-19] have suggested that optimal tissue cell adhesion and spreading occurs at intermediate wettability (water contact angle between 60 degrees and 80 degrees). Nevertheless, surfaces that are poorly wettable still support appreciable cell adhesion [12]. Similarly, on the biomaterials, in the absence of staphylococci, U2OS cells showed the best adhesion on hydrophilic HTA and PMMA and appreciable cell adhesion on hydrophobic FEP (see Fig. 3). The percentage adhesion of tissue cells with respect to surface wettabilities of biomaterials followed the same trend as demonstrated earlier [20-23]. It is surprising that the poorly wettable polymer PE was capable of supporting significant cell attachment and spreading. This was also reported by Lydon *et al.* [23]. in the previous study. U2OS cells showed no adhesion and spreading on a polymer – brush coating.

A combined bacterial and tissue cell adhesion and growth study on biomaterial surfaces is novel. This study showed that in the presence of staphylococci, U2OS cell

adhesion in terms of numbers of adhering cells, reduced significantly on all biomaterial surfaces evaluated, as compared to a control, i.e. in the absence of adhering bacteria ($p<0.05$). Spreading of adhering tissue cells was less affected by the presence of bacteria except on hydrophobic FEP. This demonstrates that tissue cell interactions with biomaterials are hampered by bacterial presence on all biomaterials, establishing a tight race between bacteria and tissue cells for the biomaterial surface. Clinically, this is the reason why antibiotics need to be used in order to substantially reduce the risk of post-operative infection or BAI [24]. The polymer – brush coating showed both reduced bacterial adhesion and tissue cell adhesion and spreading, which makes it unsuitable for implant coatings, albeit for a different reason than valid for the biomaterial surfaces evaluated. Therefore, this study emphasizes the need for the development of bi-functional surfaces, discouraging bacterial adhesion and growth and simultaneously supporting tissue cell adhesion and spreading.

Conclusion

This study demonstrates that tissue cell interactions with biomaterials were hampered by biofilm formation on biomaterial surfaces and in fact most on a hydrophobic FEP surface. This indicates that the race for the biomaterial surface a tight one, as can be inferred from the relatively high rate of occurrence of BAI. As such, neither hydrophobic nor hydrophilic surfaces aid in a decisive way to determine the outcome of the race for the surface. Polymer-brush coatings appear less promising in determining the race for the surface than expected on the reductions observed in bacterial adhesion and growth on polymer-brush coatings, because they do not support tissue cell adhesion and spreading. Surfaces, on which the race for the surface can be won with certainty by the tissue cells, do not yet exist. Biomaterials

engineering should aim for bi-functional surfaces, discouraging bacterial adhesion and growth, but at the same time supporting tissue adhesion and spreading. Hitherto, these two requirements have not been met by any of the biomaterials surfaces currently used for biomedical implants and devices.

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Bacterial Strain-Specific Effects on Mammalian Cell Growth on Poly(methyl methacrylate)

Introduction

Biomaterial-associated infection (BAI) is a serious problem in modern medicine. BAI is often difficult to treat, as the biofilm mode of growth protects the infecting organisms against the host defense system and antibiotics [1]. In most cases, the final outcome is removal of the infected implant. Biomaterial implants can become contaminated in different ways. The best documented route is direct contamination of the implant surface during surgery (peri-operative contamination) or contamination during hospitalization. Whether or not microbial contamination eventually results in BAI, depends on the outcome of the so-called 'race for the surface' between successful tissue integration of the implant surface and biofilm formation [2]. If this race is won by tissue cells, then the implant surface is covered by a cellular layer and less vulnerable to biofilm formation. On the other hand, if the race is won by bacteria, the implant surface will become colonized by bacteria and tissue cell functions are hampered by bacterial virulence factors and toxins [2,3]. Since microorganisms are frequently introduced on an implant surface during surgery, microorganisms have a head start in this race for the surface. In the concept of the race for the surface, full coverage of an implant surface *in vivo* by a viable tissue cell layer, intact cell membrane and functional host defense mechanisms resist biofilm formation [4].

Previously, we proposed an *in vitro* model to experimentally determine the influence of peri-operative bacterial contamination on the race for the surface, in which adhesion, spreading and growth of mammalian cells on a biomaterial surface is compared in the absence or presence of adhering bacteria [5]. The outcome of the competition between contaminating *Staphylococcus epidermidis* ATCC 35983 and U2OS cells on glass appeared to be dependent on the number of bacteria adhering prior to mammalian cell seeding and the absence or presence of fluid flow. Cells lost

the competition in the absence of flow due to accumulation of bacterial toxins, but were able to grow under flow due to the continuous supply of fresh medium to and removal of endotoxins from the interface on all commonly used biomaterial surfaces [6]. Coverage of the surface by U2OS cells in the presence of adhering *S. epidermidis* was reduced however, as compared to cell surface coverage in the absence of adhering bacteria.

In general, *S. epidermidis* and *Staphylococcus aureus* are the most frequently isolated pathogens from infected biomaterial implant surfaces. Additionally isolated organisms include *Escherichia coli* and *Pseudomonas aeruginosa* [2,7]. Almost 50% of infection associated with catheters, artificial joints and heart valves are caused by *S. epidermidis* [8], whereas *S. aureus* is detected in approximately 23% of infections associated with prosthetic joints [8]. *P. aeruginosa* is the causative organism of around 12% of hospital acquired urinary tract infections, 10% of bloodstream infections and 7% of hip joint infections [9].

It is known that bacterial virulence is a contributing factor in the pathogenesis of BAI [10], but hitherto this aspect has not been included in our studies on the race for the surface, and experiments have been confined to *S. epidermidis* ATCC 35983. Therefore the aim of this study is to compare the influence of different strains of *S. epidermidis*, *S. aureus* and *P. aeruginosa* on the outcome of the competition between adhering bacteria and mammalian cells for the biomaterial surface in a peri-operative contamination model.

Materials and Methods

Biomaterial. Poly(methyl methacrylate) (PMMA) (Vink Kunststoffen, Didam, The Netherlands) was used as a substratum. Samples were rinsed thoroughly with ethanol

(Merck, Darmstadt, Germany) and washed with sterile ultrapure water before use to yield a water contact angle of 73 ± 3 degrees [6].

Mammalian cell culturing and harvesting. U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-low glucose supplemented with 10% fetal calf serum (FBS), 0.2mM ascorbic acid-2-phosphate (AA2P), denoted in the paper as "DMEM+FBS". Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, they were passaged at 70 – 90% confluency using trypsin/EDTA.

Bacterial growth conditions and harvesting. The bacterial strains used in this study were *S. epidermidis* ATCC 35983, *S. epidermidis* ATCC 35984, *S. epidermidis* 3399, *S. aureus* ATCC 12600, *S. aureus* A20734, *S. aureus* 7388, *P. aeruginosa* DN7348, *P. aeruginosa* 01, *P. aeruginosa* ATCC 27853. First, a strain was streaked on a blood agar plate from a frozen stock and grown overnight at 37°C. The plate was then kept at 4°C. For each experiment, a colony was inoculated in 10 ml of tryptone soya broth (TSB; OXOID, Basingstoke, England) and cultured for 24 h. This culture was used to inoculate a second culture, which was grown for 17 h prior to harvesting. Bacteria were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were sonicated on ice (3 x 10 s) in sterile potassium phosphate buffer (PBS, 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) in order to break bacterial aggregates. This suspension was further diluted in sterile PBS to a concentration of 3 x 10^6 bacteria per ml. Prior to the experiments, growth and biofilm formation of all bacterial strains in modified culture

medium (98% DMEM+FBS and 2% TSB [5]) was confirmed by culturing bacteria in this medium for 48 h.

Competitive assay for mammalian cell growth and biofilm formation. The competitive assay was studied on the PMMA bottom plate of a parallel plate flow chamber (175 x 17 x 0.75 mm^3), as described in detail before [5]. The flow chamber was equipped with heating elements and kept at 37°C throughout the experiments. Bacterial and U2OS deposition were observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Olympus BH-2 (Olympus, Germany) with a 40x objective for bacteria and 10x objective for mammalian cells.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, and before the addition of the bacterial suspension, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, a bacterial suspension in PBS was perfused through the chamber at the same shear rate and phase-contrast images were obtained. As soon as the desired density of adhering bacteria (10^3 cm^{-2}), was reached, flow was switched to sterile PBS to remove the bacterial suspension from the tubes and chamber. Subsequently, a U2OS cell suspension (1.2×10^5 cells ml^{-1}) in modified culture medium (98% DMEM+FBS and 2% TSB) was allowed to enter the flow chamber. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped for 1.5 h in order to allow U2OS cells to adhere and spread on the substratum surface. Subsequently, phase contrast images (nine images, 900 x 700 μm each) were taken and the number of adhering cells per unit area as well as the area per spread cell were determined using Scion image software. Finally, modified culture medium supplemented with 2% HEPES was perfused

through the system at a low shear rate of 0.14 s^{-1} for 48 h and phase-contrast images were obtained continuously.

Immuno-cytochemical staining and determination of cell surface coverage. After 48 h of flow, the surfaces were prepared for immuno-cytochemical staining to assess the mammalian cell morphology and spreading. For fixation, surfaces with adhering bacteria and U2OS cells were placed in a Petri dish with 30 ml of 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5 min, the fixation solution was replaced by 30 ml of fresh CS for another 5 min. Subsequently U2OS cells were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS and stained for 30 min with 5 ml PBS containing 49 µl DAPI and 2 µg ml^{-1} of TRITC-Phalloidin. The U2OS cells on the surfaces were washed four times in PBS and examined with confocal laser scanning microscopy (CLSM, Leica DMRXE with confocal TCS SP2 unit). Images (nine images on different locations, 900 x 700 µm each) were taken. The number of adhering U2OS cells per unit area and the average area per spread cell were determined using Scion image software to yield the total coverage of the substratum surface by mammalian cells.

Statistics. Data are presented as a mean with standard deviation. Statistical ANOVA analysis was performed followed by a Tukey's HSD post-hoc test and $p < 0.05$ was considered significant.

Results

Immediately after seeding, U2OS cell adhesion and spreading was observed in the absence and presence of adhering bacteria on PMMA. After 1.5 h, the average number of adhering U2OS cells on the PMMA surface was 2.5×10^4 cells cm^{-2} with an average area of 500 μm^2 per cell. The spreading of U2OS cells on the PMMA surface after 1.5 h was not significantly different in the absence or presence of adhering bacteria, regardless of the strain involved. After 18 h of growth, U2OS cell death was observed on PMMA in the presence of adhering *S. aureus* and *P. aeruginosa* strains, whereas no cell death was observed in the presence of adhering *S. epidermidis* strains (Fig. 1). The simultaneous growth of U2OS cells and *S. epidermidis* was subsequently observed for a period of 48 h.
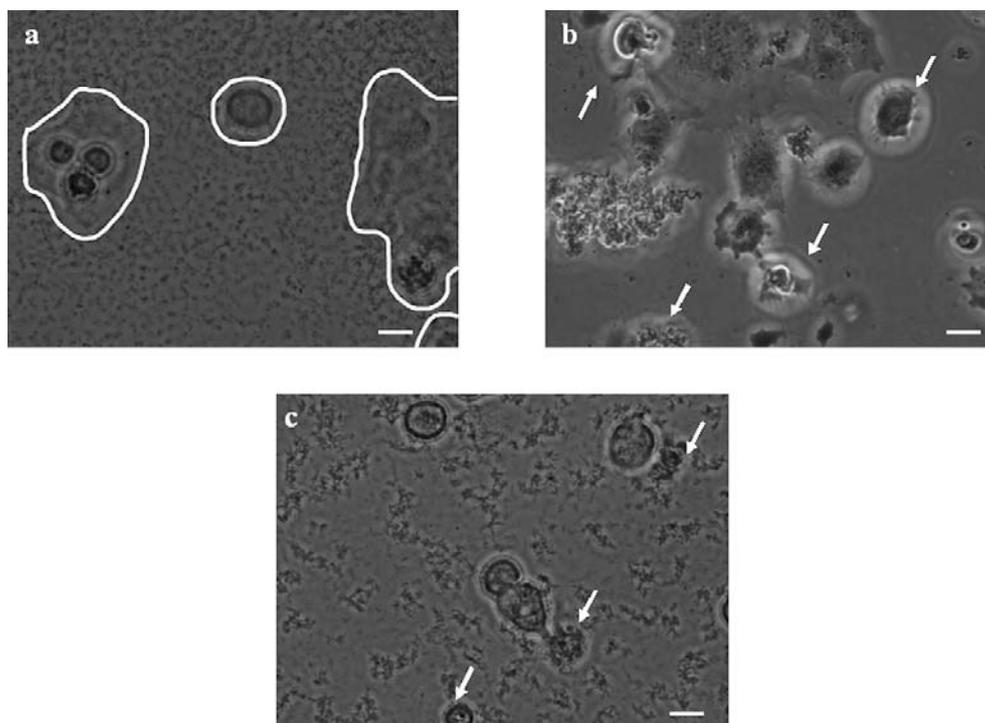
Fig. 1. Phase contrast images of U2OS cells after 18 h of growth in the presence of adhering bacteria (a – *S. epidermidis* ATCC 35983, b – *S. aureus* ATCC 12600 and c – *P. aeruginosa* ATCC 27853) on PMMA surfaces. Fig. 1a, U2OS cells are differentiated by a contour line from *S. epidermidis* biofilm. White arrows in Figs. 1b and 1c indicate U2OS cell death. The bar denotes 10 μm.

After 48 h of simultaneous growth of cells and bacteria, the adhering U2OS cells were immunocyto-stained for CLSM analysis to derive the number and spread area of adhering U2OS cells in the absence and presence of the different *S. epidermidis* strains. No U2OS cells were detected after 48 h of growth in the presence of adhering *S. aureus* and *P. aeruginosa*. In Fig. 2 it can be seen that the percentage increase in number of adhering U2OS cells was significantly reduced (p < 0.01) due to the presence of *S. epidermidis* as compared to the absence of adhering bacteria. The reduction in U2OS cell adhesion was larger (p < 0.01) in the presence of *S. epidermidis* 3399 and *S. epidermidis* ATCC 35984 as compared to *S. epidermidis* ATCC 35983 (p < 0.05). Adhering U2OS cells showed no significant difference in spreading on PMMA in the presence of adhering *S. epidermidis* as compared to the absence of adhering staphylococci (Fig. 3).
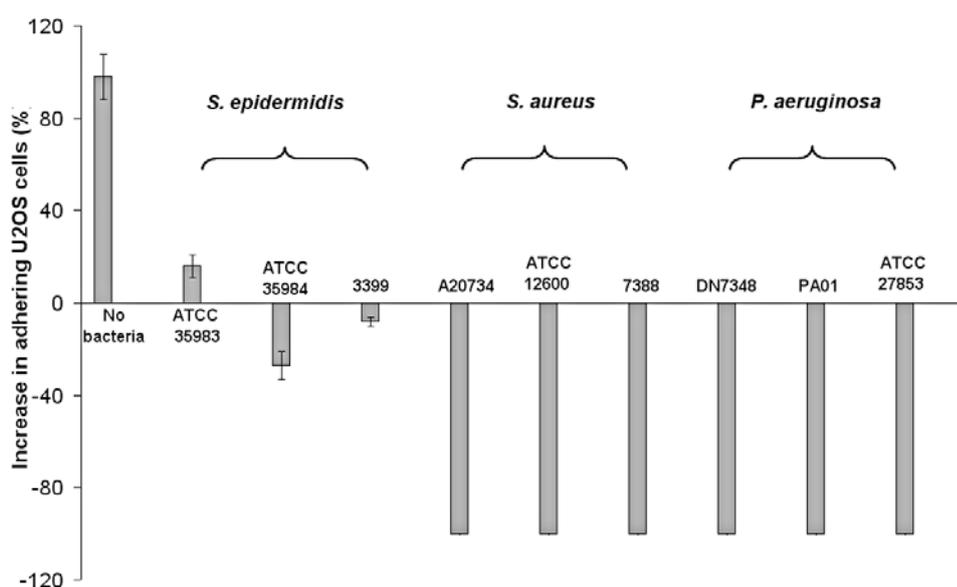
Fig. 2. Percentage increase in the number of adhering U2OS cells after 48 h of growth with respect to their initial number immediately after seeding at 1.5 h on PMMA in the absence (no bacteria) and presence of adhering bacteria. Error bars represent the standard deviations over three replicates, with separately cultured bacteria and mammalian cells. Cell number in the presence of bacteria is significantly different (p < 0.01) from the cell number in the absence of bacteria.
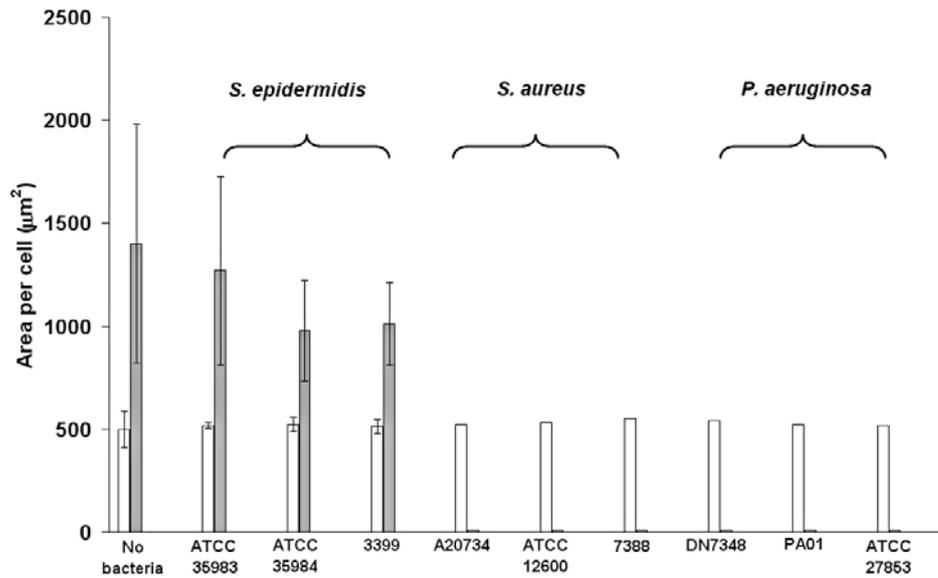
Fig. 3. Average area per adhering U2OS cell immediately after seeding at 1.5 h (□) and after 48 h (■) of growth on PMMA in the absence (no bacteria) and presence of adhering bacteria. Error bar represents the standard deviation over three replicates, with separately cultured bacteria and mammalian cells.

