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Deconvoluting the Effects of Surface Chemistry and Nanoscale Topography: *Pseudomonas aeruginosa* Biofilm Nucleation on Si-based Substrates

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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 hours 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.

Keywords: Biofilm nucleation, nanoscale, silicon, surface chemistry, surface topography,

Pseudomonas aeruginosa

Introduction:

Outside of the laboratory, bacteria are seldom found in high-density monocultures, suspended in nutrient rich environments. 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—catheterization, 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 SiO₂ and Al₂O₃ 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 TiO₂ 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 chemistry on both biomolecule (protein) and bacterial adsorption, we cannot draw consistent conclusions about the effect of nanotopography on biofilm nucleation and growth.

As outlined in a review of work in this area, an ideal method for generating surfaces with nanoscale topography would be highly reproducible, would create surfaces that are uniform in *both* their topography and surface chemistry, and would allow for generation of large surface areas both quickly and cheaply [12]. The use of anisotropic chemical etchants offers promise for generating surfaces that satisfy these criteria, since the lateral resolution required to create true nanoscale (< 100 nm) topography is challenging to achieve with more traditional lithographic methods.

This approach has been used to create nanostructured Ti with varying topographies [21] and works on other metals as well. In that particular study, though, there is little chemical detail about the nature of impurities (e.g. S and F) deposited by the etching process. The authors suggest that different oxide layer thickness on the different surfaces influences cell proliferation on those surfaces, but there is no way to tell if these differences in oxide thickness are accompanied by changes in surface chemical termination [21].

Anisotropic etching of silicon surfaces is well understood [22] and has been exploited to examine the interaction of cells and bacteria with nanoscale topography on this readily-available and chemically flexible surface. Etching of Si(100) in base has been used in combination with photolithography to generate specific features (such as grooves) that were then used to study cell behavior [23], but in this case the generation of specific patterns was still by size-limited lithography. In a more recent study nanoscale “sponges” were created on oxidized silicon wafers (orientation unspecified) by Ag nanoparticle-assisted etching, followed by functionalization with 3-aminopropyltrimethoxysilane (APTMS) and perfluorodecyltrichlorosilane (FDTS) [24.] This approach holds promise because of the ability to generate reproducible nanotopography and then to control the chemical termination of that topography with well-defined silane chemistry. However, the characterization of the resulting surfaces by static contact angle provides evidence that the surface chemistry in this example is not as well-defined as one might hope. For instance, “pristine oxidized” Si substrates that would normally be extremely hydrophilic exhibited water contact angles of 29°, and the

nanosponge APTMS- functionalized surfaces were hydrophilic, with a much lower contact angle than the flat APTMS-coated surfaces: the opposite of what one would expect from the introduction of surface roughness [24]. Some of the inconsistencies here may arise from the use of tri- (rather than mono-) functionalized silanes, which can result in polymerization and generally less reproducible surfaces [25].

In this study we have exploited a different anisotropic chemical etching process to generate Si(100) surfaces with and without nanoscale topography. These surfaces--both the initial flat surfaces and the “rough” ones with nanoscale features--are either rendered hydrophilic by aqueous oxidation or hydrophobic by reaction with a monochlorosilane reagent that has been shown to generate highly reproducible monolayers [25].

The nanoscale topography used in this work is created by etching of hydrogen-terminated Si(100) in deoxygenated, ultrapure water. As demonstrated in a combined scanning tunneling microscopy (STM) and infrared (IR) spectroscopy study of this process, etching in this manner for ~24 hours creates surfaces that are covered with “hillocks” that are ~50 nm in diameter [26]. While further studies have provided more detailed understanding of both the etching process that leads to these features and the exact chemical termination of the H:Si(100) surface that results [27,28], in this work we simply rely on the ability of this reaction to reproducibly create surfaces with regular nanoscale features. This process does not require access to a clean room or to lithographic techniques; the production of regular nanotopography simply requires a standard chemical cleaning process (detailed below) followed by room-temperature etching in deoxygenated water.

