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Bacterial response to spatially organized microtopographic surface patterns with nanometer scale roughness

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ABSTRACT

In this study, the influence of nanometer scale roughness on bacterial adhesion and subsequent biofilm formation has been evaluated using spatially organized microtopographic surface patterns for four major opportunistic pathogens of the genus *Staphylococcus* (*S. epidermidis* and *S. aureus*) responsible for associated-biofilm infections on biomedical devices. The results presented demonstrated that regardless of the strain employed the initial adhesion events to these surfaces are directed by cell-surface contact points maximisation and thus, bacterial cells actively choose their position to settle based on that principle. Accordingly, bacterial cells were found to preferably adhere to the square corners and convex walls of recessed surface features rather than the flat or concave walls of equal protruding features. This finding reveals, for the first time, that the particular shape of the surfaces features employed potentially determined the initial location of the adhering cells on textured surfaces. It was further shown that all surfaces patterns investigated produce a significant reduction in bacterial adhesion (40–95%) and biofilm formation (22–58%). This important observation could not be related to physical constrains or increased solid surface hydrophobicity, as previously suggested by other authors using engineered topographies with microscale surface roughness. It is evident that other causes, such as nanoscale surface roughness-induced interaction energies, might be controlling the process of bacterial adhesion and biofilm formation on surfaces with well-defined nanoscale topography.

1. Introduction

Bacterial cells tend to associate with material surfaces forming self-organized multicellular structures called biofilms [1–3]. The formation of these living structures poses a real and serious problem in modern society from both an economic and health point of view. In a wide variety of industrial settings, for example, microbial colonization of pipelines, filters, heat exchangers, separation membranes or food processing equipment largely decreases production rates, increases operating costs, and causes major contamination problems [4,5]. In the medical field, the adherence and proliferation of bacterial cells onto surgically implanted and non-implanted devices, such as joint replacements, cardiac valves, voice prostheses, catheters, contact lenses and endotracheal tubes, is at the onset of severe and persistent hospital-acquired infections that come at high cost and burden for the patients, their families, and the public healthcare systems [6–8].

It is well-established that biofilm formation involves an initial critical step in which planktonic cells adhere to the material surfaces, after which bacterial cells proliferate and grow into complex biofilms [9]. Given that mature biofilms are able to function as a physical and physiological barrier against chemical treatments, antibiotic therapy, and the human host defence mechanisms, prevention of the initial adhesion step is highly desirable over biofilm treatment [10–12]. The initial adhesion phase is primary controlled by the physico-chemical properties of the interacting surfaces [12–17]. Thus, the influence of surface charge, hydrophobicity and roughness on bacterial adhesion has been extensively investigated in the last decades. Several studies have been able to successfully explain the initial affinity of a number of bacterial strains and species to different materials based on these surfaces properties [18–20], but discrepancies between experimental observations and theoretical expectations are also frequently observed. Negatively charged bacteria, for example, have been found to adhere onto negatively charges surfaces [21,22], and reports on the influence of surface hydrophobicity and roughness on bacterial adhesion often

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reveals inconclusive and/or contradictory results [23–27].

An alternative strategy to prevent bacterial adhesion and colonization has relied on the generation of antimicrobial surface coatings. To this end, a number of natural and synthetic antimicrobial molecules, such as antibiotics, cationic peptides, silver ions and quaternary ammonium compounds, have been effectively incorporated into the surface of a wide variety of polymers and several other materials [28–32]. These compounds, known to interfere with specific processes that are essential for cell growth and/or division, are typically linked to or entrapped within the material surfaces. The first scenario assures a high local concentration of antimicrobial agents, reducing bacterial exposure to sub-inhibitory concentrations and thereby the development of antimicrobial resistance. The second scenario is beneficial because it not only allows for the interaction of the compounds with the surface-associated cells, but also with any potential pathogen suspended in the surrounding area. Nevertheless, the amount of surface incorporated agents into these coatings is finite and subject to depletion, and the initially massive release of antimicrobial compounds typically provided by these coatings has been found to be followed by a slow release of inappropriate, i.e. sub-inhibitory concentrations [33–39]. Hydrophilic polymers, such as poly(ethylene glycol), hyaluronic acid and poly-N-vinylpyrrolidone, have also been proposed and successfully applied to passively hinder bacterial adhesion [40,41]. The mode of action of these covalently linked brush-like structures is to function as a steric barrier, i.e. a physical separation distance between the cells and the underlying substratum surface. However, coatings that rely on chemically surface bound polymers or antimicrobial compounds are often compromised by the non-specific adsorption of proteins, surfactants and many other molecules secreted by the cells, which eventually masked their physical or chemical functionality [42–44].