In the concept of the race for the surface, the total cell surface coverage of the substratum surface by tissue cells is considered determinant for the fate of a biomaterial implant. The surface coverages by U2OS cells at 1.5 h after seeding and after 48 h of growth are shown in Fig. 4. The surface coverage by adhering U2OS cells 1.5 h after seeding varied between 11% and 16% regardless of the absence or presence of adhering bacteria. The cell surface coverage increased significantly after 48 h in the absence and in the presence of adhering *S. epidermidis*. In the presence of adhering *S. aureus* and *P. aeruginosa*, reductions in cell surface coverages were observed after 18 h of growth, indicating U2OS cell death and no U2OS cells were detected on the PMMA surface after 48 h (Fig. 5).
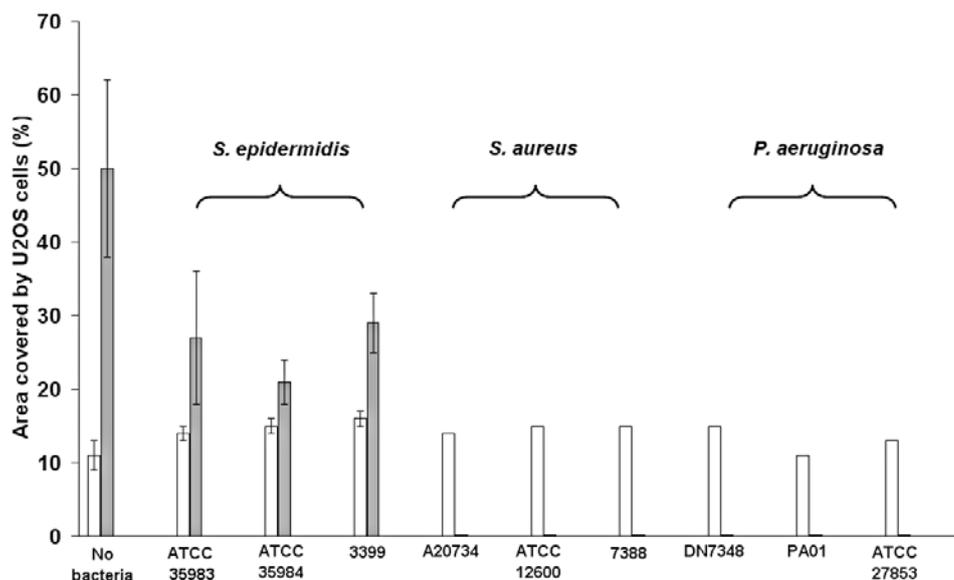
Fig. 4. Surface coverage by adhering U2OS cells immediately after seeding at 1.5 h (□) and 48 h (■) of growth on PMMA in the absence (no bacteria) and presence of adhering bacteria. Error bar represents the standard deviation over three replicates, with separately cultured bacteria and mammalian cells. Surface coverage by U2OS cells after 48 h in the presence of adhering bacteria are significantly different ($p < 0.05$) from the absence of bacteria.
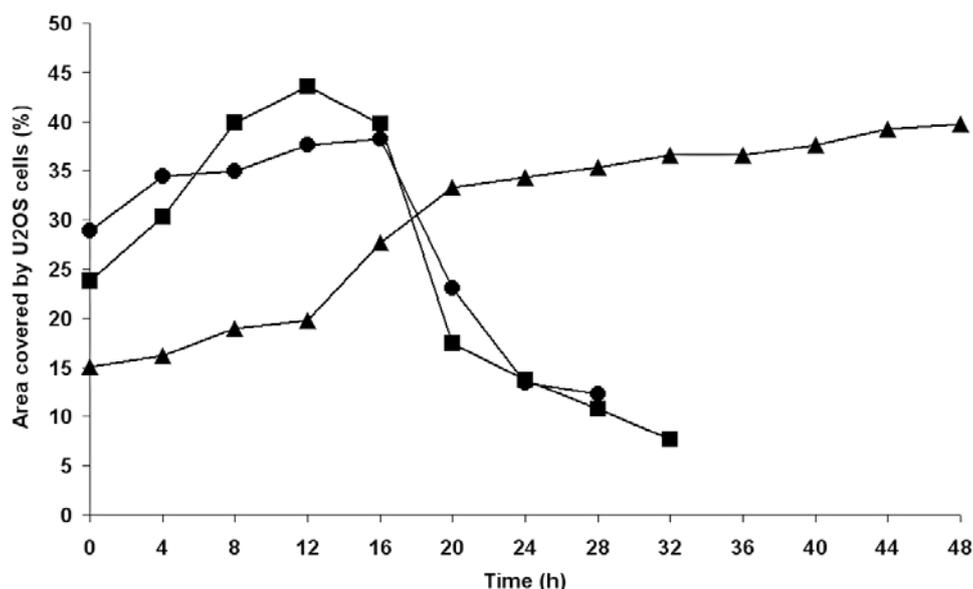
Fig. 5. An example of surface coverage by adhering U2OS cells as a function of time on PMMA in the presence of adhering bacteria ((▲) – *S. epidermidis* 3399, (■) – *S. aureus* A20734, (●) – *P. aeruginosa* DN 7348).

Discussion

This paper presents the first experimental comparison of the influence of different

pathogens on the outcome of the race between adhering bacteria and mammalian cells

for a biomaterial surface. In our *in vitro* model, bacteria were allowed to adhere prior to cell adhesion and spreading, which mimics the situation of peri-operative bacterial contamination of implant surfaces. The number of bacteria adhering on the PMMA surface prior to initiating mammalian cell adhesion and spreading was set to 10^3 cm^{-2}. In the past, it has been documented that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on a wound is about 270 cm^{-2}. The bacterial counts are generally higher during periods of activity and when more people are present in the operation theatre [11]. More recent, through the use of modern, better ventilated operation theatres (20 changes of air per hour) and impermeable patient and personnel clothing, peri-operative bacterial contamination may well be less [12]. However, many surgical procedures in which implants are introduced in the body last much longer than 1 h. Therefore, the level of bacterial contamination chosen in our experiments is probably realistic of a worst case scenario. Despite these low numbers, peri-operatively introduced organisms, particularly when of low virulence, can survive on an implant surface for prolonged periods of time and later, during periods of host immune depression, they proliferate and establish an infection with clinical symptoms [13].

In the concept of the race for the surface, full coverage of a biomaterial surface *in vivo* protects an implant against bacterial colonization [4]. Previously, in our model for the 'race for the surface', all common biomaterial surfaces, including PMMA, allowed *S. epidermidis* ATCC 35983 biofilm formation with a negative impact on the coverage of the biomaterial surface by mammalian cells [6]. Yet, PMMA showed better mammalian cell adhesion and spreading in the presence of adhering *S. epidermidis* ATCC 35983 than other commonly used biomaterials [6]. The present study confirms that U2OS cells are able to adhere, spread and grow in the presence of

different *S. epidermidis* strains, but not in the presence of adhering *S. aureus* or *P. aeruginosa*. The latter strains cause death of all adhering U2OS cells within 18 h, despite removal of bacterial toxins by flow.

These observations are in line with clinical findings that BAI due to *S. aureus* and *P aeruginosa* usually progresses much more aggressively than BAI caused by *S. epidermidis*. *S. aureus* appears more frequently in acute infections, within 4 weeks of surgery, of complicated total joint arthroplasty compared to *S. epidermidis*. *S. epidermidis* is most commonly implicated in delayed septic loosening of total joint prostheses [14] or even in presumed a-septic loosening [15], indicating its low virulence with only minor clinical symptoms of infection. Pseudomonas is also much more virulent than *S. epidermidis,* which is ascribed to the more aggressive endotoxins in the slime. Pseudomonas slime injected into mice produces liver and renal dysfunction and death within a short time, while no such effect was seen with *S. epidermidis* slime [16,17]. In vascular grafts, pacemakers and orthopedic devices, *S. epidermidis* is mostly found in low grade infections and a common cause of late infections. In patients with low grade hip implant infections, only 5% of the patient had a temperature of 37.8°C or higher and only 20% had wound drainage [18]. Buchholz and co-workers [19] showed that 84% of 64 patients with an infection by a low virulent organism, were free from infection 2 years later. Alternatively, when *S. aureus* was the causal organism, recurrent infection occurred in 28% of the patients [19], and almost 50% of the patients with a Gram-negative bacterial infection (*Pseudomonas*) experienced recurrent infection. This suggested a direct correlation between the clinical outcome and the causative bacterial strain, in line with our *in vitro* results.

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 severely tissue damaging toxins [8,9,20]. In *S. epidermidis* infections, biofilm formation is considered the only virulence factor and therefore infections are usually sub-acute or chronic [21-23]. Amongst the *S. epidermidis* strains involved in this study, *S. epidermidis* ATCC 35983 was considered not to produce slime, while *S. epidermidis* 3399 and *S. epidermidis* ATCC 35984 were slime producers [24]. Interestingly, a significant reduction in number of adhering U2OS cells was observed in the presence of *S. epidermidis* ATCC 35984 and *S. epidermidis* 3399 compared to *S. epidermidis* ATCC 35983 in direct correlation to the slime producing capability of these strains.

This study demonstrates that U2OS cells are bound to lose the race for the surface in the presence of adhering, highly virulent *S. aureus* or *P. aeruginosa* strains, whereas spreading and growth of adhering U2OS cells, although impaired, still occurs in the presence of adhering *S. epidermidis* strains. These results are in line with the clinically documented progression of BAI by the different species included in this study and highlight the potential use of our *in vitro* model for studies investigating the effect of pathogen virulence on host tissue cells in BAI.

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Bacterial Biofilm Formation *versus* Mammalian Cell Growth on Titanium-Based Mono- and Bi-Functional Coatings

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Introduction

Biomaterial-associated-infections (BAI) remain a major cause of failure of biomaterial implants. Biomaterial implants may become contaminated with microorganisms during implant surgery (peri-operative contamination) or during hospitalization [1], causing the onset of BAI. Microorganisms involved in BAI are protected from antibiotics due to their biofilm mode of growth. Consequently, infected implants often have to be removed and a new implant can only be inserted once complete eradication of the infected surrounding tissue has been performed. Successful tissue integration depends on the outcome of the race for the surface between microorganisms and tissue cells [1]. If this race is won by tissue cells, then the biomaterial surface is covered by a cellular layer and is less vulnerable to biofilm formation. On the other hand, if the race is won by bacteria, the implant surface will become colonized by bacteria, and tissue cell functions are hampered by bacterial virulence factors [2]. Since microorganisms are frequently introduced on an implant surface during surgery, microorganisms often start the race for the surface before tissue integration can even occur [2].

Biofilm formation occurs on all currently used biomaterials. Microbial adhesion to biomaterials is determined by the physicochemical properties of the implant surface [3]. Thus, modification of an implant surface may be able to prevent bacterial adhesion and biofilm formation. Several surface modification methods [4-6], such as hydrophilic polymer brush-type surfaces (in particular poly(ethylene glycol) PEG), have been developed to prevent bacterial adhesion and biofilm formation [7]. However, such non-adhesive polymer coatings also resist adhesion, spreading and growth of mammalian cells [6,8], which are required for successful tissue integration.

In order to prepare bi-functional coatings which prevent microbial adhesion while supporting tissue cell growth, the biologically inert poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG) copolymer was modified with the arginine-glycine-aspartic acid (RGD) peptide sequence [8]. The RGD peptide is known as one of the major recognition sites of integrin receptors through which mammalian cells connect to their extracellular matrix molecules [9]. In earlier studies, reduced bacterial adhesion on bi-functional PLL-*g*-PEG/PEG-RGD-modified surfaces has been demonstrated separately from their ability to support mammalian cell growth [7,10]. However, a simultaneous study on bacterial biofilm formation and mammalian cell growth, i.e. an actual "race for the surface", has not been performed due to lack of proper methodology. Recently, an *in vitro* experimental methodology to investigate bacterial biofilm formation and mammalian cell growth in a single experiment was developed [11]. The outcome of the competition between *Staphylococcus epidermidis* and U2OS cells appeared to be dependent on the number of bacteria present prior to cell seeding and the absence or presence of fluid flow. Mammalian cells lost the competition in the absence of flow due to the accumulation of bacterial toxins, but were able to grow under flow due to the continuous supply of fresh medium to and removal of endotoxins from the interface. A further study of the race for the surface on different biomaterials demonstrated that mammalian cell interactions with biomaterials were hampered by bacterial biofilm formation on all commonly used biomaterial surfaces [12].

This study aimed at evaluating titanium oxide surfaces modified with biopassive PLL-*g*-PEG and bioactive PLL-*g*-PEG/PEG-RGD with respect to biofilm formation and simultaneous mammalian cell adhesion, spreading and growth. In

particular the hypothesis that the bi-functional, bioactive surface may favour mammalian cell interaction over biofilm formation, is tested.

Materials and Methods

TiO₂-coated glass slides. Microscope glass slides (76 × 26 mm) were purchased from Menzel GmBH+Co KG, Braunschweig, Germany. The glass slides were sputter-coated with a 21 nm thick TiO_2 layer (reactive magnetron sputtering, Paul Scherrer Institute, Villigen, Switzerland).

PLL-*g*-PEG and PLL-*g*-PEG/PEG-RGD. PLL-*g*-PEG and PLL-*g*-PEG/PEG-RGD were purchased from Surface Solutions, AG, Switzerland. PLL-*g*-PEG is a copolymer with a polycationic PLL backbone onto which non-functionalized (methoxy-terminated) PEG chains are grafted. Grafting ratio, expressed as *g*, represents the ratio between number of lysine units and number of grafted PEG chains (*g* = Lys/PEG). PLL-*g*-PEG/PEG-RGD describes a copolymer with a PLL backbone onto which two different types of PEG chains are grafted, i.e. non-functionalized PEG and RGD-functionalized PEG [8].

The molecular weights of PLL, non-functionalized PEG and RGD-functionalized PEG, are 20 kDa, 2 kDa, and 3.4 kDa, respectively. The grafting ratio (*g*) of both copolymers PLL-*g*-PEG and PLL-*g*-PEG/PEG-RGD is 3.5. For the latter, 8% of all PEG chain carry the terminal, covalently linked peptide GCRGYG**RGD**SPG.

