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Conditions of lateral surface confinement that promote tissue-cell integration and inhibit biofilm growth

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ABSTRACT

Surfaces with cell adhesiveness modulated at micro length scales can exploit differences between tissue/bacterial cell size, membrane/wall plasticity, and adhesion mechanisms to differentially control tissue-cell/material and bacteria/material interactions. This study explores the short-term interactions of *Staphylococcus aureus* and osteoblast-like cells with surfaces consisting of cell-adhesive circular patches (1–5 μm diameter) separated by non-adhesive electron-beam patterned poly(ethylene glycol) hydrogel thin films at inter-patch distances of 0.5–10 μm. Osteoblast-like U2OS cells both bind to and spread on the modulated surfaces, in some cases when the cell-adhesive area comprises only 9% of the total surface and in several cases at least as well as on the continuously adhesive control surfaces. In contrast, *S. aureus* adhesion rates are 7–20 times less on the modulated surfaces than on the control surfaces. Furthermore, the proliferation of those bacteria that do adhere is inhibited by the lateral confinement imposed by the non-adhesive boundaries surrounding each patch. These findings suggest a new approach to create biomaterial surfaces that may promote healing while simultaneously reducing the probability of infection.

1. Introduction

The underlying objective of any tissue-contacting biomedical device is to promote healing after injury or disease. Such devices are designed to interact with tissue cells in a variety of ways, including supporting tissue-cell adhesion and tissue integration. The scientific and clinical communities have, however, long recognized that implantable devices provide foreign surfaces to which bacteria can adhere and colonize. Hence, in addition to their primary healing function, tissue-contacting biomedical devices also bring a heightened probability of introducing a chronic infection with potentially severe consequences to the patient. An important challenge facing next-generation biomaterials is thus to preserve or enhance the ability of a device to facilitate desirable tissue interactions while simultaneously inhibiting bacterial colonization [1,2].

Many approaches have been explored to inhibit the bacterial colonization of synthetic implant surfaces. Foremost among these are antifouling surface coatings that resist protein and cell adhesion, such as poly(ethylene glycol) [PEG]. PEGylated surfaces, for example, have been extensively studied [3–5], and a number of analogous gel-like coatings are increasingly being explored. Surfaces with micro/nano-patterned topography have also been shown to hinder bacterial adhesion and alter biofilm development [6–9], and topographic modulations at even smaller length scales can force individual bacteria to adopt positions and orientations defined by the surface patterning [10]. While successful at reducing bacterial colonization, tissue-cell interactions with these same surfaces are either simultaneously inhibited or have not been studied.

Surfaces with adhesiveness laterally modulated over microscopic length scales present an alternate between the extremes of fully adhesive or fully non-adhesive surfaces with which to differentially control cell/material and bacteria/material interactions. There is good evidence that tissue cells are able to adhere to and spread on such modulated surfaces. Chen et al. [11], for example, showed that individual endothelial cells are able to bind to a

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surface modulated at sub-cellular length scales with adhesive patches patterned on otherwise non-adhesive surface. The cells straddle the non-adhesive surface areas and bind to one or more adhesive patches depending on the patch size and spacing. Spatz et al. [12–14] have done similar experiments that explore fibroblast adhesion and spreading on surfaces with square adhesive patches having edge sizes of 0.1–3 μm and patch spacings equal to the patch size. Malmstrom et al. [15] used colloidal lithography to pattern surfaces with randomly distributed adhesive patches ranging in diameter from 0.1 to 1.0 μm and showed that mammalian breast cancer cells can adhere to them all by bridging across the non-adhesive surface. The adhesion is less effective as the patch diameter decreases, an effect they attribute to a disruption of focal adhesion formation.

Less is known about how bacteria respond to surfaces with laterally modulated adhesiveness. Wang et al. [16,17] have recently studied surfaces whose adhesiveness is modulated by submicron non-adhesive microgels and showed that bacterial adhesion is reduced as the surface concentration of microgels increases. At the opposite extreme, cell-adhesive patches patterned on an otherwise non-adhesive surface introduce a form of spatial confinement that can both localize where bacteria adhere as well as limit the physical size of growing colonies [18]. Furthermore, the fact that adjacent but separately growing bacterial colonies are able to communicate via the diffusion of quorum-sensing agents [19,20] indicates that the proximity of growing colonies on a surface may influence both their individual and collective development into biofilms. The idea that spatial confinement influences biofilm development has been further reinforced by recent experiments, which demonstrate quorum-sensing behavior in individual bacteria when they are confined within small droplets of growth medium on a surface [21–23].