By achieving high levels of surface control and reproducibility, we are able to examine the effects of both surface chemistry and nanotopography on the nucleation of biofilms of *P. aeruginosa*, while effectively separating the two effects by varying one surface characteristic at a time. It is worth noting that, as a previous study illustrated, the exact details of nanotopography can influence the behavior of cells and organisms on that topography, and therefore characterization of surface features beyond simple roughness measurements is critical for interpreting results from different surfaces [29]. In this case, while the topographic features of our surface are better described by the high resolution of STM and have been discussed elsewhere [26], we focus on the fact that our method ensures that we are comparing the same features across the two different types of surface chemistry.

Materials and Methods:

Substrate Preparation

Samples for all experiments were cut by hand as 13 mm squares from Si(100) wafers (University Wafer, Boston, MA: CZ-type, single-sided polished, N-type, resistivity 0-100 Ω -cm, thickness 500 ± 20 μ m). For flat surface experiments the wafers were used as received, with a layer of native oxide. All water used in sample preparation was deionized (DI) water (18.2 M Ω) from a Barnstead Nanopure benchtop deionizer (Thermo Scientific) fed with house reverse osmosis water.

Flat, hydrophilic samples were prepared by immersion in a standard SC-2 clean [30] (4:1:1 volume ratio of DI water: hydrogen peroxide (H_2O_2 , 30%, ACS grade, VWR): hydrochloric acid (HCl, 70%, Fisher Scientific Co., ACS grade)) at 70-80°C for 10 min. Samples were rinsed thoroughly with DI water and dried with compressed air before use. This preparation results in a relatively contaminant-free, hydroxylated SiO_2 layer that is extremely hydrophilic (water contact angle less than 5°). Silica (SiO_2) is well known to be negatively charged at neutral pH, and therefore the samples we will call “hydrophilic” throughout this study should also be assumed to be negatively charged.

To prepare flat, hydrophobic surfaces the hydrophilic samples from above were subjected to an additional silanization step to create a homogeneous alkyl monolayer. After the SC-2 clean, the samples were thoroughly rinsed with deionized water, dried at 120°C for 30 min and subsequently transferred to a sealed Schlenk tube. Samples were held in a glass sample holder above ~1 mL of n-octyldimethylchlorosilane (Gelest Inc., used as received), and the tube was heated in a silicone oil bath to 70°C to allow the surfaces to react with vapor-phase silane. This method has been shown previously to create a higher-quality monolayer than reaction with solution-phase silane [25]. After 24 h the samples were sonicated in a toluene (Fisher Scientific Co., ACS grade) bath for 5 min to remove excess silane and then rinsed with toluene, ethyl alcohol (95% ACS grade, Pharmco-AAPER) and DI water. A somewhat more tightly packed monolayer can be generated by a more vigorous cleaning process prior to silanization [25], e.g. piranha clean, but previous work in our lab [31] showed that the less energetic SC-2 clean preserves more of the nanoscale topography that we will be comparing to these flat surfaces; therefore SC-2 clean was used prior to silanization throughout this study.

Surfaces with nanoscale topography (referred to as “rough”) were prepared from the same

Si(100) wafers with a 900-1,000 Å thick sacrificial thermal oxide [32]; removal of this oxide layer as described below results in a pristine surface that has not been subjected to polishing damage. The starting substrate for preparation of the rough surfaces was a flat, hydrogen-terminated Si surface prepared via a modified RCA clean [30]: a 10-min SC-1 clean (4:1:1 volume ratio of DI water: hydrogen peroxide: ammonium hydroxide (NH_4OH , 14.8 M, EMD Chemicals, ACS grade)) at 70-80°C; 60 s etch in buffered hydrofluoric acid (BOE, Transene Buffered HF Improved, Transene Inc.) to remove the sacrificial oxide; a 10-min SC-2 cleaning at 70-80°C, and a final 30 s etch in concentrated hydrofluoric acid (HF, 47-52%, J.T. Baker). Immediately after the final HF step the samples were submerged in DI water sparged with argon (Ivey Gas, 99.999%) to deoxygenate it. The water was deoxygenated for 30 min prior to sample immersion and then continuously throughout the etching process.