Surface preparation and polymer adsorption. The copolymers were dissolved at a 0.25 mg ml⁻¹ concentration in HEPES II buffer (10 mM HEPES and 150 mM NaCl,

adjusted to pH 7.4 by addition of NaOH). The polymer solutions were filter-sterilized (0.22 μm filter), aliquoted and stored at -20°C until use. Prior to surface modification, TiO_2-coated glass slides were ultrasonicated for 10 min in a cleansing solution (300 mM hydrochloric acid and 1% detergent, Cobas Integra®, Roche Diagnostic, Indianapolis, USA), rinsed with ultra pure water, followed by 10 min ultrasonication in 2-propanol to remove the adventitious macroscopic contamination and blow-drying under a stream of nitrogen. Subsequently, the TiO_2-coated glass slides were cleaned by means of oxygen plasma treatment for 2 min (Plasma cleaner/sterilizer PDC-32G, Harrick scientific products Inc., USA). Copolymer solutions were subsequently placed onto the pre-cleaned substrates completely covering their surfaces. Copolymer adsorption was allowed to proceed for 1 h to provide a complete monolayer on the surface, followed by extensive washing with HEPES II buffer, ultrapure water, and finally blow-drying under a stream of nitrogen. Data on conformation of the used copolymers adlayers and interaction with proteins have been described elsewhere [13].

The copolymer-modified substrates were placed in a clean sample holder and stored at 4°C until use. The surfaces are denoted as "TiO_2" for bare titanium oxide surface, "PEG" for PLL-*g*-PEG-modified and "PEG-RGD" for PLL-*g*-PEG/PEG-RGD-modified surface. The surfaces were sterilized using 70% ethanol and washed with sterile ultrapure water before use.

Bacterial growth conditions and harvesting. *S. epidermidis* ATCC 35983, originally isolated from human blood of a patient with an infected intravascular catheter, and known to produce polysaccharide integrin adhesin [14], was used throughout this study. First, the strain was streaked on a blood agar plate from a

frozen stock and grown overnight at 37°C. The plate was then kept at 4°C. For each experiment, a colony was inoculated in 10 ml of tryptone soya broth (TSB; OXOID, Basingstoke, England) and cultured for 24 h. This culture was used to inoculate a second culture, which was grown for 17 h prior to harvesting. Bacteria were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were ultrasonicated on ice (3 x 10 s) in sterile PBS (10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) in order to break bacterial aggregates. This suspension was further diluted in sterile PBS to a concentration of 3×10^6 bacteria per ml.

Mammalian cell culturing and harvesting. U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagles Medium (DMEM)-low glucose supplemented with 10% fetal calf serum (FBS) and 0.2 mM of ascorbic acid-2-phosphate (AA2P) without antibiotics, denoted "DMEM+FBS". Osteoblasts were maintained at 37°C in a humidified 5% CO_2 atmosphere, and passaged at 70 – 90% confluency using trypsin/EDTA.

Bacteria and tissue cell competitive adhesion assays. Competitive adhesion assays were performed on the bottom plate of a parallel plate flow chamber (175 x 17 x 0.75 mm^3) prepared from the biomaterial surfaces with or without polymer coatings under investigation, as described in detail elsewhere [11]. Importantly, the flow chamber was equipped with heating elements and kept at 37°C throughout the experiments. Bacterial and U2OS cell deposition were observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast Leica DM2000 microscope (Leica

Microsystems Ltd, Germany) with a 30x objective for bacteria and 10x objective for mammalian cells.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, to ensure all air bubbles were removed from the system. Prior to the addition of bacterial suspension, sterile PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Subsequently, the bacterial suspension in PBS was perfused through the chamber at the same shear rate for 2 h. Images were obtained continuously and the number of bacteria per unit area were analyzed in real-time by using proprietary software based on the Matlab Image processing Toolkit (The MathWorks, MA, USA). The flow with shear rate of 11 s^{-1} was then re-initiated to deliver sterile PBS to remove unattached bacteria from the tubes and flow chamber, after which a U2OS cell suspension (6×10^5 cells ml^{-1}) in modified culture medium, consisting of 98% DMEM+FBS and 2% TSB which is suitable for the simultaneous growth of U2OS cells and *S. epidermidis* [11], was allowed to enter the flow chamber. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, the flow was stopped for 1.5 h in order to allow tissue cells to adhere and spread on the substratum. Subsequently, phase contrast images (nine images on different locations, 900 x 700 μm^2 each) were taken to determine the number of adhering cells per unit area and the area per spread cell using Scion image analysis software. Finally, modified culture medium supplemented with 2% HEPES was perfused through the system at a low shear rate of 0.14 s^{-1} for 48 h, and phase-contrast images were collected continuously. After 48 h of growth, the shear stress was increased to 5.6 s^{-1} for 30 min, i.e. a value that is lower than the one used during initial bacterial adhesion (11 s^{-1}). Subsequently, U2OS cells and bacterial biofilm

growth were monitored continuously. Control experiments with only U2OS cells (in

the absence of *S. epidermidis*) were performed using the above procedure.

Finally, the adhering U2OS cells were stained with TRITC-Phalloidin for

quantitative analysis. Briefly, the substrate surfaces with adhering bacteria and tissue

cells were fixed with 30 ml of 3.7% formaldehyde in cytoskeleton stabilization buffer

(CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5

min, the fixation solution was replaced with 30 ml of fresh CS for another 5 min.

Subsequently, U2OS cells were incubated in 0.5% Triton X-100 for 3 min, rinsed

with PBS, stained for 30 min with 5 ml PBS containing 49 µl DAPI and 2 $\mu g\ ml^{-1}$ of

TRITC-Phalloidin, washed four times in PBS and examined with confocal laser

scanning microscopy (CLSM, Leica DMRXE with confocal TCS SP2 unit equipped

with a water immersion lens). Images (nine images on different locations, 900 x 700

μm^2 each) were taken and the number of adhering cells per unit area and the average

area per spread cell were determined.

Statistics. Experiments on different surfaces were performed in triplicate. Data are

represented as a mean with standard deviation. For statistical analysis ANOVA was

performed followed by a Tukey's HSD post-hoc test and a *p*-value < 0.05 was

considered to be significant.

Results

Bacterial adhesion. Initial adhesion of *S. epidermidis* after 2 h of flow at a shear rate of 11 s^{-1} was significantly reduced to $3.6\pm1.8 \times 10^3$ cm^{-2} and $6.0\pm3.9 \times 10^3$ cm^{-2} on both PEG and PEG-RGD coatings respectively, compared to $1.3\pm0.4 \times 10^5$ cm^{-2} on the bare TiO$_2$ surface (Fig. 1). This demonstrates that in the presence of RGD-peptide sequences, the non-adhesive functionality of the PEG brush surface is still maintained.
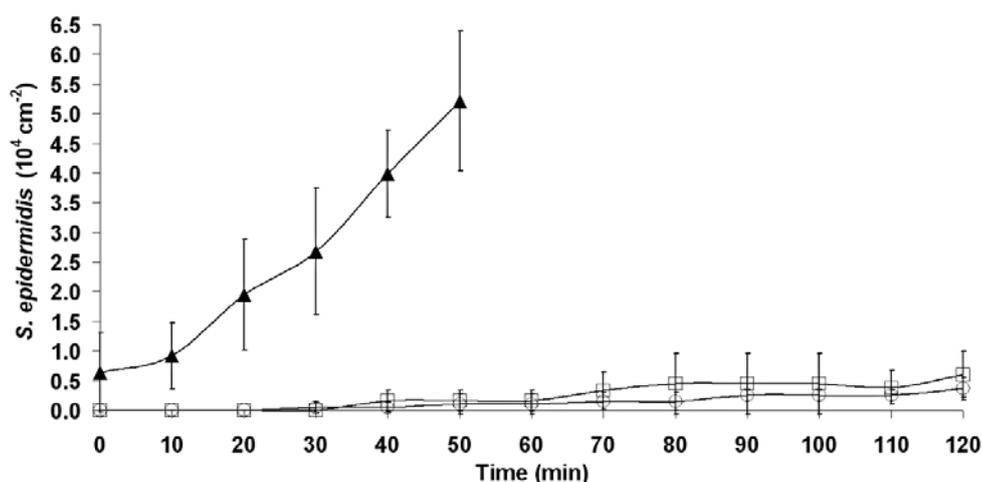
Fig. 1. The number of adhering *S. epidermidis* ATCC 35983 as a function of time in a parallel plate flow chamber (shear rate 11 s^{-1}) on mono-functional PEG coating (○) and a bi-functional PEG-RGD coating (□) as well as on a bare TiO$_2$ surface (▲). Error bars represent the standard deviation over three replicates with separately cultured bacteria.

U2OS cell adhesion and spreading in the absence and presence of adhering *S. epidermidis*. Immediately after seeding, U2OS cell adhesion and spreading was observed in the absence and presence of adhering *S. epidermidis* on TiO$_2$ surfaces and PEG-RGD coatings, but not on the PEG coatings. At 1.5 h, there was no significant difference in the number of adhering U2OS cells on the different surfaces (Table 1), but cell spreading was greater on PEG-RGD coatings, as compared to PEG coatings and TiO$_2$ surfaces (p<0.01), irrespective of absence or presence of *S. epidermidis* bacteria (Table 1).

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Table 1: Number of cells, area of spread cells and surface coverage of U2OS cells on TiO_2, PEG and PEG-RGD surfaces after initial seeding at 1.5 h and after 48 h of growth at a shear rate of 0.14 s^{-1} and subsequent application of an elevated shear rate (5.6 s^{-1}) for 30 min, in the absence and presence of adhering *S. epidermidis*. ± indicates the standard deviation over three independently prepared and measured samples.

Surfaces	Absence of *S. epidermidis*						Presence of *S. epidermidis*					
	U2OS cell number 10^3 cm^{-2}		Area/cell µm^2		Surface coverage by U2OS cells %		U2OS cell number 10^3 cm^{-2}		Area/cell µm^2		Surface coverage by U2OS cells %	
	1.5 h	48 h	1.5 h	48 h	1.5 h	48 h	1.5 h	48 h	1.5 h	48 h	1.5 h	48 h
TiO_2	51±4	59±9	550±48	1020±114	27±2	63±10	45±3	39±6	620±55	940±210	27±2	38±6
PEG	43±3	*	460±60	*	19±1	*	42±4	*	480±40	*	19±2	*
PEG –RGD	43±4	56±9	875±50	1180±82	36±4	69±11	43±3	50±9	1070±35	1024±96	44±4	54±10

* no U2OS cells were detected on the surface.

After 48 h, the adhering *S. epidermidis* had grown into a biofilm on all surfaces, but simultaneously U2OS cell adhesion and spreading was observed on TiO_2 surfaces and PEG-RGD coatings (Fig. 2). On PEG coatings, U2OS cells that were loosely adhered retained a rounded morphology up to 48 h (Fig. 2e). Upon application of an elevated shear rate (5.6 s^{-1}), biofilms detached from PEG and PEG-RGD coatings and partially from TiO_2 surfaces, whereas U2OS cell detachment only occurred from the PEG coating (Fig. 2g,h and i).
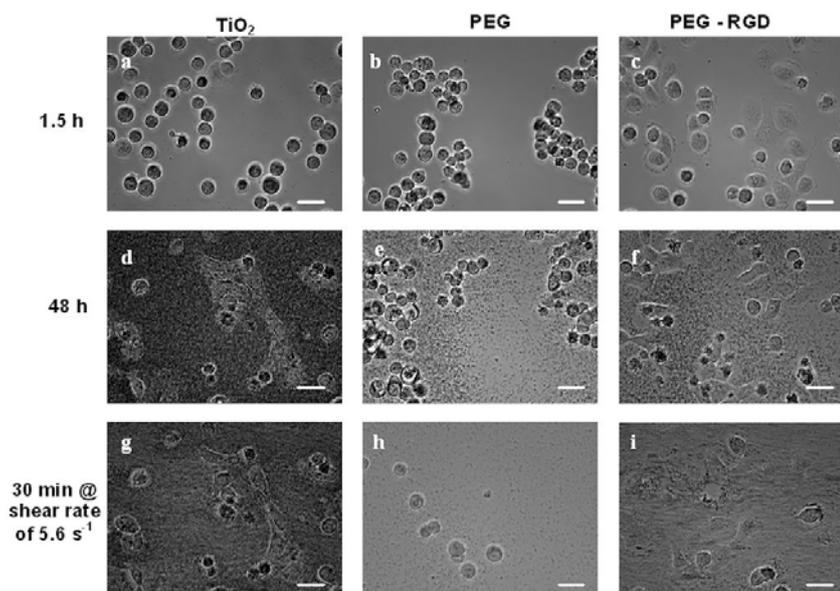
Fig. 2. Phase-contrast images of U2OS cell adhesion and spreading after seeding at 1.5 h, after 48 h of growth at low shear (0.14 s^{-1}) in the presence of adhering *S. epidermidis* as well as after an elevated shear (5.6 s^{-1}) for 30 min at the end of the experiment, on PEG and PEG-RGD coatings, and on TiO_2 control surfaces. At 1.5 h, well spread U2OS cells were observed on PEG-RGD (c) compared to PEG coating (b) and TiO_2 control surfaces (a), for which cell spreading was less. At 48 h, biofilm formation and U2OS cell spreading were observed on TiO_2 control surface (d) and PEG-RGD coating (f), whereas only biofilm formation and no cell spreading were seen on PEG coating (e). Upon application of an elevated shear, detachment of biofilm and U2OS cells was observed on PEG coatings (h), while only biofilm detachment occurred on PEG-RGD coating (i) and TiO_2 surface (g). All images were taken at the same magnification. The bar denotes 10 μm.

After 48 h of growth and subsequent application of a higher shear, adhering U2OS cells were immunocyto-stained for CLSM analysis (Fig. 3) to derive the number and spread area of adhering U2OS cells in the absence and presence of staphylococci. In Fig. 4 it can be seen that on a percentage basis, the number of

adhering U2OS cells was significantly reduced on TiO$_2$ surfaces in the presence of *S. epidermidis* as compared to the control, i.e. in the absence of adhering bacteria ($p < 0.05$). In the presence of *S. epidermidis*, the percentage adhering U2OS cells decreased on TiO$_2$ surfaces in contrast to that on bi-functional PEG-RGD coatings (Fig. 4) which showed an increase. U2OS cells showed no significant difference in spreading on PEG-RGD coating as compared to a TiO$_2$ surface in the presence or absence of adhering *S. epidermidis* (Fig. 5 and Table 1).

Surface coverage of U2OS cells in the absence and presence of adhering *S. epidermidis*. Immediately after seeding at 1.5 h, a significant increase in the surface coverage of U2OS cells was observed on PEG-RGD coatings compared to TiO$_2$ surfaces and PEG coatings, irrespective of the absence or presence of adhering *S. epidermidis* (Table 1).
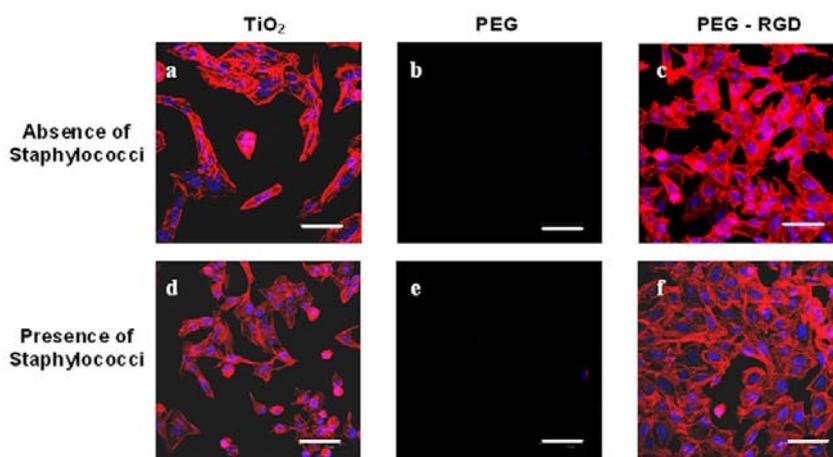
Fig. 3. CLSM images of U2OS cells after 48 h of growth at low shear (0.14 s^{-1}) and subsequent application of an elevated shear (5.6 s^{-1}) for 30 min on TiO$_2$ surfaces, PEG and PEG-RGD coatings in the absence (a-c) and presence (d-f) of adhering *S. epidermidis*. U2OS cells were stained with 5 ml PBS containing 49 μl DAPI and 2 μg ml^{-1} of TRITC-phalloidin. All images were taken at the same magnification. The bar denotes 75 μm.

