

Effects of *Staphylococcus epidermidis* on osteoblast cell adhesion and viability on a Ti alloy surface in a microfluidic co-culture environment

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ABSTRACT

A microfluidic device was used for real time imaging of MC3T3-E1 murine calvarial pre-osteoblasts (osteoblasts) in response to very small numbers of *Staphylococcus epidermidis* inoculated on the surface of a polished TiAl6V4 alloy in a serum-based medium. The Ti alloy surface was integrated to a poly(dimethylsiloxane) fluidic housing with eight 10 μ l channels for high-throughput, cross-contamination-free co-culture. In the absence of *S. epidermidis* osteoblasts were able to adhere, spread, proliferate and remain viable on the Ti alloy surface during a 25 h culture period. With 10^2 or 10^5 colony forming units (cfu) ml^{-1} *S. epidermidis* inoculated on the alloy surface osteoblast adhesion, spreading and proliferation were not adversely affected during the early stages of culture. However, osteoblasts became damaged by the end of culture, as *S. epidermidis* actively proliferated in the co-culture channels and formed small clusters on the alloy surface. These observations suggest that the small numbers of *S. epidermidis* did not necessarily compete with osteoblasts for the alloy surface during initial host cell development, but rapid proliferation of the bacteria might have changed the microenvironment, making it unfavorable to sustain the viability of osteoblasts. The results provide a new insight in projecting the potential utility of the microfluidic co-culture approach to developing physiologically and clinically relevant in vitro models of orthopedic implant-associated bacterial infection.

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1. Introduction

Biofilm-associated infection is one of the leading causes of biomedical implant failure. For orthopedic devices infection rates vary from a few percent for hips and knees to as much as 15% for open wound trauma rods, with *Staphylococcus aureus* and *Staphylococcus epidermidis* accounting for about 34% and 32% of infections, respectively [1]. Biofilm formation on an abiotic implant surface occurs as a result of a small number of bacteria adhering to the surface. For example, only 10^2 colony forming units (cfu) of *S. aureus* are required to infect 95% of subcutaneous implants in guinea pigs, while 10^8 cfu is typically needed without an implant [2]. Due to the ineffectiveness of conventional antibiotic treatments, the standard care for implant infection involves the surgical removal of the infected device and the surrounding tissue, followed by further surgery to implant a new device. The two stage revision procedure is costly and is accompanied by significant patient trauma, immobility and possible death.

Despite the severity of the device-associated infection problem, it appears that there is no clear solution on the horizon. For exam-

ple, progress has been slow in developing coatings that contain antimicrobial agents [3,4]. The current situation has been summarized by Götz [5]: “Many in vitro results with new materials look very promising at the beginning, but the in vivo situation is frequently disillusioning”. Clearly, there is a need to re-think the conventional paradigm by which infection-resistant biomaterials are developed and evaluated.

Microfluidics technology has been developed for a wide range of exciting applications, from nanomaterial self-assembly to high throughput proteomic studies to point of care medical diagnostics [6,7]. Growing efforts have also been made to create physiologically relevant in vitro culture conditions using microfluidic tools for studying cell–material interactions and tissue regeneration [8]. This development can systematically and quantitatively augment the capability to control the level of in vitro complexity in simulating in vivo-like situations. Our long-term interest is to explore the potential of microfluidic devices as new tools in developing more physiologically and clinically relevant in vitro models for studying implant-associated bacterial infection.

We have previously studied the effects of hydrodynamics on *S. epidermidis* biofilm formation in poly(dimethylsiloxane) (PDMS)-based microchannels [9]. Monolayered colonies were produced at high flow velocity and high fluid shear locations, in contrast to

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the formation of multilayered biofilm structures at low velocity and low shear locations. We also observed that short-term treatments with antibiotics were ineffective in eradicating biofilms from PDMS microchannels unless a novel anti-biofilm agent (Dispersin B) was introduced.

This investigation was prompted by our curiosity about the response of osteoblast cells to bacteria present on the surface of biomaterials. The objectives of this paper were to: (1) design and fabricate a microfluidic device which allows real time imaging of osteoblasts on the surface of a clinically relevant Ti alloy (TiAl6V4) substrate; (2) evaluate the adhesion and viability of osteoblasts in response to a small number of *S. epidermidis* inoculated on the alloy surface. The results from this investigation were used to project the issues, challenges and potential utility associated with developing a microfluidic-based in vitro model of implant-associated infection.

2. Materials and methods

2.1. Microfluidic device design

Soft lithography based on PDMS was used to fabricate a microfluidic device integrated with a Ti alloy substrate. The device was designed to contain eight rectangular microchannels of $800\ \mu\text{m}$ width \times $200\ \mu\text{m}$ depth \times $6\ \text{cm}$ length (Fig. 1a) of high throughput capability. The total internal volume of each channel was $10\ \mu\text{l}$. The overall design features and fabrication procedures for the device were largely dictated by the requirement of real time, non-invasive visualization of osteoblast adhesion and growth on a non-transparent Ti alloy surface. An upright confocal laser scanning microscope (CLSM) (Nikon E1000 with Nikon C1-Plus confocal system, Melville, NY) was used to visualize cells on the Ti alloy surface through an $\sim 300\ \mu\text{m}$ PDMS top and the channel space that was filled with culture medium (Fig. 1a and b). In order to directly image osteoblasts on the non-transparent Ti alloy surface integrated into the device, the PDMS top (Fig. 1a) was made as thin as possible to minimize laser beam penetration length. As shown in Fig. 1b, an upright microscope was used to image cells on the Ti alloy surface through the thin PDMS top and the channel over a total distance of $\sim 500\ \mu\text{m}$. However, we were not able to directly image bacteria on the alloy surface with this visualization ap-

proach, due to the working distance limitations of high magnification lenses.