Once the H-terminated rough surfaces are prepared, they can be further reacted to create the same hydrophilic and hydrophobic surface chemistry previously described for the flat surfaces. For rough, hydrophilic surfaces the H-terminated samples were removed from the deoxygenated water and then subjected to a 10-minute SC-2 clean at 70-80°C. This process generates a thin (~20 Å) oxide layer that is similar to a clean native oxide; following rinsing in DI water the surface is hydroxylated and hydrophilic. To generate rough, hydrophobic surfaces the SC-2 cleaned rough surfaces were again dried at 120 °C for 30 min and exposed to vapor-phase n-octyldimethylsilane as described above.

Surface characterization

Dynamic contact angle was used to verify the quality of monolayers formed on both flat and rough surfaces; surface roughness on the scale present in our samples also impacts surface wettability [20], so these measurements simultaneously provide evidence for the persistence of rough surface topography after functionalization of the rough, hydrophobic surfaces. Both advancing (θ_A) and receding (θ_R) angles of DI water were measured manually with a Ramé-Hart Model 100-00 contact angle goniometer. Five droplets of approximately 1-2 μL each were measured on each sample. Both the rough and flat hydrophilic surfaces have θ_A and θ_R values less than 5°, so specific values are not reported for these surfaces.

Atomic force microscopy (AFM) was also used to confirm the persistence of nanoscale topography between the hydrophilic and hydrophobic rough surfaces. AFM images were acquired in tapping mode with Si₃N₄ tips on a Digital Instruments Multimode AFM. The rms surface roughness was calculated from 1 μm x 1 μm images and is presented along with contact angle data in Table 1. Representative images of all four surface types (flat and rough, hydrophilic and hydrophobic) are presented in Figure 1. The precise details of the geometry of the rough surface, which is characterized by complex crystallographic facets, is better captured by the higher-resolution scanning tunneling microscopy images acquired by Hines and coworkers [26]; for the purposes of the current work we note that, even after silanization, these surfaces are accurately characterized as being rough on the nanoscale. Both the increased θ_A values and the larger contact angle hysteresis ($\theta_A - \theta_R$) for the rough hydrophobic surface as compared to the flat one are also consistent with the effects of nanoscale topography on the wettability of the rough surface.

Bacterial strain, culture conditions and sample preparation

The bacterial strain used in this study was *Pseudomonas aeruginosa* NIH3/pMQ80-7, a gentamicin resistant strain capable of expressing eGFP. The original bacterial strain was obtained from ATCC [33] and subsequently transformed with a pMQ80 plasmid harboring gentamycin resistance and an arabinose-inducible GFP. *P. aeruginosa* was streaked from a -80°C bacterial stock on a Luria-Bertani (LB; Becton, Dickinson and Company, Sparks, MD) agar plate containing 30 μg/mL gentamicin. Prior to each attachment experiment, a bacterial colony grown on the LB agar plate prepared was grown overnight, at 37°C with shaking (105 rpm), in 25 mL of LB broth containing 10μg/ml final gentamycin concentration. The resulting cell density was adjusted to an OD₆₀₀=1.3 and subsequently diluted 100-fold to ensure a similar number of initial cells (~10⁵ cells) exposed to our surfaces during biofilm growth experiments.

In our bacterial attachment and biofilm growth studies substrates prepared as described above were placed face-down atop sterile silicone O-rings that were fitted into the wells in 12-well flat-bottom microtiter polystyrene plates (Corning Inc). We chose to place the relevant surfaces face down in the wells in order to distinguish actual the actual attachment that precedes biofilm formation from simple passive deposition of bacteria. Each well contained 2 mL LB broth supplemented with