More recently, spatially organized microtopographic surface patterns have been proposed as a novel, chemical-free alternative to limit and control the ability of bacterial cells to attach to and colonize surfaces. The first example of topographic inhibition of a fouling organism was presented by Carman et al., whose group engineered a patterned surface inspired on the skin of sharks named as Sharklet topography (i.e. 2 μm wide rectangular-like periodic structures of 4–16 μm in length spaced at 2 μm and 3 μm height/depth) that was found to significantly reduce (up to 85%) the settlement of a green alga spore compared to equivalent smooth surfaces [45]. This same group later demonstrated that their bioinspired surface topography was also able to reduce the attachment and colonization of the human pathogens *Staphylococcus aureus* and *Escherichia coli* [46,47]. In a similar approach, several other groups using patterned surfaces featuring a wide range of different geometries (i.e. close-packed circular pillars and wells, honeycomb-like patterns, protruding/recessing square features, parallel channels, etc) have subsequently corroborated the significant impact of these highly structured, engineered microscale topographies on bacterial adhesion and proliferation for a number of unrelated bacterial strains [48–54]. Remarkably, a common characteristic of most patterns employed is their microscale surface roughness, i.e. they contained surface features whose height/depth was always comparable or larger than the size of the cells. By virtue of their height/depth, these features have been proposed to act as a physical barrier against bacterial proliferation, as proposed by Chung et al. and Reddy et al. evaluating the proliferation of *S. aureus* and *E. coli* on several variations of the Sharklet topography. [46,47]. Later on, Friedlander et al. and Xu et al. working with square arrays of round pillars demonstrated that the incorporation of air pockets in the space between features notably reduces the surface area accessible to bacteria of the genus *S. epidermidis* and *E.coli*, resulting in a decreased probability of interaction with, and attachment to, the material surfaces [49,51].

The mechanisms by which nanoscale surface roughness modulate cell adhesion and proliferation remain, however, largely unexplored. The literature often provides contradicting results in this matter, but it is essential to note that most reported studies involved materials whose randomly roughened surfaces were mostly undefined in topographical terms [55–57]. In fact, the adhesion of several *Pseudomonas* strains, for instance, has been shown to be higher on nanorough than conventional (smooth) titanium [58]. A different group, however, attributed antifouling properties to similar nanorough titanium surfaces evaluating the adhesion of *Pseudomonas* and several *Staphylococcus* strains [59]. Thus, the purpose of this study is to further investigate the influence of surface topography on bacterial adhesion and biofilm formation by means of engineered topographies (containing well-defined features) with nanometer scale roughness. Particularly, the surfaces employed exhibit topographical features of different size and shape (i.e. protruding and receding square and circular features and parallel channels) with lateral dimensions (length/width and interspace) that are larger than the size of the cells and vertical dimensions that, unlike most related studies, lie on the nanometer scale (i.e significantly smaller than the size of the cells). These surfaces were generated using soft lithography in polydimethylsiloxane (PDMS), a widely used elastomer for its good biocompatibility and stability in pharmaceutical and medical applications [60,61]. Adhesion and biofilm formation assays were performed in a nutritionally rich medium using four major opportunistic pathogens from the genus *Staphylococcus* (*S. epidermidis* and *S. aureus*), common source of medical-device related and hospital-acquired infections [62,63].

2. Materials and methods

2.1. Sample preparation

Samples with spatially organized microtopographic surface patterns were generated via soft lithography using PDMS and two commercially available silicon masters (Budget Sensors, Bulgaria) following the procedure described in a previous study [64]. The silicon masters employed, i.e. HS-100MG and HS-20MG, contain both a centre area of 1 × 1 mm that comprises protruding and recessing 6 × 6 μm square features with a interstitial space of 4 μm, protruding a recessing circular features of 3 μm in diameter with 2 μm spacing, and 2 μm-wide ridges separated by 3 μm-wide channels. The depth/height of all features was 117 nm (HS-100MG) and 21.1 nm (HS-20MG), according to manufactureŕs specifications. The fidelity of the replicated surface patterns has been previously evaluated in our group through light and atomic force microscopy (AFM): regularly spaced virtually defect-free geometrical features were observed with dimensions (length, height/depth, and interspace) that lie within a range of 0.6 and 7% of the manufactureŕs specifications [64]. Nevertheless, prior to use, all samples employed were subjected to optical inspection (OlympusBX41, Olympus, Spain), whilst the dimension and geometry of the topographical surface patterns was again verified for randomly selected samples using an AFM 5500 system (Agilent Technologies, USA) operating in contact mode. To serve as a control, smooth samples were produced by casting the polymer over flat silicon wafers (Crystec, GmbH, Germany). A summary of the physical characteristics of the patterned surfaces employed and the flat control samples are displayed in Table 1.

2.2. Bacterial strains, adhesion experiments and biofilm formation

Four different Gram-positive strains were used in this study: the biofilm-negative *Staphylococcus epidermidis* ATCC 12228 (*S. epidermidis* 8), and the biofilm-forming strains *Staphylococcus epidermidis* ATCC 35983 (*S. epidermidis* 3), *Staphylococcus aureus* ATCC 25923 (*S. aureus* 23) and *Staphylococcus aureus* ATCC 29213 (*S. aureus* 13). All strains were maintained in blood agar plates (OXOID) and cultured in Trypticase Soy Broth (BBL™, Becton, Dickinson and Company, Sparks, USA) at 37° C on a rotary shaker (150 rpm) for 24 h. These cultures were used to inoculate a second culture that was grown for 10 h to early stationary phase. Bacterial cells were then re-suspended for