The question of how micro-patterned cell adhesiveness affects the surface interactions both with tissue cells and with bacteria raises the hypothesis that there is a window of patch sizes and patch spacings that promotes tissue-cell interactions and also inhibits bacterial colonization. This study thus reports on experiments that test how tissue cells and staphylococcal bacteria interact with surfaces that have circular adhesive patches with diameters of 1, 2, 3, or 5 μm patterned within otherwise non-adhesive surface.

2. Materials and methods

2.1. Surface patterning

Microgel patterned glass slides were prepared using established procedures [24,25]. Glass microscope slides were sonicated in ethanol (96%), cleaned in a Piranha solution (3:1 98% sulfuric acid and 30% H_2O_2) for 30 min, rinsed with deionized water, dried, and exposed to low-pressure O_2 plasma (~300 mTorr, 1.75 W) for 10 min. The slides were then silanized with 2% [v/v] vinyl-methoxy siloxane homopolymer (Gelest Inc., Morrisville, PA) in ethanol for 10 min, rinsed, dried, and baked at 110 °C for 2 h. After cooling, thin films of PEG (~100 nm thick) were spin cast onto these substrata using a solution of 2 wt% PEG (6 kDa; Fluka) in tetrahydrofuran. Samples were stored under vacuum (~10^{-3} Torr) until they were used for patterning. E-beam patterning used a Zeiss Auriga Scanning Electron Microscope (SEM) with a Schottky field-emission electron source with a point dose of 10 fC and an incident electron energy of 2 keV. Such irradiation conditions convert the PEG homopolymer precursor into microgels grafted to the underlying substrate [25]. The e-beam position and dwell time were controlled using a Nabity Nanometer Pattern Generation System. Individual PEG microgels with diameters of about 400 nm were created by single point irradiation, and the spacing between adjacent irradiation points was set to 50 nm so individual microgels would overlap to form continuous thin-film gels. After patterning, the slides were rinsed in deionized water for 30 min to remove unirradiated PEG and expose the silanized glass in the unirradiated areas.

Arrays of adhesive patches were patterned on an otherwise non-adhesive surface by creating areas of PEG microgel films containing circular patches of exposed silanized glass. Patch diameters, α, of 1, 2, 3 or 5 μm with inter-patch (patch-edge to patch-edge) distances, β, of $\alpha/2$, α and 2α were studied. These two parameters were combined to create surfaces with adhesive area fractions, χ, of 0.09, 0.20, and 0.35 where the adhesive area fraction is defined as:

$$\chi = \frac{\pi\left(\frac{\alpha}{2}\right)^2}{(\alpha+\beta)^2} \quad (1)$$

Twelve 200 μm × 200 μm patch arrays were patterned on a single glass slide, with the arrays separated from each other by 100 μm-wide strips of silanized glass. After patterning, substrata were stored under vacuum (~10^{-3} Torr). Light microscopic images of the arrays were taken using a Nikon Eclipse E1000 upright optical microscope. The final cross-linked PEG film thickness was determined by atomic force microscopy (AFM; Bruker Bioscope Catalyst).

Fibronectin (Fn) was adsorbed onto exposed silanized glass surfaces by immersing the patterned slides in a 25 μg/mL solution of human Fn (Sigma–Aldrich BV, Zwijndrecht, The Netherlands) in phosphate buffered saline (PBS, 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) for 30 min at room temperature and then washed three times with PBS. To confirm the preferential Fn adsorption on the exposed silanized glass, a slide was immersed in PBS containing 1% BSA for 1 min to block non-specific protein adsorption, rinsed three times in PBS, exposed to a primary antibody (rabbit-anti-human fibronectin Ab, polyclonal, dilution 1:400 in PBS) for 30 min, rinsed, and then exposed to a fluorescent secondary antibody (FITC-conjugated donkey-anti-rabbit IgG, dilution 1:100 in PBS). After a final rinse, the patterned glass slide was placed in a petri dish filled with PBS and examined, while fully hydrated, by confocal laser scanning microscopy (CLSM, Leica DMRXE with confocal TCS SP2 unit). Fn-treated silanized glass is henceforth referred to here simply as glass unless otherwise indicated.