After 48 h of growth at low shear and subsequent application of an elevated shear for 30 min, TiO_2 surface showed a significant decrease in surface coverage in the presence of adhering staphylococci compared to the control, i.e. the absence of adhering staphylococci. On PEG coatings, no adhering U2OS cells were detected on the surface (Fig. 3b,e). Conversely PEG-RGD coatings in the presence of adhering staphylococci showed no significant difference in surface coverage of U2OS cells compared to cells cultured in the absence of *S. epidermidis*.
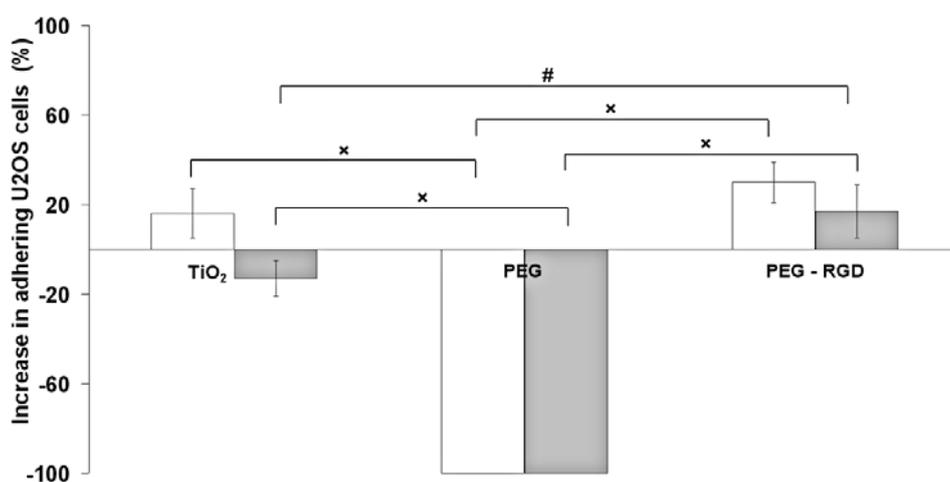
Fig. 4. The percentage increase in the number of adhering U2OS cells after 48 h of growth at low shear (0.14 s^{-1}) and subsequent application of an elevated shear (5.6 s^{-1}) for 30 min with respect to their initial number immediately after seeding at 1.5 h on TiO_2 surfaces and PEG and PEG-RGD coatings in the absence (□) and presence (■) of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviations over three replicates, with separately cultured bacteria and tissue cells.
× denotes significance at $p < 0.01$, # denotes significance at $p < 0.05$.
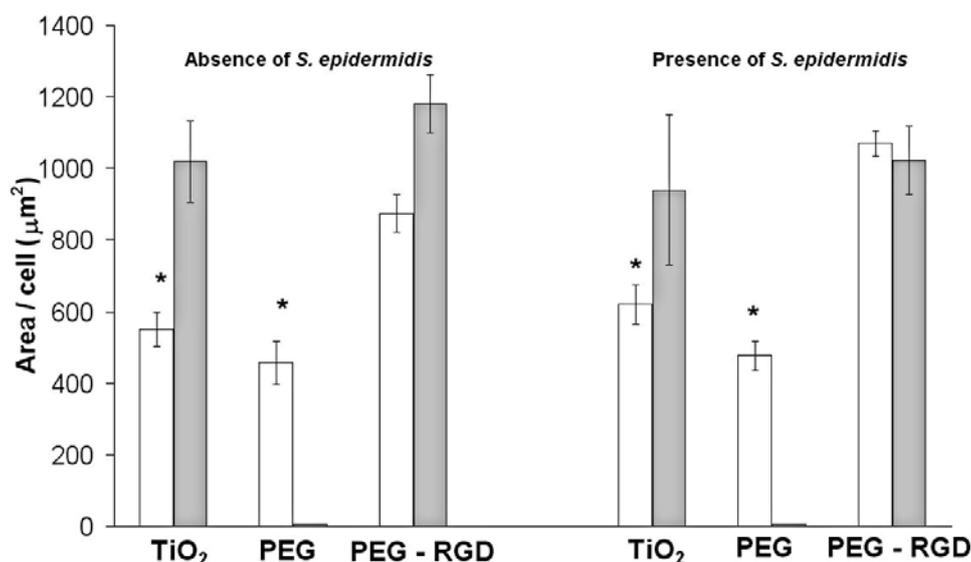
Fig. 5. The average area per adhering U2OS cell immediately after seeding at 1.5 h (□) and after 48 h of growth at low shear (0.14 s^{-1}) and subsequent application of an elevated shear (5.6 s^{-1}) for 30 min (■) on TiO$_2$ surfaces, PEG and PEG-RGD coatings in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviations over three replicates, with separately cultured bacteria and tissue cells.
* denotes significance at differences at $p < 0.01$ compared with PEG-RGD.

Discussion

This paper is the first to experimentally demonstrate the advantage of bi-functional versus mono-functional coatings in the prevention of infection on implant surfaces by conducting the race for the surface between bacteria and mammalian cells, as eventually determining the fate of a biomaterial implant. Bi-functional coatings clearly remained non-adhesive to *S. epidermidis*, but at the same time supported mammalian cell adhesion and spreading to a greater extent than mono-functional coatings and a control surface, in this case a bare TiO$_2$ surface. Titanium is widely used in dental and orthopaedic implants, heart valves and vascular stents [15]. Titanium devices implanted in the body are covered with a layer of oxide, responsible for the favourable biocompatibility of titanium implants. Tissue cells reactions on TiO$_2$ and pure titanium are very similar [16].

In our *in vitro* model, bacteria were allowed to adhere for 2 h prior to cell adhesion and spreading, which is considered to mimic the clinical situation where implants become contaminated prior to implantation. The number of bacteria adhering on PEG and PEG-RGD was 10^3 cm^{-2} and 10^5 cm^{-2} for TiO$_2$, representing a reduction in initial bacterial load on polymer brush coatings of two log-units. Note that in this study we deliberately chose to fix the time for bacterial adhesion, and not to contaminate all surfaces with the same number of organisms. This choice allows to compare coatings and surfaces for their performance during a fixed surgical period. In the past, it has been documented that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on the wound is about 270 per cm^2 [17]. The bacterial counts are generally higher during periods of activity and when more people are present in the operation theatre. More recent, through the use of modern, better ventilated operation theatres (20 changes of air per hour) and impermeable patient and personnel clothing, peri-operative bacterial contamination may well be less [18]. However, many surgical procedures in which implants are introduced in the body last much longer than 1 h. Therefore, the bacterial adhesion densities chosen in our experiments represent a moderate to worst case scenario.

Significant reduction of bacterial adhesion on PEG and PEG-RGD-coated surfaces compared to that on bare TiO$_2$ surface was previously reported [7]. In earlier studies [19,20], it was reported that the presence of a peptide covalently attached to PEG reduced bacterial adhesion similar to PEG coatings. In agreement with previous studies our results show that *S. epidermidis* showed no significant difference in adhesion on PEG coatings compared to PEG-RGD coatings indicating that the *S. epidermidis* ATCC 35983 bacteria do not recognize the RGD, unlike mammalian cells recognize RGD via integrin receptors.

Cell adhesion to a biomaterial surface is a prerequisite for successful tissue integration. Mammalian cells bind to the biomaterial surface through adsorbed proteins and one of the main binding sites in these proteins is the RGD peptide sequence [21]. On PEG coatings, hardly any protein adsorbed resulting in an inhibition of cell adhesion and spreading [6,22]. Similarly, in our experiments U2OS cells remained rounded on mono-functional PEG coatings up to 48 h. Such anti-fouling behaviour is mainly due to steric repulsion between the hydrated uncharged PEG chains and proteins [23]. However cell adhesion to mono-functional PEG-coatings can be achieved by introducing specific bioligands, such as a cell-interactive peptide (RGD) linked covalently to the non-fouling PEG, producing a bi-functional PEG-RGD coating. Our results show an enhanced mammalian cell adhesion to the bi-functional PEG-RGD coatings, in which already within 1.5 h after cell seeding, the U2OS cells showed near maximum spreading, indicating recognition of the RGD-sequence by adhesion receptors in the cell membrane [24]. The enhanced cell attachment and spreading depends on the RGD-peptide surface density [25]. The RGD-peptide surface density of around 5 pmol RGD peptide per cm^2 applied here was well above reported densities for cell adhesion of over 0.6 pmol RGD peptide per cm^2 [26]. The former was calculated [27] based on the copolymer composition (RGD grafting density, NMR) and the adsorbed copolymer mass from *in situ* monitoring of the copolymer adsorption using Optical Waveguide Lightmode Spectroscopy (OWLS) (data not shown).

In the concept of the race for the surface, a full coverage of a biomaterial *in vivo* by a viable tissue cell layer, intact cell membranes and functional host defense mechanisms resist bacterial colonization [28]. Previously, in our model for the 'race for the surface', all biomaterial surfaces allowed bacterial adhesion and biofilm

growth with a negative impact on the coverage of the biomaterial by mammalian cells [12]. Similarly, the presence of adhering *S. epidermidis* decreased the surface coverage of U2OS cells on TiO_2 surface compared to their control, i.e. the absence of *S. epidermidis*. In contrast to the TiO_2 surface, the presence of RGD-peptides at the surface significantly improved U2OS cell adhesion and spreading in the presence of *S. epidermidis,* eventually resulting in similar surface coverage than the control, i.e. the absence of *S. epidermidis*. Considering that cell adhesion and spreading are the signs of cellular well-being, this is highly indicative for a protective effect of cell binding to RGD in the presence of bacteria [29]. Even though complete coverage of the biomaterial surface was not obtained after 48 h, significantly improved U2OS cell adhesion and spreading on PEG-RGD coatings could eventually lead to complete coverage of U2OS cells on biomaterial surfaces over longer periods of time. In the concept of the race for the surface, one may speculate that PEG-RGD coated implants will become more easily integrated by host tissue cells than other biomaterial surfaces and thus better protect the implant against infection.

PEG coatings have been shown to be resistant to protein adsorption, biofilm formation and cell attachment *in vitro* over periods of up to 4 weeks [8, 30-32]. Depending on the exact nature of the coating, degradation of this biocompatible polymeric monolayer by hydrolytic/enzymatic activity has been reported. Another concern is the mechanical robustness of the PEG coatings, and if handled with surgical instruments, coatings may be easily damaged. Optichem® [32] and nano-particle based polymer brush coatings [31] are mono-functional non-adhesive coatings with greater robustness than can be obtained with monolayer coatings, like the PEG-RGD coating evaluated here. The results of the present study, however, aid in directing such further development of monolayer bi-functional coatings into more

robust ones, suitable for clinical use. The early protection by bi-functional coatings is believed to be clinically relevant, as it could prevent an implant from becoming colonized during the important period of actual implantation, which is critical for the long-term success of an implant [33,34].

Conclusion

This study demonstrates that biopassive, mono-functional PEG coatings and bioactive, bi-functional PEG-RGD coatings have potential to reduce bacterial adhesion and prevent firm adhesion of biofilms compared to common biomaterial surfaces. Moreover, in contrast to mono-functional PEG coatings, bi-functional PEG-RGD coatings allow cell adhesion and spreading. At the same time, adhesion and spreading of mammalian cells is not detrimentally affected by the presence of adhering *S. epidermidis*. Bi-functional coatings thus have a strong potential to reduce the risk of infection in applications requiring tissue integration, such as in dental and orthopaedic implants.

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Mammalian Cell Growth *versus* Biofilm Formation on Biomaterials Surfaces in a Post-operative Contamination Model *In vitro*

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Introduction

Biomaterial-associated infections (BAI) can develop from peri-operative microbial contamination of implant surfaces during implantation, immediately post-surgery during hospitalization or by late haematogenous spreading from infections elsewhere in the body. Both peri-operative and post-operative contaminations can cause BAI many years after implantation, as bacteria can stay dormant on an implant surface for several years [1,2]. Microorganisms involved in BAI are resistant to antibiotics and the host immune system due to their biofilm mode of growth, and biomaterial implants with a biofilm have to be removed in most cases [3-5]. 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 biofilm growth [6]. If this race is won by tissue cells, then the biomaterial surface is fully integrated by tissue cells and less vulnerable to bacterial biofilms. On the other hand, if the race is won by bacteria, the implant surface will become colonized by bacteria and tissue cell functions are hampered by bacterial virulence factors and toxins [6,7]. In the concept of the race for the surface, a full surface coverage of a biomaterial *in vivo* by a viable tissue cell layer, intact cell membrane and functional host defense mechanisms resist bacterial colonization [8].

Previously an *in vitro* experimental model for peri-operative bacterial contamination of implant surfaces was forwarded and the effects of bacterial presence on adhesion, spreading and growth of mammalian cells were determined in a single experiment [9]. The outcome of the race for the surface between contaminating *Staphylococcus epidermidis* and mammalian cells on glass appeared to be dependent on the number of bacteria present prior to mammalian cell seeding and the absence or presence of fluid flow. Cells lost the race for the surface in the absence of flow due to

accumulation of bacterial toxins, but were able to grow under flow due to the continuous supply of fresh medium to and removal of endotoxins from the interface on all commonly used biomaterial surfaces [10].

In the concept of the race for the surface, as forwarded by the late orthopedic surgeon A.G. Gristina, tissue integration is an important protective factor against bacterial contamination of an implant surface [8]. However, the degree of tissue coverage required to effectively protect an implant surface against bacterial contamination is unknown due to lack of a suitable experimental model. Therefore, the aim of this study is to describe an *in vitro* model for post-operative bacterial contamination of implant surfaces and investigate the effects of different degrees of mammalian cell coverage on the balance between cell growth and bacterial biofilm formation.

Materials and Methods

Biomaterial surface. Poly (methyl methacrylate) (PMMA) (Vink Kunststoffen, Didam, The Netherlands), a commonly used biomaterial, was used as a substratum surface. Samples were rinsed thoroughly with ethanol (Merck, Darmstadt, Germany) and washed with sterile ultrapure water before use.

Mammalian cell culturing and harvesting. U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-low glucose supplemented with 10% fetal calf serum (FBS), 0.2 mM of ascorbic acid-2-phosphate (AA2P) and denoted as "DMEM+FBS". Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged at 70 – 90% confluency using trypsin/EDTA.

Bacterial growth conditions and harvesting. *S. epidermidis* ATCC 35983, originally isolated from human blood of a patient with an infected intravascular catheter, was used throughout this study. First, the strain was streaked on a blood agar plate from a frozen stock and grown overnight at 37°C. The plate was then kept at 4°C. For each experiment, a colony was inoculated in 10 ml of tryptone soya broth (TSB; OXOID, Basingstoke, England) and cultured for 24 h. This culture was used to inoculate a second culture, which was grown for 17 h prior to harvesting. Bacteria were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were sonicated on ice (3 x 10 s) in sterile PBS in order to break bacterial aggregates. This suspension was further diluted in sterile PBS to a concentration of 3 x 10^6 bacteria per ml.

Competitive assay for mammalian cell growth and biofilm formation. The competitive assay was studied on the PMMA bottom plate of a parallel plate flow chamber (175 x 17 x 0.75 mm^3), as described in detail before [9]. The flow chamber was equipped with heating elements and kept at 37°C throughout the experiments. Bacterial and U2OS cell deposition were observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Olympus BH-2 (Olympus, Germany) with a 40x objective for bacteria and 10x objective for mammalian cells.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, the U2OS cell suspension in modified culture medium, consisting of 98% DMEM+FBS and 2% TSB suitable for the simultaneous growth of U2OS cells and *S. epidermidis* [9], was allowed to enter the flow chamber. Once the entire volume of

buffer inside the chamber was replaced by the U2OS cell suspension, flow was stopped for 1.5 h in order to allow cells to adhere and spread on the substratum. Subsequently, phase contrast images (nine images, 900 x 700 μm each) were taken and the number of adhering cells per unit area and the area per spread cell were determined using Scion image software. Subsequently, modified culture medium supplemented with 2% HEPES was perfused through the system at a low shear rate of 0.14 s^{-1} for 24 h. Experiments with three different U2OS cell densities (1.2×10^5 cells ml^{-1}, 6×10^5 cells ml^{-1} and 13×10^5 cells ml^{-1}) were performed to attain different degrees of initial cell coverage after 24 h prior to seeding of bacteria. After 24 h of U2OS cell growth, bacterial suspension in PBS was perfused through the chamber at shear rate of 11 s^{-1} and phase-contrast images were obtained as a function of time. As soon as the desired density of adhering bacteria (10^3 cm^{-2}, which is a relevant number in implant contamination [11]), was reached (around 20 min), flow was switched to sterile PBS to remove unattached bacteria from the tubes and flow chamber, after which modified culture medium supplemented with 2% HEPES was perfused through the system at a low shear rate of 0.14 s^{-1} for another 24 h. HEPES was added in order to compensate for the absence of 5% CO_2 during cell growth in the flow chamber.

Immuno-cytochemical staining and determination of U2OS cell surface coverage.
After simultaneous growth of bacteria and U2OS cells, surfaces were prepared for immuno-cytochemical staining to assess the mammalian cell morphology and spreading. For fixation, surfaces with adhering bacteria and U2OS cells were placed in a Petri dish with 30 ml of 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5 min, the fixation solution was replaced by 30 ml of fresh CS for another 5 min.

