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Regular Article

Deconvoluting the effects of surface chemistry and nanoscale topography: *Pseudomonas aeruginosa* biofilm nucleation on Si-based substrates

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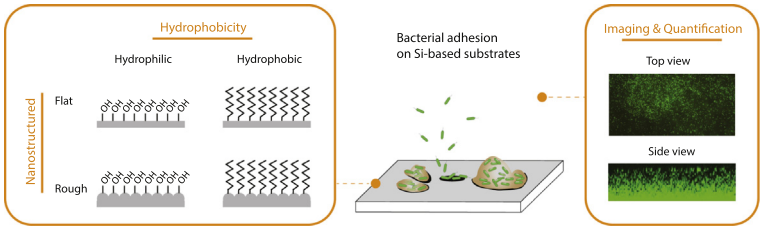
GRAPHICAL ABSTRACT



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ABSTRACT

Hypothesis: The nucleation of biofilms is known to be affected by both the chemistry and topography of the underlying substrate, particularly when topography includes nanoscale (<100 nm) features. However, determining the role of topography vs. chemistry is complicated by concomitant variation in both as a result of typical surface modification techniques. Analyzing the behavior of biofilm-forming bacteria exposed to surfaces with systematic, independent variation of both topography and surface chemistry should allow differentiation of the two effects.

Experiments: Silicon surfaces with reproducible nanotopography were created by anisotropic etching in deoxygenated water. Surface chemistry was varied independently to create hydrophilic (OH-terminated) and hydrophobic (alkyl-terminated) surfaces. The attachment and proliferation of Psuedomonas aeruginosa to these surfaces was characterized over a period of 12 h using fluorescence and confocal microscopy.

Findings: The number of attached bacteria as well as the structural characteristics of the nucleating biofilm were influenced by both surface nanotopography and surface chemistry. In general terms, the presence of both nanoscale features and hydrophobic surface chemistry enhance bacterial attachment and colonization. However, the structural details of the resulting biofilms suggest that surface chemistry and topography interact differently on each of the four surface types we studied.

1. Introduction

Outside of the laboratory, bacteria are seldom found in high-density monocultures, suspended in nutrient rich environments.

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Instead, in most clinical and natural environments, bacteria live in biofilms, attached to surfaces under restricted nutrient conditions. While a great deal of effort has been expended on characterizing the molecular, physiological, cellular and clinical characteristics of biofilms, less is known about the details of the initial interactions between surfaces and bacteria [1–3]. These interactions regulate the early attachment stages of the biofilm, and in so doing, profoundly influence the speed and architecture of subsequent biofilm development [4].

A clearer understanding of the initial stages of biofilm attachment has obvious applied implications. In many circumstances—c atheterization, prosthetic implants, ventilators—the prospect of engineering surfaces that would inhibit or prevent bacterial attachment provides obvious clinical benefits [5]. Conversely, other applications, including the construction of scaffolds for cell growth or the promotion of commensal biofilm formation to limit pathogen invasion, might benefit from surfaces that enhance cell adhesion. In both cases a better understanding of how various surface features govern cell/surface interactions will allow for better design and control of these interactions, sometimes portrayed as "the race for the surface [6,7]".

This study focuses on the attachment of *Pseudomonas aeruginosa* to silicon-based surfaces. Pseudomonas has long been seen as model organism for the exploration of biofilm formation. It is an opportunistic pathogen that has been implicated in a number of clinically relevant infections, including catheter-associated infections, post-surgical infections of implants and prostheses, contact lens-associated eye infections, and pulmonary infection in cystic fibrosis patients [8,7]. The transition from planktonic to biofilm growth modes involves a regulated set of changes in gene expression that coincide with the initial attachment stage [9–11]. Here, we explore the effect of different surface topographies and chemistries on the extent of biofilm formation, as well as on the architecture of the resulting biofilm. We rely on the fluorescence conferred by the inducible plasmid-encoded GFP carried by our Pseudomonas NIH3 strain to undertake a microscopic examination and quantification of the Pseudomonas biofilm, using both fluorescence and confocal microscopy.