2.2. Device fabrication and Ti alloy integration

Fabrication of the microfluidic device is schematically illustrated in Fig. 1c. A positive relief of the microchannels was patterned on a silicon wafer by photolithography with a photoresist material (SU8 2025, Microchem, Newton, MA). A 20:1 mixture of a PDMS base polymer and its curing agent (Sylgard 184, Dow-Corning, Somerville, NJ) was poured on the patterned silicon wafer, briefly spun at 300 rpm. and cured at $70\ ^\circ\text{C}$ for 1 h to create the $300\ \mu\text{m}$ thick PDMS top. 5 mm thick inlet and outlet PDMS pieces were bonded to the thin PDMS top by plasma treatment. The entire PDMS assembly was then peeled off from the mold and holes were created for fluidic connections. Ti alloy (TiAl6V4) substrates were provided by Stryker Orthopaedics (Mahwah, NJ). The substrate surface was polished to $\sim 0.05\ \mu\text{m}$ in roughness, sterilized by γ -irradiation and encased in air-tight packaging by the supplier. An adhesive-mediated bonding method was developed for Ti alloy substrate integration. A PDMS precursor with a 5:1 base polymer to curing agent ratio was used as the adhesive. The precursor was spun on a glass slide at 6000 rpm. for 60 s to form an $\sim 50\ \mu\text{m}$ thick adhesive layer. The patterned PDMS assembly was (1) placed on the adhesive-coated glass, (2) released from the glass, (3) brought to the Ti alloy surface, and (4) bonded by curing at $100\ ^\circ\text{C}$ overnight. The Ti alloy substrates were used immediately after opening the air-tight packages without any further cleaning or sterilization.

2.3. *S. epidermidis* inoculum preparation

S. epidermidis strain NJ9709 [10], isolated from the surface of an infected intravenous catheter, was used. Inoculum preparation followed procedures described previously [9]. The final inoculum was prepared in phosphate-buffered saline (PBS) and filtered through a $5\ \mu\text{m}$ syringe filter to produce a single cell suspension. The resulting concentration was confirmed to be approximately $1 \times 10^7\ \text{cfu ml}^{-1}$ by the Petroff–Hausser counting chamber (Electron Microscopy Sciences, Hatfield, PA). This original inoculum was further diluted to 1×10^2 and $1 \times 10^5\ \text{cfu ml}^{-1}$ in PBS.

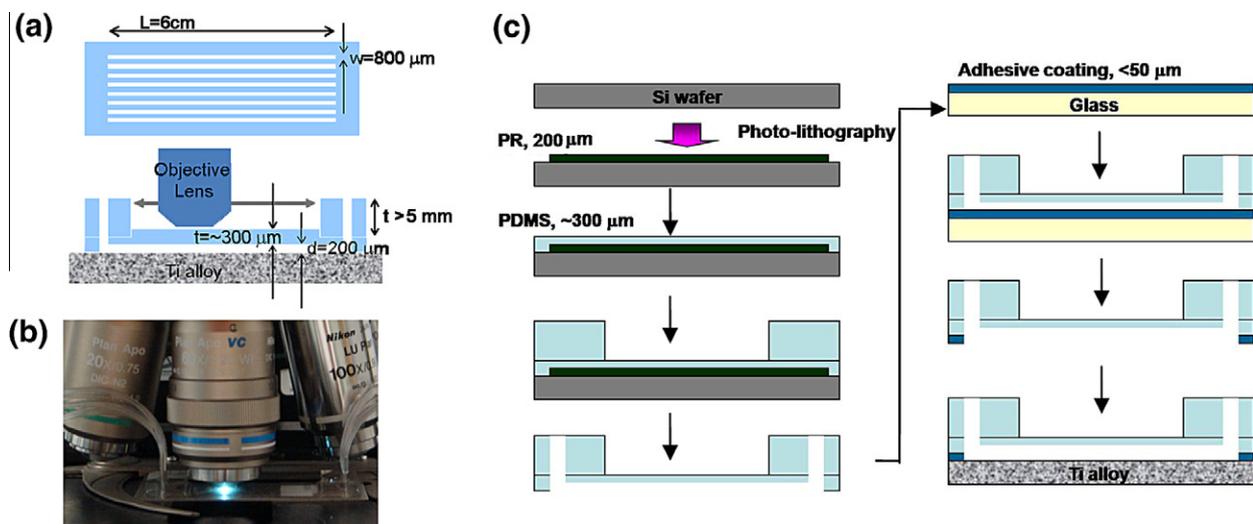


Fig. 1. Design and fabrication of the microfluidic device. (a) Schematics of top and side views. (b) Photograph of device operation. (c) Fabrication procedures including: (i) Si wafer, (ii) photoresist (PR) coating and patterning via photolithography (iii) PDMS precursor spin coated and cured, (iv) 5 mm thick inlet and outlet supporting pieces, which are separately prepared, attached via plasma treatment, (v) entire PDMS unit peeled from the mold and punched for fluidic connection, (vi) $50\ \mu\text{m}$ adhesive layer spin coated on a glass slide, (vii and viii) adhesive layer transferred to entire PDMS unit by stamping and (iv) PDMS unit bonded to the substrate by curing the adhesive at $100\ ^\circ\text{C}$ overnight.

ID	Title	Pages
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