Subsequently U2OS cells were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS and stained for 30 min with 5 ml PBS containing 49 µl DAPI and 2 µg ml^{-1} of TRITC-phalloidin. The cells on the surfaces were washed four times in PBS and examined with confocal laser scanning microscopy (CLSM, Leica DMRXE with confocal TCS SP2 unit). Images (nine images on different locations, 900 x 700 µm each) were taken and the number of adhering cells per unit area and the average area per spread cell were determined using Scion image software to yield the total coverage of the substratum surface by mammalian cells.

Statistics. Data are presented as mean ± standard deviation of nine images. Statistical ANOVA analysis was performed followed by a Tukey's HSD post-hoc test and a *p*-value of < 0.05 was considered significant.

Results

U2OS cells were allowed to adhere and spread for 24 h followed by *S. epidermidis* adhesion to mimic post-operative infection. Subsequently, the simultaneous growth of mammalian cells and *S. epidermidis* was observed for 24 h.

1.5 h after U2OS cell seeding, the average numbers of adhering U2OS cells on the PMMA surface were 2.5×10^4 cells cm^{-2}, 8.2×10^4 cells cm^{-2} and 17×10^4 cells cm^{-2} for the different seeding densities, with an average area of the spread cells between 380 µm^2 and 540 µm^2 per cell. After 24 h of U2OS cell growth, *S. epidermidis* were allowed to adhere at a shear rate of 11 s^{-1} until 10^3 bacteria per cm^2 were counted. The adhering U2OS cells were not affected during initial adhesion of *S. epidermidis*.

After 24 h of simultaneous growth of U2OS cells and *S. epidermidis*, the number of adhering *S. epidermidis* was significantly higher ($p < 0.01$) on PMMA with the lower cell seeding density (2.5×10^4 cells cm^{-2} on average) as compared to the number of adhering *S. epidermidis* on PMMA with higher U2OS cell seeding densities. There was no significant difference in number of adhering *S. epidermidis* on PMMA with U2OS cell seeding densities of 8.2×10^4 cells cm^{-2} and 17×10^4 cells cm^{-2} (Fig. 1).
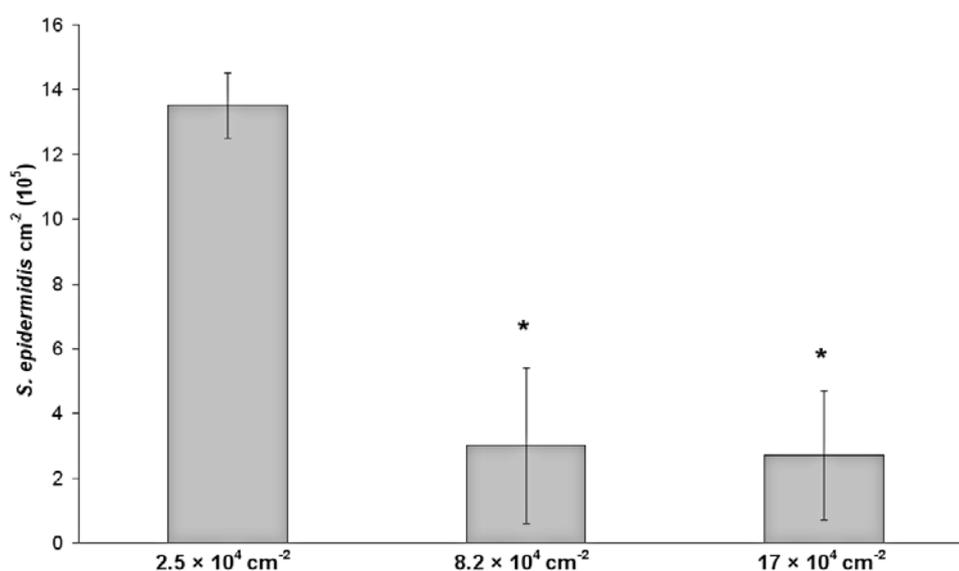
Fig. 1. The number of adhering *S. epidermidis* ATCC 35983 after 24 h of simultaneous growth of bacterial and U2OS cells on PMMA with different initial numbers of U2OS cells present. Error bars represent the standard deviations ($n = 9$). * Significantly different ($p < 0.01$) from the lowest U2OS cell seeding density (2.5×10^4 cells cm^{-2}).

The adhering U2OS cells were immunocyto-stained for CLSM analysis in order to derive their number and spread area after 48 h (Fig. 2). In Fig. 3 it can be seen, that the %increase in number of adhering cells was significantly reduced due to the presence of adhering staphylococci in all cases as compared to the control, i.e. in the absence of adhering staphylococci ($p < 0.01$) . The average area per spread cell was approximately 1000 μm^2 at the two lower cell seeding densities, but at the highest cell

seeding density of 17×10^4 cells cm^{-2}, U2OS cells spread to only 460 μm^2 per cell, similar to control (Fig. 4).
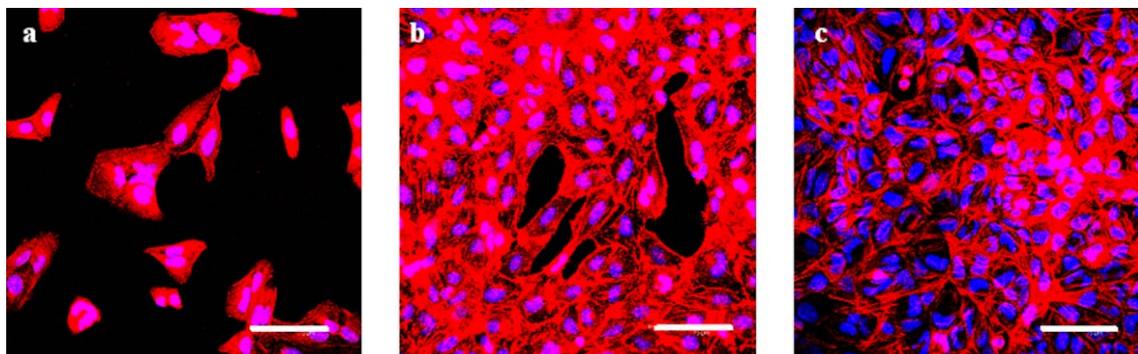
Fig. 2. CLSM images of U2OS cells seeded to a density of (a) 2.5×10^4 cells cm^{-2}, (b) 8.2×10^4 cells cm^{-2} and (c) 17×10^4 cells cm^{-2} after 48 h of growth in the presence of adhering *S. epidermidis* ATCC 35983 on PMMA. U2OS cells were stained with 5 ml PBS containing 49 μl DAPI and 2 μg ml^{-1} of TRITC-phalloidin. The bars denote 75 μm.
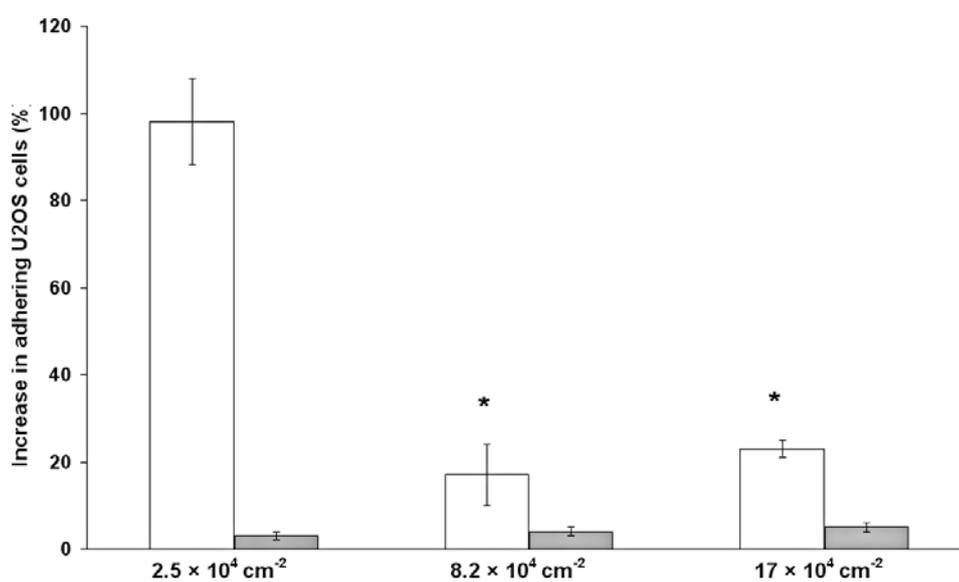
Fig. 3. Percentage increase in the number of adhering U2OS cells after 48 h of growth with respect to their initial number immediately after seeding at 1.5 h on PMMA in the absence (□) and presence (■) of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviations (n = 9). *Significantly different (p < 0.01) from the lowest U2OS cell seeding density (2.5×10^4 cells cm^{-2}).

In the concept of the race for the surface, the total cell surface coverage of the substratum by host tissue cells is determinant for the fate of an implant. The surface coverage of U2OS cells at 1.5 h after seeding and after 48 h of growth are shown in Fig. 5. After 1.5 h of U2OS cell seeding, the average surface coverages by U2OS cells on PMMA surface were 12%, 33% and 65% for seeding densities 2.5×10^4 cells cm^{-2}, 8.2×10^4 cells cm^{-2} and 17×10^4 cells cm^{-2}, respectively. After 24 h of U2OS cell growth, a slight increase of 5% - 10% in surface coverage was observed (data not shown). After 48 h of growth in the absence of *S. epidermidis*, a significant increase ($p < 0.01$) in surface coverage by adhering cells was observed compared to 1.5 h. In the presence of *S. epidermidis*, a significant reduction ($p < 0.01$) in surface coverage by adhering cells was observed at the lowest cell density (2.5×10^4 cells cm^{-2}) as compared to the control, i.e. in the absence of adhering staphylococci. At the higher cell densities, cell surface coverage was similar in the absence or presence of adhering staphylococci.
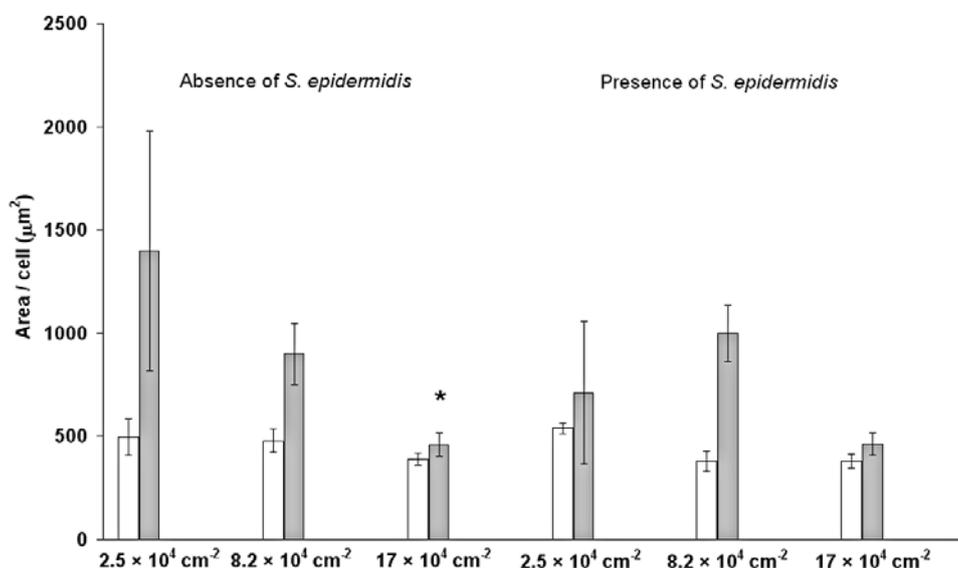
Fig. 4. Average area per adhering U2OS cells immediately after seeding at 1.5 h (□) and after 48 h (■) of growth on PMMA in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviations (n = 9). *Significantly different ($p < 0.05$) from the lowest U2OS cell seeding density (2.5×10^4 cells cm^{-2}).
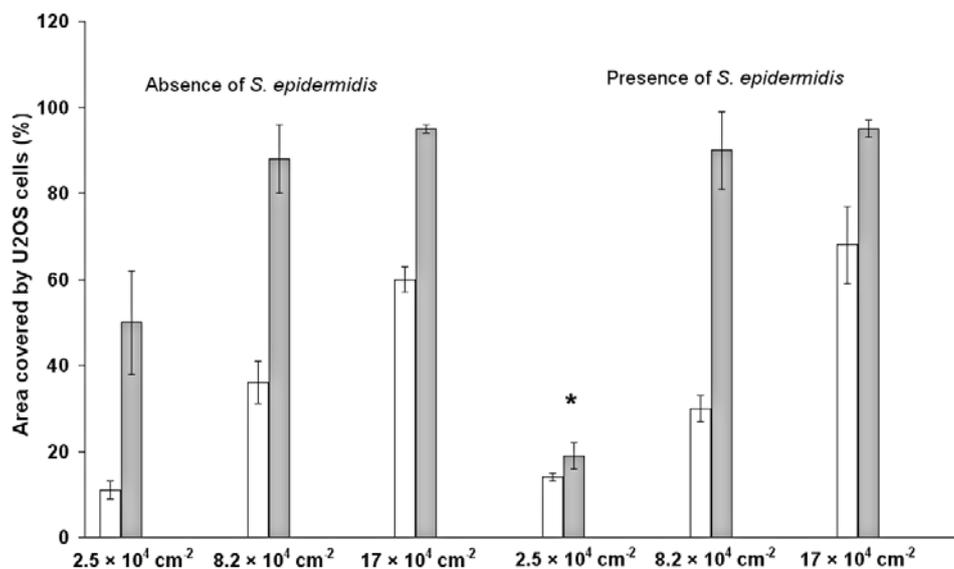
Fig. 5. Surface coverage by adhering U2OS cells immediately after seeding at 1.5 h (□) and 48 h (■) of growth on PMMA in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviations (n = 9). *Significantly different (p < 0.01) from the control (absence of staphylococci).

Discussion

This paper presents the first experimental *in vitro* study on the race between bacteria and tissue cells on PMMA in a post-operative bacterial contamination model of implant surfaces. Below a threshold coverage of the substratum surface by adhering mammalian cells, contaminating *S. epidermidis* ATCC 35983 negatively impacted mammalian cell growth, but once cell surface coverage exceeded a critical value, contaminating *S. epidermidis* ceased to negatively impact cell growth. The bacterial challenge chosen in the current experiments was low and is similar to peri-operative contamination levels [11,12]. Kaduragamuwa *et al.* [11] showed in a murine model that low doses of bacteria, introduced either peri-operatively or post-operatively, established a stable infection resembling the clinical situations. Therefore, the bacterial challenge concentration used is considered relevant.

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The current study was conducted with *S. epidermidis*. Clinically, *S. epidermidis* is one of the main causative organisms for BAI in particular chronic prosthetic joint infections [13,14]. *S. epidermidis* can yield early post-operative contamination as long as wound closure is incomplete. Alternatively, late haematogenous spreading may occur from infections elsewhere in the body to an implant surface. Notorious in this aspect are abscesses underneath the skin, developing for instance after minor injuries. Also dental treatment is known to be a cause of post-operative contamination of implant surfaces, as even routine inspection of the dentition by a dentist or mouth hygienist may give rise to bacteraemia [15,16]. BAI due to *S. epidermidis* is usually low grade, since the organism lacks the genes to produce toxins and tissue damaging exoenzymes, that are produced by, for instance *Staphylococcus aureus* [17,18], another causative strain of BAI [13,14]. *S. aureus* infections are therefore more aggressive than *S. epidermidis* ones, but at the same time are more readily noticed and treated. In a sense this makes the low-grade BAI due to *S. epidermidis* more troublesome, which motivates our current choice for *S. epidermidis* to set up our post-operative contamination model.

PMMA is well-know for its use in ophthalmological, orthopedic and dental applications. In orthopedic applications, PMMA-based bone cements are extensively used for fixation of total joint replacements, as the material supports cell adhesion and spreading [19-21]. In a peri-operative contamination model, PMMA showed better tissue cell adhesion and spreading in the presence of adhering *S. epidermidis* than other commonly used biomaterials [10].

