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Microbial biofilm growth vs. tissue integration: "The race for the surface" experimentally studied

Guruprakash Subbiahdoss [a], Roel Kuijer [a], Dirk W. Grijpma [a,b], Henny C. van der Mei [a,*], Henk J. Busscher [a]

[a] Department of Biomedical Engineering, University Medical Center Groningen, Groningen and University of Groningen, P.O. Box 196, 9700 AD Groningen, The Netherlands

[b] Department of Polymer Chemistry and Biomaterials, Faculty of Science and Technology, Institute for Biomedical Technology (BMTI), University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

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Abstract

Biomaterial-associated infections constitute a major clinical problem. Unfortunately, microorganisms are frequently introduced onto an implant surface during surgery and start the race for the surface before tissue integration can occur. So far, no method has been forwarded to study biofilm formation and tissue integration simultaneously. The aim of this study is to describe an in vitro method to investigate this "race for the surface". First, a suitable growth medium was prepared that allowed both bacterial and tissue growth in a parallel plate flow chamber. Staphylococci were deposited on the glass bottom plate of the flow chamber in different surface densities, after which U2OS osteosarcoma cells were seeded. U2OS cells did not grow in the absence of flow, possibly due to poisoning by bacterial endotoxins, but under flow both staphylococci and U2OS cells grew. The number of adhering cells and area per spread cell were determined after 48 h in relation to the initial number of bacteria present. Both the number and spread area per cell decreased with increasing density of adhering staphylococci. This demonstrates that the outcome of the race for the surface between bacteria and tissue cells is dependent on the number of bacteria present prior to cell seeding.

Keywords: Race for the surface; Biofilm; Tissue integration; Biomaterial

1. Introduction

Biomaterials play a major role in modern medicine for the restoration of function, frequently used examples being prosthetic joints or heart valves. Biomaterial-associated infections (BAIs) 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]. BAIs are 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 (perioperative 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

* Corresponding author. Tel.: +31 50 3633140; fax: +31 50 3633159.
E-mail address: h.c.van.der.mei@med.umcg.nl (H.C. van der Mei).

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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 vs. 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. 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, so 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 evaluated either 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.

2. Materials and methods

2.1. 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, 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.

2.2. 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 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, UK) 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*g* for 5 min at 10 °C and washed twice with sterile ultrapure water. Subsequently, the harvested bacteria were sonicated on ice (3 × 10 s) in sterile phosphate-buffered saline (PBS, comprising 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.

2.3. Development of a 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 the growth rates of both *S. epidermidis* and U2OS cells were determined.

To determine U2OS cell growth, 1 ml of 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.

2.4. 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.

2.5. 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 30× 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 10× magnification after 48 h and analyzed using Scion image software.

2.6. 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 30× objective for bacteria and 10× 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 but 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 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 four 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, six images (900 × 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.

2.7. Statistics

Experiments for each density of adhering bacteria under both static and flow conditions were carried out in triplicate. Data are represented as a mean with standard deviation. Statistical analysis of variance was performed followed by Tukey's HSD post hoc test and a p-value of <0.05 was considered significant.

3. Results

3.1. 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% or 4% TSB compared to cells grown in 100% DMEM + FBS. For higher percentages of TSB, a 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 was 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).

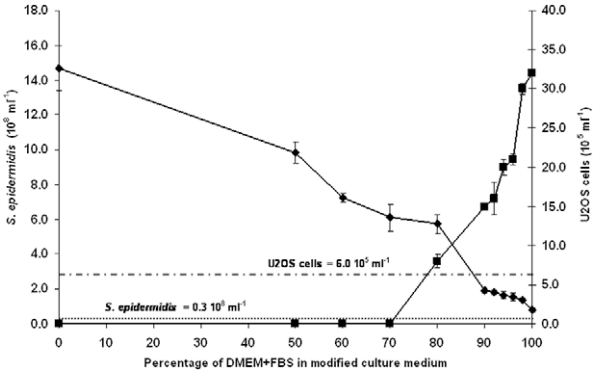

Fig. 1. Growth of U2OS cells (■) and *S. epidermidis* ATCC 35983 (♦) as a function of the percentage of DMEM + FBS in combination with TSB medium. Horizontal lines indicate the number of U2OS cells (dash-dotted) and bacteria (dotted) prior to growth. Growth was registered after 48 h.

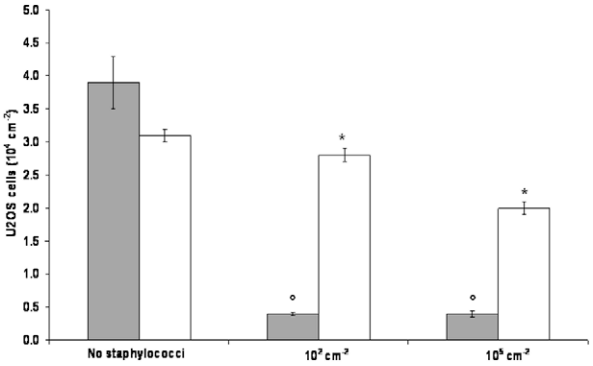

Fig. 2. Number of U2OS cells seeded on a glass substratum after 48 h under static conditions (■) and under medium flow (□) and in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates, with separate bacterial and cell cultures. *,○Significantly different ($p < 0.05$) from the control (absence of staphylococci).

3.2. Adhesion of U2OS cells in the presence of S. epidermidis: static vs. flow conditions

The response of U2OS cells to the presence of adhering staphylococci under static conditions vs. 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 compared to the control ($p < 0.05$). 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 compared to under 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, confirming that bacterial endotoxins are responsible for cell death during the race for the surface under static conditions. Fluorescent dead–live stain, comprising 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.