Both the chemistry (specific functional group termination) and topography of surfaces influence the interactions of cells with those surfaces. The particular sensitivity of eukaryotic cells to surface topography defined as nanoscale (typically features with lateral dimensions <100 nm) has been attributed to the fact that such topography mimics the scale of features within the extracellular matrix; the origin of bacterial responses to such surfaces is perhaps less obvious. In both cases, the desire to explore and better understand the nature of these interactions has prompted the development of numerous approaches to generating nanoscale topography [12]. These approaches include, but are not limited to, polymer-based techniques (de-mixing and block copolymer phase separation); deposition techniques such as molecular beam epitaxy; and use of both mechanical and chemical means of roughening (or smoothing) pre-existing surfaces.

Numerous studies highlight the complexity of cell/surface interactions. In one study, *Pseudoalteromonas issachecnkonii* was found to exhibit increased bacterial density on a glass surface etched in buffered HF/HCl to produce nanoscale features [13]. The same authors saw analogous results for other bacteria (*E. coli, P. aeruginosa* and *S. aureus*) on the same surface [14]. Introduction of nanotopography by deposition of films of SiO_2 and Al_2O_3 nanoparticles also appeared to enhance the attachment and clustering of *Pseudomonas fluorescens* [15]. However, another study that varied titania surface topography systematically via supersonic cluster beam deposition showed *inhibition* of biofilm formation for both *E. coli* and *S. aureus* on surfaces with greater nanoscale roughness [16]. These examples--where in some cases, nanotopography seems to inhibit the proliferation of bacteria on the surface and in other cases to encourage it--suggest that the effect of nanotopography on bacterial attachment to surfaces is neither simple nor necessarily consistent across surface types. What is perhaps less obvious from these examples is that nanotopography itself is complicated by its interplay with the chemistry of the surfaces whose topography has been altered. Given that all surfaces intrinsically exhibit both topography and chemistry, how can we disentangle the relative contribution of each to changes in cell behavior [17]?

In fact, as many studies have shown, decoupling topography from surface chemistry is quite challenging. The characterization of surface chemistry between surfaces with different topographies within the same study is often accomplished by some combination of elemental analysis (e.g. X-ray photoelectron spectroscopy and/or X-ray fluorescence) and evaluation of macroscopic surface wettability and/or surface free energy by contact angle goniometry. While these techniques do provide some information about the consistency of chemistry across surfaces that are in many cases subject to different processing, they generally do not provide the kind of molecular-level detail that can, for instance, demonstrate that the chemical terminations are identical for two surfaces with different topography.

As further evidence of this complexity, while the study cited above on Ti surfaces suggested that nanoscale roughness inhibits biofilm growth [16], another study on four different Ti surfaces found that while some types of nanoscale features did indeed inhibit biofilm growth, some types of features seemed to promote biofilm growth among a range of different bacteria, including *Pseudomonas aeruginosa* [18]. In this latter study the authors noted that their surfaces contained different amounts of crystalline vs. amorphous TiO_2 coatings, and also that the electrochemical procedures used to generate some topographies appear to have caused small amounts of fluorine contamination. In cases where nanostructures *inhibited* biofilm growth, the authors in this second study [18] observed the same enhancement of protein (fibronectin in this case) adsorption that was proposed to inhibit bacterial attachment in the other Ti work [16]. However, in this study the particular nanostructures that seemed to *promote* bacterial attachment displayed the same enhanced fibronectin adsorption. In other words, not only does nanoscale topography appear sometimes to inhibit and sometimes to promote biofilm growth, but the relationship between bacterial adhesion and protein conditioning is not consistent, even though pre-adsorption of proteins and other biomolecules has been proposed to play an important role in determining the extent of surface colonization.

Even when nanotopography is generated by methods that seem to offer a higher degree of chemical control, precise characterization of the resulting surfaces is still a formidable challenge. For example, in one study where a polymer de-mixing approach was used to generate a range of different sizes of nanoscale features, static contact angle measurements showed a (small) increase in wettability for surfaces with greater nanoscale roughness [19]. a result inconsistent with the generally accepted effect of surface roughness on surface wettability [20]. In this case the authors proposed that their surface chemistry is constant across different topographies because their annealing process causes segregation of one polymer (polystyrene) to the surface of the films, but without more detailed characterization it is impossible to ensure that the molecular conformation of the surface polymers is constant across these surfaces, or to rule out the possibility that some unplanned surface contamination has taken place. The contact angle data suggest that surface chemistry is varying, but it is impossible know exactly how. In general, without a more detailed understanding of the ways surface chemistry varies along with topography, and of the combined impacts of topography and