In the concept of the race for the surface, complete surface coverage of a biomaterial *in vivo* by viable tissue cells, combined with functional host defense mechanisms resist the negative consequences of bacterial contamination [8]. Rapid

and complete surface coverage of mammalian cells restricts the biomaterial surface area available for bacterial adhesion and biofilm formation. Dexter *et al.* [22] suggested that an optimal concentration of seeded 3T3 fibroblasts and conditions to stimulate cell adhesion and surface coverage without stimulating bacterial adhesion could probably reduce infection. However, the consequence of the combined presence of mammalian cells and bacteria on a surface was not monitored. In this study, the presence of *S. epidermidis* ATCC 35983 showed negative effects on cell surface coverage by U2OS cells at the lowest initial cell density (2.5×10^4 cells cm^{-2}) as compared to cell surface coverage in the absence of adhering staphylococci. In contrast, surface coverage of U2OS cells after seeding higher initial cell densities was not influenced by the presence of *S. epidermidis*. Therewith this study provides direct experimental evidence for the Gristina postulate [8] that *in vivo* tissue integration protects implant surfaces against bacterial colonization. Moreover, our study defines a critical cell surface coverage needed in order to protect an implant surface against *S. epidermidis* biofilm formation. We anticipate that this critical cell surface coverage level will depend on the biomaterial surface characteristics as well as on the infecting strain. With this model system we present a tool to assess the 'race for the surface' and compare different biomaterials, coatings and bacterial strains.

Conclusion

An *in vitro* method is presented to study the effects of post-operative bacterial contamination on the interaction of mammalian cells with biomaterial implants. A critical mammalian cell surface coverage was found, above which contaminating *S. epidermidis* ATCC 35983 no longer had a negative impact on mammalian cell growth. Thus following the concept of the race for successful implantation of a

biomaterial, survival of the implant will be more solidly assured if an implant is rapidly integrated by tissue to above this critical cell surface coverage. This new method and the concept of critical cell surface coverage will allow better evaluation of biomaterial coatings prior to animal experiments or human trials, than based on separate studies of microbial adhesion to or mammalian cell interactions with such coatings.

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A New Method to Study the Simultaneous Interaction Between Bacteria, Macrophages and Osteoblasts on a Biomaterial Implant Surface

Introduction

Biomaterial-associated infections (BAI) are a widespread complication that threatens the longevity and functionality of indwelling biomaterial implants and devices and the consequences in terms of medical care are severe. Despite of improved techniques and highly sterile conditions in the operating theatre, peri-operative contamination by microorganisms suspended in the air and from the skin flora continues to be the most common pathway for the contamination of biomaterial implants and devices [1,2].

Staphylococcus aureus and *Staphylococcus epidermidis* are microorganisms frequently isolated from BAI. *S. epidermidis* is found in almost 50% of the infections associated with catheters, artificial joints and heart valves, while *S. aureus* is seen in around 23% of the infections associated with prosthetic joints [3]. These commensals from the skin adhere to the biomaterial surface and grow to form a biofilm. Bacteria in their biofilm mode of growth are frequently more resistant to antibiotic treatment and the host immune system than their planktonic counterparts. Hence, removal of an infected implant or device is often the only remedy for a BAI. Surrounding tissue, however, may remain compromised by bacterial presence for prolonged periods of time after removal of the biomaterial [4,5] which severely lowers the prospects of a secondary implant or device, since bacteria in tissue constitute a new source for BAI to develop.

Whether or not BAI will occur, ultimately depends on the interaction between the biomaterial, the bacterium involved and host cells. Host cells and bacteria battle to proliferate and colonize a biomaterial surface. The general assumption is that on surfaces with a high affinity for tissue cells, bacterial biofilm formation will be limited, thus decreasing the risk of a BAI. Contrary, if bacteria grow and colonize

faster than tissue cells, bacterial toxins and virulence factors can impair cell functions leading to BAI [6].

In a healthy host, the host immune system comes to the aid of tissue cells [7]. 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 inflammation process and eventually foreign body reactions [7]. During infection, macrophages detect bacteria via cell surface receptors that bind to bacterial ligands and opsonines [8]. Subsequently, macrophages ingest pathogens and activate cellular functions such as proliferation, secretion of proteins and cytokines, and respiratory burst to destroy phagocyted microorganisms and recruit other cells from the adaptive immune system [9]. However, it has been shown that the presence of a foreign body may impair the host immune system and very low numbers of adherent bacteria are already sufficient to create a BAI [6].

Currently, biomaterials research is strongly focused on the design of novel functional coatings that reduce the risk of BAI by inhibiting bacterial adhesion and stimulating tissue cell adhesion [10,11]. However, in the evaluation of these coatings, bacterial and tissue cell adhesion are often considered as independent phenomena and not as simultaneous events [10,11]. Furthermore, the response of the immune system to bacterial colonization on these coatings is generally ignored. Recently, a novel *in vitro* method has been forwarded to determine the influence of bacterial presence on the outcome of the competition between bacteria and tissue cells in their attempt to colonize or integrate a biomaterial surface [12].

In this study we present an extension of this model [12] to include also the influence of macrophages on the outcome of the race for the surface between adhering bacteria and tissue cells in a single experiment.

Materials and Methods

Biomaterial. Poly (methyl methacrylate) (PMMA) (Vink Kunststoffen, Didam, The Netherlands) was used as a substratum. Samples were rinsed thoroughly with 70% ethanol (Merck, Darmstadt, Germany) and washed with sterile ultrapure water before use. Water contact angles on thus cleaned PMMA were 73 ± 3 degrees, in line with literature [13].

Tissue cell culturing and harvesting. U2OS osteosarcoma cells were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-low glucose supplemented with 10% fetal calf serum (FBS, non-heat inactivated), 0.2 mM of ascorbic acid-2-phosphate (AA2P). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged at $70 - 90\%$ confluency using trypsin/EDTA.

Macrophages culturing and harvesting. J774 murine macrophages were routinely cultured in Dulbecco's modified Eagles Medium (DMEM)-high glucose supplemented with 10% fetal calf serum (FBS, non-heat inactivated) and denoted in the paper as "optimal medium". Macrophages were maintained at 37°C in a humidified atmosphere with 5% CO_2, and passaged at $70 - 80\%$ confluency by scraping.

Bacterial growth conditions and harvesting. The bacterial strains used in this study were *S. epidermidis* ATCC 35983 and *S. aureus* ATCC 12600. These strains were cultured on blood agar plates and grown aerobically overnight at 37°C, and the plates were kept at 4°C, never longer than two weeks. For each experiment, one colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England)

and incubated for 24 h at 37°C in ambient air. This culture was used to inoculate another culture in 200 ml TSB that was incubated overnight at 37°C prior to harvesting. Bacteria were harvested by centrifugation (5 min at 5000 g at 10°C) and washed twice with sterile ultrapure water. Bacteria were sonicated intermittently on ice (30 s) in sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 6.8) to break bacterial aggregates, and resuspended in 200 ml sterile PBS to a concentration of 3×10^6 bacteria per ml. Prior to the experiments, growth and biofilm formation of *S. epidermidis* ATCC 35983 and *S. aureus* ATCC 12600 in optimal medium was confirmed by culturing bacteria in optimal medium for 24 h.

***In vitro* multiple cell type culture assays.** The competition between bacteria and U2OS cells for the colonization of PMMA in the absence of macrophages (control) and in the presence of macrophages was assessed under laminar flow on the bottom plate of a parallel plate flow chamber ($175 \times 17 \times 0.75$ mm^3). Bacterial and cell deposition were observed real-time using a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Olympus BH-2 (Olympus, Germany). The flow chamber was equipped with heating elements and kept at 37°C throughout the experiments.

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove all air bubbles from the system. Once the system was filled, and before the addition of bacterial suspension, PBS was allowed to flow through the system at a shear rate of 11 s^{-1}. Then, a bacterial suspension in PBS was perfused through the chamber at the same shear rate and phase-contrast images were obtained. As soon as the desired density of adhering bacteria (10^3 cm^{-2}), was reached, flow was switched to sterile PBS to remove unbound bacteria and the bacterial

suspension from the tubes and chamber. Subsequently, a cell suspension consisting of U2OS cells (6×10^5 cells ml^{-1}) and J774 macrophages (12×10^5 cells ml^{-1}) in optimum medium was added to the flow chamber. Once the entire volume of buffer inside the chamber was replaced by the cell suspension, flow was stopped for 1.5 h in order to allow U2OS cells and macrophages to adhere and spread on the substratum surface. Ultimately, "optimum medium" supplemented with 2% HEPES was perfused through the system without recirculation at a shear rate of 0.14 s^{-1} for 24 h and phase-contrast images were obtained continuously at 2 min intervals. Biofilm growth was assessed in real-time by determining the numbers of adhering bacteria per unit area using proprietary software based on the Matlab Image processing Toolkit (The MathWorks, MA, USA).

At the end of the assay, surfaces were prepared for qualitative analysis to assess U2OS cell and macrophages morphology and spreading. Cells adhering to PMMA were fixed with citrated-acetone-formaldehyde fixative solution for 30 s and stained with an alkaline-dye mixture (Sigma-Aldrich, Germany) (Naphtol AS-BI phosphate, sodium nitrite, fast blue BB base) for 15 min. The samples were subsequently rinsed with demineralized water and counterstained for 2 min with neutral red solution. Then the samples were rinsed once again with demineralized water, allowed to dry and phase-contrast images were taken on different places of the sample. Differentiated U2OS osteosarcoma cells stained purple/blue (alkaline phosphatase-positive) and macrophages were orange stained.

Results

Bacteria were allowed to adhere to the biomaterial surface prior to U2OS cell and macrophage adhesion, mimicking a peri-operative contamination after which bacteria,

U2OS cells and macrophages were allowed to grow simultaneously for 24 h. Events are illustrated as follows.

Migration of macrophages towards bacteria and phagocytosis. The number of bacteria adhering to the PMMA surface prior to U2OS cells and macrophages adhesion was set to 10^3 cm^{-2}, using the image analysis system Subsequently, U2OS cells and macrophages were allowed to adhere to the surface and the simultaneous interactions of bacteria, macrophages and U2OS cells were observed by phase-contrast microscopy. Figure 1 shows macrophage migration in the presence of U2OS cells towards adhering bacteria and subsequent phagocytosis. Macrophage migration towards bacteria and phagocytosis was similar on PMMA colonized by *S. epidermidis* and *S. aureus*.
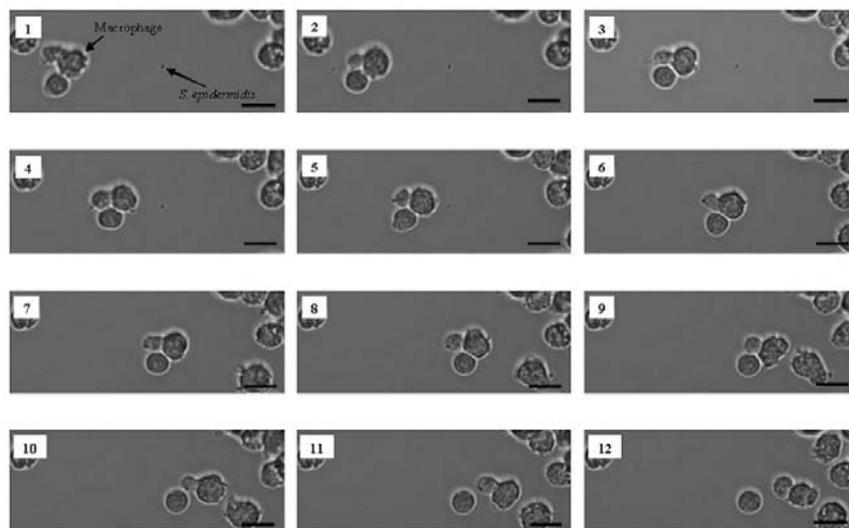
Fig. 1. Phase-contrast images of macrophage activity toward *S. epidermidis* ATCC 35983 on a PMMA surface in the presence of U2OS cells: macrophage migration towards *S. epidermidis* (images 1-5), bacterial clearance by phagocytosis (images 6-7) and further migration (images 8-12). The bar denotes 50 µm.

Bacterial biofilm formation in the absence and presence of macrophages. Biofilm growth was assessed over time by determining the numbers of bacteria adhering to PMMA at different time points during the simultaneous growth of bacteria, U2OS cells and macrophages (Fig. 2). In the presence of macrophages, reduction in the numbers of adherent bacteria, both for *S. epidermidis* and *S. aureus*, was observed as compared to controls (absence of macrophages). This effect was observed up to 20 h of growth for *S. epidermidis* and up to 14 h for *S. aureus*. Thereafter macrophage burst and release of ingested bacteria was observed.
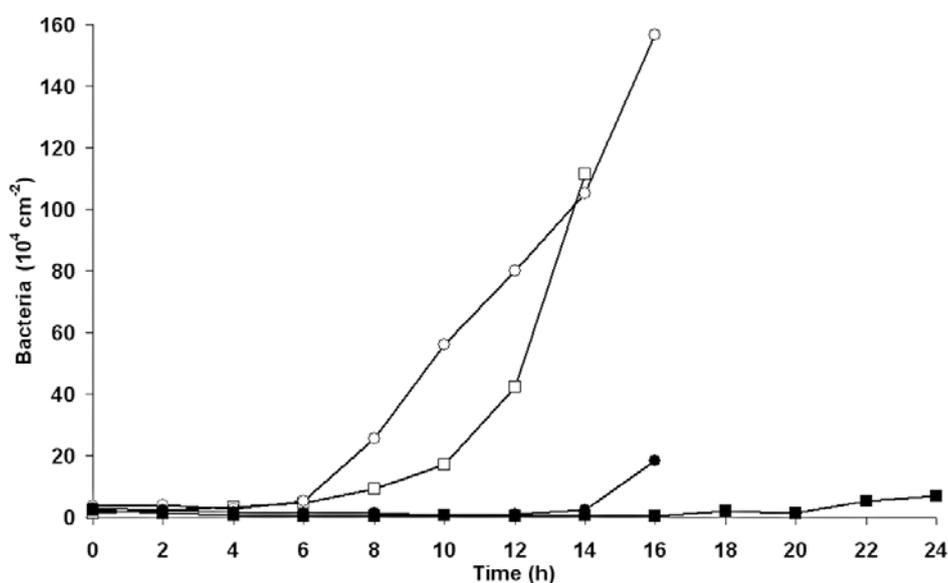
Fig. 2. The numbers of adhering bacteria on PMMA as a function of time during the simultaneous growth of bacteria and U2OS cells in the absence and presence of macrophages in a parallel plate flow chamber (shear rate 0.14 s^{-1}).
S. epidermidis in the absence of macrophages (□), *S. aureus* in the absence of macrophages (○), *S. epidermidis* in the presence of macrophages (■) and, *S. aureus* in the presence of macrophages (●).

Bacterial-tissue cell interactions in the absence and presence of macrophages. Immediately after seeding, U2OS cell adhesion and spreading on PMMA was observed independently of whether macrophages were present or not. After 24 h of simultaneous growth, U2OS cell death was observed in the presence of a *S. aureus*

biofilm irrespective of the absence or presence of macrophages. On the other hand, colonizing *S. epidermidis* did not significantly affect U2OS cells and their adhesion and spreading were similar both in the absence and presence of macrophages (see Fig. 3).
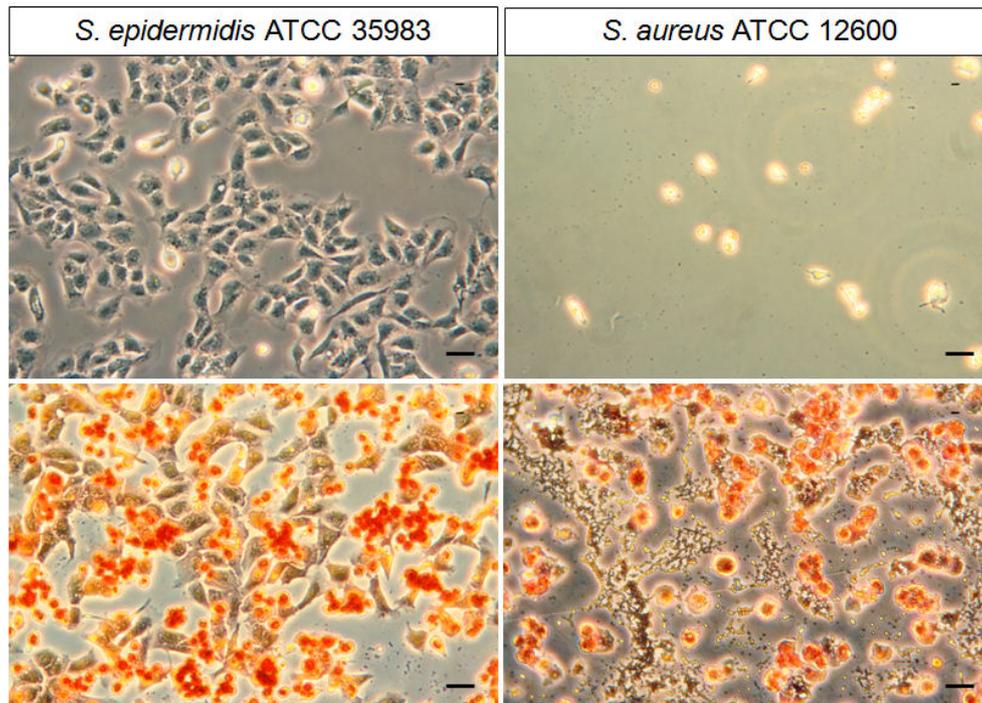
Fig. 3. Phase-contrast images of adhered cells to PMMA after 24 h of simultaneous growth of U2OS and *S. epidermidis* ATCC 35983 or *S. aureus* ATCC 12600 in the absence (upper images) and presence (lower images) of macrophages. Macrophages are orange-stained. The bar denotes 50 μm.