3.3. 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 spread. For this reason, the adhering U2OS cells were stained and analyzed by CLSM (see Fig. 5).

U2OS cells showed the greatest 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 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.

4. 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, as many of the coatings that are currently being 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. This is stimulating the development of bifunctional coatings that support cell spreading and repel microorganisms at the

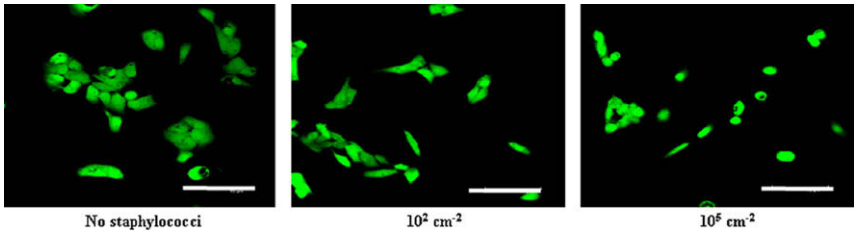

Fig. 3. CSLM live–dead images of U2OS cells after 48 h on glass under medium flow in the absence and presence of adhering staphylococci. Bar denotes 75 μm.

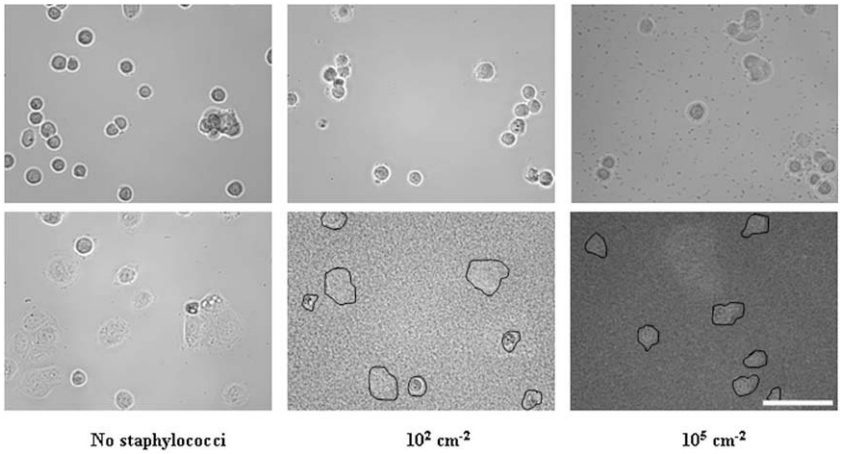

Fig. 4. U2OS cells adhesion and spreading after 1.5 h (top series) and 48 h (bottom series) under medium flow on a glass substratum in the absence and presence of adhering staphylococci. Bar denotes 75 μm. U2OS cells are differentiated by a contour line from *S. epidermidis* biofilm.

same time, which necessitates the use of a methodology such as the one described in this paper. 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 continued to adhere in the presence of adhering staphylococci and spread more when the density of adhering staphylococci was lower. We have thus 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 perioperative 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 of 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 flows continuously 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 the exact flow rates as occurring in vivo are unknown.

Several researchers have predicted 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 it is the right way to study the possible fate of a biomaterial implant in vitro. Many biomaterial coatings have been identified as being non-adhesive to bacteria or supporting tissue integration separately [6,13–15,17,18], but the combination of both bacteria and tissue cells on the same biomaterial surface has not been studied before. Shi et al. [16], for instance, promoted a surface com-

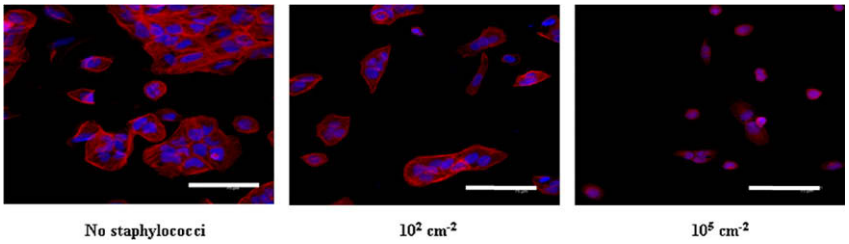

Fig. 5. CSLM images of U2OS cells after 48 h on glass under medium flow in the absence and presence of adhering staphylococci. U2OS cells were stained with 5 ml PBS containing 49 μl DAPI and 2 μg ml^{-1} of TRITC–phalloidin. Bar denotes 75 μm.

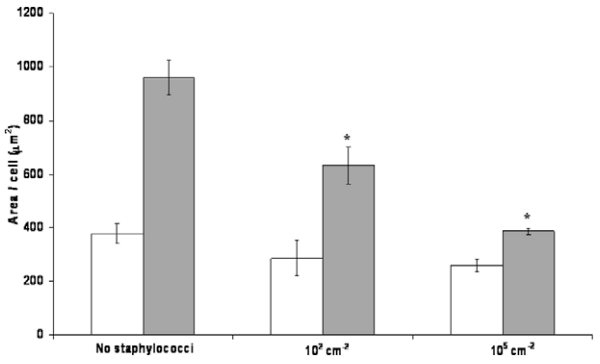

Fig. 6. Average area per spread U2OS cell after 1.5 h (□) and 48 h (■) of growth on glass under medium flow in the absence and presence of adhering *S. epidermidis* ATCC 35983. Error bars represent the standard deviation over three replicates with separate bacterial and cell cultures. *Significantly different ($p < 0.05$) from the control (absence of staphylococci).

posed of chitosans and arginine–glycine–aspartic acid (RGD) peptide sequences. In separate experiments, it was shown that these combined surfaces discouraged bacterial adhesion but enhanced cell attachment and alkaline phosphatase activity. However, the effects of the presence of bacteria, 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 the "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.

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

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