2.2. Tissue-cell culture

U2OS osteosarcoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS) and 0.2 mM ascorbic acid-2-phosphate (AA2P), denoted as DMEM + FBS. U2OS cells were maintained in tissue culture polystyrene flasks (TCPS, Greiner Bio-One) at 37 °C in humidified air with 5% CO_2 and harvested at 90% confluence using trypsine/ethylenediamine–tetra-acetic acid. The harvested cells were diluted to 6 × 10^5 cells/mL in DMEM + FBS. U2OS is an immortalized human cell line derived from osteosarcoma cells and was chosen from a broad selection of human osteoblastic cell lines used previously [26]. It has been demonstrated that osteosarcoma cell lines exhibit meaningful osteoblastic phenotypes [27].

U2OS cell adhesion and spreading on patterned surfaces were studied by *in situ* digital phase-contrast microscopy (Olympus BH-2; 10× objective) in a parallel-plate flow chamber (175× 17 × 0.75 mm^3) and by *ex situ* immunofluorescence imaging. The flow chamber was maintained at 37 °C throughout the experiments. Once fully filled and free of air-bubbles, a U2OS cell suspension was introduced in the chamber. Flow was stopped for 1.5 h to allow the cells to settle and adhere. Phase-contrast images were taken at this time point from each patterned array and also from the unpatterned glass to determine the initial surface coverage of adhered cells. DMEM + FBS supplemented with 2% HEPES buffer was then perfused through the chamber at 0.14 s^{-1} shear rate. After 48 h of additional culture beyond the 1.5 h settling period the substrata were removed and fixed in 3.7% formaldehyde in cytoskeleton stabilization buffer (CS; 0.1 M Pipes, 1 mM EGTA, 4% (w/v) PEG 8000, pH 6.9). After fixation, samples were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS, and stained for 30 min with 4 μg/mL of DAPI and 2 μg/mL of TRITC–Phalloidin. Slides were examined by fluorescence microscopy (Leica DM4000B). Each of these experiments was performed in triplicate. The total surface coverage of adhering cells on the patterned surfaces was determined by Scion image software. The surface coverage during the spreading period was defined as:

$$\Phi = \frac{\phi_{48} - \phi_{1.5}}{\phi_{1.5}} \quad (2)$$

where ϕ_i is the area fraction of surface covered by U2OS cells after 1.5 or 48 h. A positive value of Φ indicates that the cells spread. A negative value of Φ indicates that the cells either contracted or, more likely, migrated off the patterned surface.

2.3. Bacterial culture

Staphylococcus aureus NCTC 8325-4 were provided by T.J. Foster (Moyne Institute of Preventive Medicine, Dublin, Ireland). This strain possesses fibronectin-binding proteins (FnBPs). Bacteria were inoculated on trypsine soy broth (TSB, Oxoid) agar plates and incubated overnight at 37 °C. One colony was grown in 10 mL TSB overnight with constant rotation (120 rpm) and subsequently used to inoculate 190 mL TSB. After 2 h of incubation, when *S. aureus* NCTC 8325-4 was expected to be in the exponential phase of growth with the peak of FnBPs expression, bacteria were harvested by centrifugation (6500 g, 5 min, 10 °C) and washed twice in sterile PBS. Bacterial aggregates were broken by mild, intermittent sonication on ice (3 times, 10 s, 30 W, Wibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) and re-suspended to a concentration of 3 × 10^8 bacteria/mL in PBS. Bacterial adhesion to patterned glass slides was studied in a parallel-plate flow chamber and monitored *in situ* by digital phase-contrast microscopy. After removing air bubbles in the tubing by flowing PBS, the *S. aureus* suspension was perfused through the chamber (shear rate of 11 s^{-1}) for 30 min at room temperature. After bacterial deposition, sterile PBS was flowed through the system (shear rate of 11 s^{-1}) for 30 min to remove non-