Discussion

This paper presents the first experimental model to study the simultaneous interaction of macrophages-bacteria-osteoblasts on a biomaterial surface in a single experiment. In our *in vitro* model, bacteria were allowed to adhere prior to adhesion of macrophages and U2OS cells, which mimics a peri-operative bacterial contamination of implant surfaces. The number of bacteria adhering on the PMMA surface prior to macrophages and U2OS cell adhesion was set to 10^3 cm^{-2}. In the past, it has been

documented that during a surgical procedure of 1 h, the total number of bacteria carrying particles falling on a wound is about 270 cm^{-2}. The bacterial counts were generally higher during periods of high activity and when more people were present in the operation theatre [14]. Recently, through the use of modern, better ventilated operation theatres (20 changes of air/h) and impermeable patient and personnel clothing, peri-operative bacterial contamination is likely to be reduced [15]. However, many surgical procedures in which implants are introduced in the body last longer than 1 h. Therefore, the level of bacterial contamination chosen in our experiments is probably realistic of a worst case scenario. Despite these low numbers, peri-operatively introduced organisms, particularly when of low virulence, can survive on an implant surface for prolonged periods of time and later, during periods of host immune depression, they proliferate and establish an infection with clinical symptoms [16].

The pathogenesis of BAI is complex and depends on factors such as bacterial virulence, physicochemical properties of the biomaterial and alterations in the host defense [17]. Previously, in our model for the competition between bacteria and tissue cells, all common biomaterial surfaces, including PMMA, allowed *S. epidermidis* ATCC 35983 biofilm formation with a negative impact on the coverage of the biomaterial surface by tissue cells [13]. Yet, PMMA showed better cell adhesion and spreading in the presence of adhering *S. epidermidis* ATCC 35983 than other commonly used biomaterials [13]. Our present study supports our previous observations that U2OS cells are able to adhere, spread and grow in the presence of *S. epidermidis* ATCC 35983, and extend these observations to the absence and presence of macrophages. On the other hand, in the presence of adhering *S. aureus* ATCC 12600, death of all adhering U2OS cells and macrophages within 18 h was observed

despite the suspected removal of the majority of the bacterial toxins by flow. These observations are in line with clinical findings that BAI due to *S. aureus* usually progresses much more aggressively than BAI caused by *S. epidermidis*. In *S. epidermidis* infections, biofilm formation is considered the only virulence factor and therefore infections are usually sub-acute or chronic. The low virulence of *S. epidermidis* strains compared to *S. aureus* is due to the lack of additional genes responsible for producing severely tissue damaging toxins [3,18].

In general, immune cells migrate, engulf and kill invading microorganisms [19-21]. A previous study on the interaction between macrophages and colonizing *S. epidermidis,* showed that macrophage behavior is surface dependent [22]. Macrophage migration towards bacteria and phagocytosis was enhanced on cross-linked poly(ethylene)-glycol (PEG) based polymer coatings compared to the uncoated substrata due to the weak adhesion of macrophages and bacteria to the PEG coating [22]. In our study, macrophages migrate towards the bacteria on a PMMA surface and engulfed the bacteria. The phagocytosis of bacteria by macrophages differs depending on the virulence of the strain. In the presence of low virulent *S. epidermidis*, bacterial biofilm growth was strictly reduced by the presence of macrophages up to 20 h compared to only 14 h in the case of high virulent *S. aureus* biofilm growth. These results are in line with previous studies showing that in both *in vivo* and *in vitro* the uptake rate of bacteria by macrophages was inversely proportional to the virulence of the bacteria [23,24]. Furthermore, macrophages disintegration and necrotic death has been observed *in vitro* and *in vivo* due to overloading with ingested bacteria [25,26]. In this study it was observed that after a period of time macrophages become exhausted and break open which leads to a burst release of bacteria. At least part of these bacteria appeared to be active in the flow chamber. These findings suggest that

J774 macrophages in this model are not able to kill all phagocytised bacteria. Although the viability of the released bacteria was not assessed, several studies have demonstrated that immune cells lose their ability to kill bacteria [25,21,27,28]. Leid *et al*. [29] showed that leukocytes were able to migrate to *S. aureus* biofilms but failed to phagocyte the bacteria. Neutrophils adjacent to Teflon cages, implanted in peritoneal cavities, exhibited decreased bactericidal activity and reduced superoxide production due to the increased production of *S. epidermidis* extracellular slime [30-33]. Watanabe *et al*. [28] demonstrated that engulfed *S. aureus* suppressed the production of superoxide, resulting in the prolonged survival inside the macrophages. In a murine model it was shown that high numbers of *S. epidermidis* could persist within macrophages in peri-catheter tissue without showing any signs of inflammation [17]. Also *S. epidermidis* inside macrophages were not only viable but were able to proliferate. *In vivo*, the local host defense was compromised because of the presence of biomaterials, resulting in deficient intracellular killing of pathogens by macrophages [17].

The influence of macrophages on the competition between bacteria and mammalian cells is novel. This study demonstrates that despite the presence of macrophages, mammalian cells lost the race for the surface in the presence of high virulent *S. aureus*. *In vivo*, bacteria may well survive inside the macrophages for prolonged periods of time. These bacterial will favor the development of BAI, especially when certain physical conditions of the patients disturb the balance between bacteria and the host response [17]. This model validated for bacteria-macrophages-osteoblasts interactions in a flow chamber system resembles the *in vivo* environment more closely than single-cell type cultures therewith providing an important bridge between *in vitro* and *in vivo* studies. Even though, this study was

qualitatively analyzed, we believe that this methodology supported with quantitative

data, could be a suitable tool for evaluation of biomaterials based on infection models.

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CHAPTER 8

General Discussion

Hitherto, biomaterials or functional coatings for application in dentistry or orthopedics addressing infections are evaluated *in vitro* either for their ability to resist bacterial adhesion and biofilm formation or for their ability to enhance mammalian cell adhesion and proliferation [1-6]. However, according to Anthony Gristina, *in vivo* the fate of a biomaterial implant can be depicted as a race between microbial adhesion and biofilm formation on the implant surface *versus* tissue integration [7]. Even though the concept of the race for the surface has been embraced by many researchers in the field, unfortunately there has been no *in vitro* methodology forwarded to study the actual race. *In vivo*, the processes occurring at a biomaterial surface are quite complicated and involve multiple cell types, cytokines, chemokines, and secretion of bacterial and cellular substances [8]. These processes are difficult to adequately address with current *in vitro* models, but *in vitro* experiments are indispensable in order to develop new functional materials and coatings. Moreover, well designed and predictive *in vitro* experiments are often a requirement in order to obtain approval for animal experiments and at the same time reduce the need for animal experiments.

Therefore, the goal of the studies described in this thesis was to bridge the gap between the *in vitro* and *in vivo* evaluation of biomaterials-associated infections.

Bacterial Biofilm Growth *versus* Mammalian Cell Growth

As a first step, we developed a methodology to conduct the competition between bacteria and mammalian cells on the biomaterial surface. The development of methodologies were based on the two infection models: peri-operative and post-operative contamination of biomaterial implants. In the methodology based on peri-operative infection, *Staphylococcus epidermidis* ATCC 35983 was allowed to adhere

to a biomaterial surface prior to U2OS osteosarcoma cell adhesion and spreading to mimic the clinical situation where an implant becomes contaminated prior to implantation. We observed that the U2OS cells lost the competition in the absence of flow due to the accumulation of bacterial toxins, but were able to grow under flow due to continuous supply of fresh medium and remove the bacterial toxins from the interface. *In vivo* fluid flows continuously through the net-work of fine channels of osteocytes to facilitate the diffusion of nutrients and waste products [9]. In the concept of the race for the surface, tissue integration is an important protective factor against bacterial contamination of an implant surface [7]. Therefore, another methodology was developed based on post-operative contamination to investigate the effects of different degrees of mammalian cell coverage on the balance between bacterial biofilm formation and mammalian cell growth. We observed that 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 (8.2×10^4 cells cm^{-2} and 17×10^4 cells cm^{-2}), contaminating staphylococci did not affect cell growth.

Results suggest that the methodologies based on these two infection models could be well used in the evaluation of biomaterials infections, resembling the *in vivo* situation more closely than traditional single cell type cultures.

Evaluation of Biomaterials

Biomaterials can be broadly classified into two main categories biopassive and bioactive. Biopassive materials cannot induce any interfacial biological bond between implants and host cells. Metallic materials such as cobalt-chromium-molybdenum

alloys and titanium-aluminium-vanadium alloys are used in hip replacements [10] and polymeric materials such as poly (methyl methacrylate) (PMMA), (hydroxyethylmethacrylate), polyethylene oxide (PEO), phosphoryl choline are used in the field of ophthalmology (i.e contact lenses, intraocular lenses, keratoprosthesis optics and vitreous substitutes) [11] are some examples of biopassive materials. None of the synthetic materials are truly inert but materials like phosphoryl choline and polyethylene oxide are considerably promising in down-regulating biological responses in ophthalmology [11].

Bioactive materials can attach directly with body tissues and form chemical and biological bonds during the early stages of the post implantation period. Materials such as tricalcium phosphates, hydroxyapatites and calcium sulphate are some examples of bioactive materials [10]. Materials like tricalcium phosphates are osteoconductive because osteoblasts adhere and deposit bony tissue on the surface. Current efforts are being directed towards distilling the essence of cell adhesion requirements in new biomaterials incorporating minimal peptide sequences from adhesion molecules responsible for integrin binding. Biomaterials incorporated with RGD (arginine-glycine-aspartic acid) enhances tissue integration [11].

Based on the Gristina's 'race for the surface', the competition between *S. epidermidis* ATCC 35983 and U2OS cells on different biomaterials showed that mammalian cell interactions with biomaterials were hampered by bacterial biofilm formation on all biomaterials used. Yet, PMMA showed better U2OS cell adhesion and spreading in the presence of *S. epidermidis* compared to other biomaterials (Chapter 3). This study suggest that the race for the surface depends on the physico-chemical properties of the biomaterial surface and on the density of contaminating bacteria. In a comparative study, bi-functional coatings (bioactive PLL-*g*-PEG/PEG-

RGD) showed better cell adhesion and spreading and cells were less affected by the presence of *S. epidermidis* ATCC 35983 compared to mono-functional coatings (biopassive PLL-*g*-PEG) and bare titanium oxide surfaces (Chapter 5).

Bacterial Strain-Specific Effects on the Competition Between Bacterial Biofilm Growth *versus* Mammalian Cell Growth

Many different pathogens such as *S. epidermidis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* can be isolated from infected biomaterial implant surfaces, depending on their body site [7,8,12]. After an initial focus on *S. epidermidis*, our study on the competition between bacteria and mammalian cells was extended to different bacterial strains. We observed U2OS cell death within 18 h of simultaneous growth of *S. aureus* or *P. aeruginosa* and U2OS cells despite removal of bacterial toxins by flow, whereas U2OS cells were able to adhere, spread and grow in the presence of *S. epidermidis*. These observations are inline with the clinical findings that BAI due to highly virulent *S. aureus* and *P. aeruginosa* usually progresses much more aggressively than BAI caused by low virulent *S. epidermidis*. This study highlights the importance of bacterial virulence as a factor in the pathogenesis of BAI. However, characterization and quantification of substances released by bacteria was not performed. Hence, exact parameters responsible for mammalian cell death were unknown. However, studies showed that in *S. aureus*, most extracellular and surface attached virulence factors are regulated by the *agr* (accessory gene regulator) locus, a quorum-sensing system and the expression of *agr*-regulated targets is bacterial cell density-dependent. With increase in bacterial cell density *agr* becomes active, and starts producing extracellular degradative exoenzymes and toxins [13,15]. A similar cell-to-cell signalling (quorum-sensing) system plays a major role in the *P.*

aeruginosa virulence factors [14]. In contrast to *S. aureus* and *P. aeruginosa*, *S. epidermidis* produce a very limited number of tissue damaging exoenzymes and toxins. The only toxin made by *S. epidermidis* is N-formylated alpha-helical peptide δ-toxin, which plays a major role in the construction of a structured biofilm and in the detachment from surfaces [15].

Limitations of the *In Vitro* Experimental Model

In our experiments, we have focused on the development of *in vitro* methodology for the evaluation of new functional biomaterials and coatings to prevent biomaterial-associated infection. Our *in vitro* results are inline with clinical findings and provide additional information prior to animal experiments than *in vitro* studies addressing solely bacterial or tissue cell responses to biomaterials. However, the bacterial phenotypic changes occurring *in vivo*, resistance against antibiotics and host immune system are not taken into account in our *in vitro* model, which constitutes a major limitation. Secondly, the mechanism of interaction between host and biomaterial implant cannot be studied. Nevertheless, the evaluation of biomaterials based on these methodologies provides more information than can be obtained based on traditional *in vitro* studies and therewith form a good basis for further animal experiments.

Future Research

The model provided here to study the race for the surface methodologies shows that biomaterials contaminated even with very low number of bacteria can ultimately harvest a biofilm capable of influencing mammalian cell adhesion and growth depending on the virulence factor of the pathogens (Chapter 4). Results suggest the

need for development of biomaterials or coatings with selective toxicity to bacteria and only stimulating mammalian cell adhesion and growth.

It would be interesting to answer research questions such as: i) What would be the outcome of the race for the surface if both bacteria and mammalian cells are introduced to the biomaterial surface at the same time? ii) Could a biomaterial surface completely covered by a monolayer of mammalian cells prevent bacterial colonization? ii) What would be the outcome of the race for the surface if the biomaterial surface is pre-treated with physiologically relevant proteins?

Future experiments also includes characterization and quantification of the substances released by bacteria prior to the race for the surface study in order to gain a better insight on the influence of bacteria on mammalian cells adhesion and growth. This *in vitro* methodology could be progressed towards resembling the *in vivo* environment quite closely by incorporating antibiotics, growth factors and cytokines. Furthermore, animal experiments should be performed in order to validate the *in vitro* model, but pre-screening materials and coatings with the methodology developed here may save a large number of animals.

The choice of cell type in this thesis was mainly focussed on the orthopedic applications, but this methodology could be applicable for other cell types and implant systems, such as oral implants, surgical meshes and vascular grafts.

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Summary

Biomaterial-associated infections (BAI) are the major cause of implant failure and can develop many years after implantation. BAI can develop from peri-operative microbial contamination of implant surfaces during implantation, immediately post-surgery during hospitalization or by late hematogenous spreading from infections elsewhere in the body. The microorganisms involved in BAI are resistant to antibiotics due to their biofilm mode of growth and the infected implant has to be removed in most cases.

In 1987, the orthopedic surgeon Anthony G. Gristina coined the term "the race for the surface" to describe the fate of biomaterial implants in relation with the development of BAI. The fate of a biomaterial implant was depicted as a race between microbial adhesion and biofilm growth on an implant surface versus tissue integration. 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 rapidly covered by a biofilm and tissue cell functions will be hampered by bacterial virulence factors and toxins. The concept of race for the surface has been embraced by many researchers in this field, but hitherto there has been no *in vitro* methodology forwarded to study the race.

Chapter 1 reviews the main causes of implant failure and development of BAI. 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". In the past till now, researchers predicted the outcome of the race for the surface by *in vitro* evaluation of biomaterials or functional coatings either for their ability to resist bacterial adhesion and biofilm formation or for their ability to support mammalian cell adhesion and proliferation. However, the effects of the presence of bacteria including the influence of bacterial activity and toxins on mammalian cell adhesion and proliferation remains

unknown, which could completely change the fate of a biomaterial implant according to the concept of the "race for the biomaterial surface". Therefore, the main aim of this thesis is to develop a method that could bridge the gap between *in vitro* and *in vivo* studies on BAI.

Chapter 2, as a first step towards bridging the gap between *in vitro* and *in vivo* studies, an *in vitro* experimental methodology was developed based on Anthony G. Gristina's concept of the "race for the surface". This methodology was developed based on the peri-operative contamination model in which the implant becomes contaminated prior to implantation. After developing a suitable growth medium that allowed both bacterial and mammalian cell growth in the parallel plate flow chamber, the simultaneous growth of *Staphylococcus epidermidis* ATCC 35983 and U2OS osteosarcoma cells was studied for a period of 48 h. The outcome of the competition between *S. epidermidis* ATCC 35983 and U2OS cells appeared to be dependent on the number of bacteria present prior to U2OS cell seeding and the absence or presence of fluid flow. U2OS cells lost the competition in the absence of flow, probably due to the accumulation of bacterial toxins, but were able to grow under flow due to the continuous supply of fresh medium and removal of most endotoxins from the interface.

This methodology based on the peri-operative contamination model was used to determine the influence of biomaterial surfaces with different wettabilities and a polymer brush coating on the outcome of the competition between *S. epidermidis* ATCC 35983 and U2OS cells which is described in **chapter 3**. This study demonstrated that the presence of *S. epidermidis* reduced U2OS cell growth on all surfaces, but adhering U2OS cell spread equally well in the absence and presence of staphylococci. A hydrophilic polymer-brush coating discouraged bacteria and cellular

adhesion and growth. This study highlights the non existence of a surface on which the race for the surface can be won by mammalian cells and therefore emphasizes the need for biofunctionalized surfaces that discourage biofilm formation and support mammalian cell growth at the same time.

There are different pathogens involved in BAI, such as *S. epidermidis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. It is known that bacterial virulence is a contributing factor in pathogenesis of BAI. In **chapter 4**, the influence of different strains of *S. epidermidis*, *S. aureus* and *P. aeruginosa* on the outcome of the competition between bacteria and mammalian cells for the biomaterial surface was studied. U2OS cells are able to adhere, spread and grow in the presence of adhering *S. epidermidis* strains but not in the presence of adhering *S. aureus* or *P. aeruginosa*, which cause death of all adhering U2OS cells within 18 h, despite the continuous medium flow. These observations are in line with clinical findings that BAI due to *S. aureus* and *P. aeruginosa* usually progress much more aggressively than BAI caused by *S. epidermidis*. This study highlights the potential of this *in vitro* model to be used to investigate the effect of pathogen virulence on host tissue cells in BAI.

In **chapter 5**, bacterial and cellular responses to poly(ethylene glycol) (PEG) brush coatings on titanium oxide surfaces presenting the integrin-binding peptide RGD (arginine-glycine-aspartic acid) (bioactive "PEG-RGD") were compared to mono-functional PEG brush coatings (biopassive "PEG") and bare titanium oxide (TiO$_2$) under flow. In this chapter, bacteria were allowed to adhere for a fixed period of 2 h on all surfaces prior to mammalian cell adhesion and spreading and simultaneous growth of bacteria and mammalian cells was allowed for a period of 48 h. After 2 h, staphylococcal adhesion was reduced more than 20 fold on PEG and

PEG-RGD coatings, compared to the TiO_2 surface. When allowed to grow for 48 h, biofilms were formed on all surfaces. However, biofilms on PEG and PEG-RGD coatings detached when exposed to higher shear. U2OS cells neither adhered nor spread on the biopassive mono-functional coating PEG regardless of the presence of biofilm. In contrast, in the presence of biofilm, U2OS cells adhered and spread on the bioactive PEG-RGD coating with a significantly higher surface coverage than on bare TiO_2. This study highlights the potential of bifunctional coatings to reduce the risk of infection in the applications requiring tissue integration.

In the concept of the race for the surface, as forwarded by the late orthopedic surgeon Gristina, tissue integration is an important protective factor against bacterial contamination of an implant surface. Therefore, **chapter 6** was focused on the development of an *in vitro* model based on post-operative bacterial contamination of implant surfaces. The effect of differences in mammalian cell coverage on the balance between mammalian cell growth and bacterial biofilm formation was investigated. In this study, mammalian cells were allowed to adhere and spread for 24 h followed by *S. epidermidis* adhesion to mimic post-operative infection. Subsequently, the simultaneous growth of mammalian cells and *S. epidermidis* was observed for another 24 h under low shear conditions (0.14 s^{-1}). Mammalian cell growth was severely impaired when bacteria were introduced on surfaces with a low initial cell density (2.5×10^4 cells cm^{-2}), but in the presence of higher initial cell densities (8.2×10^4 cells cm^{-2} and 17×10^4 cells cm^{-2}), contaminating staphylococci did not affect cell growth. A critical mammalian cell coverage (8.2×10^4 cells cm^{-2}) was determined, above which the contaminating *S. epidermidis* ATCC 35983 no longer had a negative impact on mammalian cell growth. This study demonstrates that a critical coverage by host cells

is needed to effectively protect a biomaterial implant against contaminating *S. epidermidis*.

The success of an implant is a complex interplay between factors such as physico-chemical properties of the biomaterial surface, bacterial virulence and alteration in the immune system. As a second step in bridging the gap between *in vitro* and *in vivo* studies, the influence of immune cells (macrophages) on the competition between bacteria and mammalian cells was studied in **chapter 7**. This study based on the peri-operative contamination model validates the interactions of multiple cell types (bacteria-macrophages-osteoblast cells) which more closely resembles the *in vivo* environment than single cell type cultures. Results showed that despite the presence of macrophages, mammalian cells lost the race for the surface in the presence of the highly virulent *S. aureus*.

In the general discussion **chapter 8**, the advantages and limitations of our novel *in vitro* methodology are discussed. Even though the results from the *in vitro* model are inline with the clinical findings, the importance of validating this *in vitro* model with animal experiments are highlighted under future research. *In vitro* methodologies based on two infection models as suggested in this thesis will allow better evaluation of biomaterials and coatings prior to animal experiments or human trials and possibly reduce the number of experimental animals.

Samenvatting

Biomateriaal-geassocieerde infecties (BAI) zijn de belangrijkste oorzaak voor het falen van implantaten en kunnen vele jaren na implantatie pas evident worden. Biomateriaal-geassocieerde infecties kunnen zich ontwikkelen na contaminatie van een implantaat met een micro-organisme tijdens de implantatie (peri-operatief), direct na een operatie tijdens de ziekenhuisopname, of veel later doordat micro-organismen vanuit wondjes elders in het lichaam zich verspreiden via de bloedbaan. Micro-organismen in biomateriaal-geassocieerde infecties groeien in een biofilm waardoor ze nagenoeg ongevoelig zijn voor antibiotica. In de meeste gevallen moet zo'n implantaat worden verwijderd.

In 1987 introduceerde de orthopeed Anthony G. Gristina de term "Race for the surface" om de ontwikkeling van een biomateriaal-geassocieerde infecties te beschrijven op een geïmplanteerd biomateriaal. De uitkomst van de 'race' tussen microbiële adhesie en groei èn integratie van het biomateriaal in het weefsel is een belangrijke indicator voor een succesvolle implantatie. Als de 'race' wordt gewonnen door weefselintegratie, dan wordt het oppervlak bedekt met cellen en weefsel en is daardoor minder aantrekkelijk voor bacteriën. Als daarentegen de 'race' wordt gewonnen door micro-organismen, dan wordt het oppervlak snel bedekt met een biofilm, die toxines produceert, waardoor het functioneren van het weefsel sterk negatief beïnvloed wordt. Het concept van de 'Race for the surface" wordt algemeen aanvaard in dit onderzoeksveld, maar tot voor kort was er geen methode beschikbaar om deze 'race' in het laboratorium te bestuderen.

Hoofdstuk 1 geeft een overzicht van de belangrijkste oorzaken voor het ontwikkelen van biomateriaal-geassocieerde infecties en daarmee het falen van implantaten. Onafhankelijk van de manier waarop zo'n infectie is ontstaan hangt het lot van het implantaat voornamelijk af van de zogenaamde 'Race for the surface'. Tot

op heden werd de uitkomst van de 'race' voorspeld door onderzoekers door in het laboratorium te meten in hoeverre biomateriaal oppervlakken of coatings bestand waren tegen microbiële adhesie en groei van een biofilm. In een tweede experiment werd dan nagegaan of cellen uit weefsels konden hechten aan de oppervlakken en er op konden groeien. De invloed van de aanwezigheid van micro-organismen op de hechting van weefselcellen en hun groei bleef echter onbekend. Het belangrijkste doel van de experimenten beschreven in dit proefschrift is het ontwikkelen van een laboratorium techniek die een brug kan vormen tussen *in vitro* en *in vivo* studies van biomateriaal-geassocieerde infecties.

In hoofdstuk 2 wordt als een eerste stap een laboratorium model beschreven waarmee Anthony G. Gristina's 'Race for the surface' kan worden bestudeerd. Als basis voor de methode diende een peri-operatief model van besmetting, waarbij het implantaat voor implantatie wordt besmet met een relatief lage dosis bacteriën. Na ontwikkeling van een geschikt groeimedium voor zowel bacteriën als weefselcellen in een parallelle plaat doorstroom kamer ('flow chamber') , werd de gelijktijdige groei van *Stafylococcus epidermidis* ATCC 35983 en U2OS osteosarcoma cellen gedurende 48 uur bestudeerd. De uitkomst van de competitie tussen *S. epiderimidis* en U2OS cellen bleek afhankelijk van het aantal bacteriën dat aanwezig was voordat de U2OS cellen werden gezaaid en van de aanwezigheid van stroming. Zonder stroming van het medium verloren de U2OS cellen de competitie, vermoedelijk door een opeenhoping van bacteriële toxines. Bij stroming van het medium bleken de U2OS cellen in staat te hechten en te groeien. De continue verversing van medium zorgde waarschijnlijk voor verwijdering van de meeste endotoxines van het oppervlak.

Dezelfde methode, gebaseerd op het peri-operatieve model van besmetting, werd gebruikt om de effecten van oppervlakte eigenschappen van biomaterialen op de

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uitkomst van de competitie tussen *S. epidermidis* ATCC 35983 en U2OS cellen te bepalen. Materialen met oppervlakken verschillend in hydrofobiciteit of bekleed met polymeer borsteltjes werden getest (Hoofdstuk 3). Deze studie toonde aan dat in de aanwezigheid van *S. epidermidis* de groei van U2OS cellen werd beperkt op alle oppervlakken, maar dat de spreiding van de cellen niet werd beïnvloed. De bekleding van het oppervlak met hydrofiele polymeer borsteltjes voorkwam hechting en groei van bacteriën en U2OS cellen bijna volledig. Deze studie geeft eens te meer aan dat er geen oppervlak is waarop de 'Race for the surface' kan worden gewonnen door weefselcellen en benadrukt de noodzaak van het ontwikkelen van bio-functionele oppervlakken, die biofilmgroei ontmoedigen en weefselcelgroei bevorderen.

Bij biomateriaal-geassocieerde infecties kunnen verschillende pathogenen betrokken zijn, zoals *S. epidermidis*, *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, etc. Het is bekend dat de bacteriële virulentie een rol speelt in de mate waarin de infectie actief is. In hoofdstuk 4 wordt een studie beschreven waarin de invloed van verschillende bacteriestammen van *S. epidermidis*, *S. aureus* en *P. aeruginosa* werd gemeten op de groei en spreiding van U2OS cellen in het model, zoals beschreven in hoofdstuk 2. U2OS cellen bleken in staat te hechten, spreiden en groeien in de aanwezigheid van hechtende *S. epidermidis*, maar niet in de aanwezigheid van hechtende *S. aureus* of *P. aeruginosa*, die alle resulteerden in dood van de U2OS cellen binnen 18 uur ondanks stroming van het medium. Deze uitkomsten komen overeen met klinische waarnemingen dat biomateriaal-geassocieerde infecties met *S. aureus* of *P. aeruginosa* veel agressiever verlopen dan infecties met *S. epidermidis*. Deze studie geeft het belang van het ontwikkelde model aan voor het bestuderen van de effecten van pathogeniciteit van infecterende micro-organismen op weefselcellen.

In de studie beschreven in hoofdstuk 5 werden bekledingen van titanium oxide oppervlakken met poly(ethylene glycol) (PEG) borsteltjes al dan niet voorzien van het integrine-bindende peptide RGD (arginine-glycine-aspartine zuur) (PEG-RGD) vergeleken in het in hoofdstuk 2 beschreven model. In deze studie kregen *S. epidermidis* ATCC 35983 gedurende 2 uur de tijd te hechten aan een oppervlak, waarna de weefselcellen werden gezaaid. De gelijktijdige groei van bacteriën en weefselcellen werd gedurende 48 uur gevolgd. Na 2 uur bleek de bacteriële hechting op de PEG- en PEG-RGD beklede oppervlakken 20-voudig gereduceerd te zijn ten opzichte van kaal titanium oxide. Na 48 uur waren op alle drie de oppervlakken biofilms te zien. De biofilms op PEG- en PEG-RGD beklede oppervlakken bleken los te laten bij hogere stroming snelheden. Onafhankelijk van de aanwezigheid van een biofilm bleken U2OS cellen niet op PEG beklede oppervlakken te hechten, laat staan te groeien. Op de 'bioactieve' PEG-RGD bekleding daarentegen, bleken de U2OS cellen goed te hechten en te spreiden ook in aanwezigheid van een biofilm. De bedekking van het oppervlak met U2OS cellen was significant hoger vergeleken met kaal titanium oxide. Met deze studie wordt aangetoond dat een 'bioactieve' coating waardevol kan zijn om het risico van een infectie terug te dringen.

In het door Anthony G. Gristina beschreven concept van de 'Race for de surface' is de integratie van het materiaal in het weefsel een belangrijke beschermende factor tegen microbiële besmetting van het oppervlak. Daarom was de studie beschreven in hoofdstuk 6 gericht op de ontwikkeling van een post-operatief bacteriële contaminatie model van het oppervlak van een implantaat. De invloed van de mate van bedekking van het oppervlak met weefselcellen op de balans tussen weefselcelgroei en *S. epidermidis* biofilm vorming werd bestudeerd. In deze studie werden verschillende aantallen weefselcellen gezaaid en konden ze hechten en

spreiden gedurende 24 uur. Daarna werd een vast aantal *S. epidermidis* gezaaid en werd de gelijktijdige groei van bacteriën en weefselcellen gevolgd in de tijd onder vloeistofstroming (0.14 s^{-1}). Weefselcelgroei werd ernstig geremd als bacteriën werden gezaaid op oppervlakken met lage aantallen weefselcellen (2.5 x 10^4 cellen cm^{-2}), maar bij hogere weefselceldichtheden (8.2 x 10^4 en 17 x 10^4 cellen cm^{-2}) hadden de aanwezige *S. epidermidis* geen effect op de celgroei. Een kritische weefselceldichtheid werd bepaald: 8.2 x 10^4 cellen cm^{-2}, waarboven aanwezige *S. epidermidis* niet langer een negatief effect op de weefselcelgroei hadden. Deze studie toont aan dat bij een zekere mate van bedekking van een biomateriaal oppervlak met gastheercellen het implantaat effectief beschermd lijkt tegen besmetting door *S. epidermidis*.

Het succes van een implantaat is een complex samenspel van factoren, zoals de fysisch-chemische eigenschappen van het oppervlak, bacteriële virulentie en de effectiviteit van het immuunsysteem. Als een tweede stap in het overbruggen van de kloof tussen *in vitro* en *in vivo* studies, werd de invloed van immuuncellen (macrofagen) op de competitie tussen bacteriën en weefselcellen bepaald. Deze studie, beschreven in hoofdstuk 7, gebaseerd op het peri-operatieve contaminatie model (hoofdstuk 2), valideert de interacties tussen meerdere celtypes (bacteriën, macrofagen, en osteosarcomacellen) en benadert meer de *in vivo* situatie dan wanneer slechts één celtype wordt gebruikt. De resultaten tonen aan dat ondanks de aanwezigheid van macrofagen de weefselcellen de 'Race for the surface' verliezen in de aanwezigheid van 'hoog' virulente *S. aureus*, gelijk de *in vivo* situatie

In de algemene discussie, hoofdstuk 8, worden de voordelen en beperkingen van onze nieuwe *in vitro* methodiek besproken. Hoewel de resultaten van de studies met de *in vitro* modellen overeenkomen met klinische bevindingen wordt het belang

van validatie van de methode door middel van proefdierexperimenten benadrukt. De beide *in vitro* modellen (peri-operatieve en post-operatieve contaminatie) beschreven in dit proefschrift zullen leiden tot een betere evaluatie van biomaterialen en coatings, voordat overgegaan wordt naar dierstudies of klinische experimenten en daarmee mogelijk het aantal voor deze doeleinden gebruikte proefdieren reduceren.

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