gentamicin (10 μ g/ml final concentration) and 2% w/v arabinose. An aliquot of 100 μ L bacterial suspension prepared as described above was dispensed into each well. The microtiter plate was allowed to incubate at 37°C for 6 or 12 hours with constant shaking at 105 rpm to prevent settling of the solution and to permit biofilm growth on the substrates. The 6 hour time point was chosen as the earliest time point tested that yielded visible and quantifiable differences in the amount of fluorescence observed; 12 hours was selected more arbitrarily as a time point that corresponded to significant biofilm development on all four surface types. Four different replicates of each of the substrate surfaces were prepared at one time; comparisons of biofilm growth on the surfaces were always made within a single experiment in order to control for possible variation in cell growth or concentration. Prior to biofilm visualization and quantification, the substrates with attached biofilm were rinsed gently with 2 mL phosphate buffered saline (PBS) three times to remove planktonic and unattached cells.

Biofilm quantification and visualization

GFP fluorescence ($\lambda_{\text{excitation}} = 485$ nm, 20 nm bandwidth 20 nm, $\lambda_{\text{emission}} = 535$ nm, 8 nm bandwidth) was measured using a Tecan Infinite M1000 plate reader and Tecan i-Control software. In each experiment four replicates were measured for each type of surface to obtain fluorescence means and error estimates. A low-resolution three-dimensional model of the biofilm topography formed on each type of surface after 6 h was simulated on MATLAB using the fluorescence data acquired from 44 reads within a circle of radius 7.05 mm centered within the larger imaging field (radius 11.05 mm).

To get a more detailed picture of the spatial characteristics of the biofilms formed on different types of surfaces, samples were also observed using an Olympus BX51 light-fluorescent microscope at x20 magnification with images captured by an Olympus DP71 digital camera system. On each substrate only the central portion of the sample was analyzed, since we observed that the edges of the surface were prone to collecting anomalously thick colonies of biofilm. This central portion, which accounted for roughly 80% of the original substrate surface, was divided into a 20 x 20 grid of rectangular image areas, each measuring 585 x 484 μ m. A random number generator was used to select the x and y coordinates for 20 out of the 400 image regions for subsequent analysis. For this

microscopy analysis, three samples were visualized for each type of constructed surface in each of the triplicate experiments.

Image analysis was performed with ImageJ image processing software (US National Institute of Health, Bethesda, MD). Images were first converted to grayscale followed by a manual adjustment of threshold to distinguish cells from the substrate background. The percentage of substrate colonized by bacterial cells was obtained using the area fraction tool.

Confocal microscopy was used to assess the thickness of biofilm at both initial and more mature stages of development. Images were captured using a Leica TCS SP5 Laser Scanning Confocal Microscopy (LSCM) equipped with an oil immersion lens (x40). The three-dimensional projection of biofilm on each sample was acquired with LAS AF software. Biofilm thickness was determined by multiplying the number of slices by the z-step size (2 μm). Images from 6 representative areas were taken for each surface type in one experiment.

Results and Discussion

We sought to determine the extent to which surface nanoscale topography and surface chemistry act to influence the nucleation of *P. aeruginosa* biofilms. We began by analyzing the total amount of bacterial adhesion as a function of time and of substrate characteristics. This analysis revealed differences in the rate of biofilm nucleation on the different substrates in the study and provided an important macroscopic measure of consistency for the more localized image analysis described below.

As the data in Figure 2 demonstrate, substrate characteristics have a larger impact on biofilm formation in the initial nucleation stages. After 6 hours of growth (Figure 2A), the total amount of fluorescence detected on the rough hydrophobic surface is significantly greater than that detected on the other three substrate types. There are no significant differences in the amount of fluorescence detected from each of the remaining three substrates (both flat and rough hydrophilic and flat hydrophobic). After 12 hours of growth, however, the difference between the rough hydrophobic surface and the other three substrates has essentially disappeared. Taken together, these results suggest that any substrate-induced differences in biofilm formation are most apparent at shorter nucleation times, with the rough hydrophobic substrate promoting the most bacterial adhesion in the

early stages of biofilm nucleation.

Maps of biofilm topography, generated by plotting these fluorescence data across one sample from each substrate type after 6 hours of exposure, are presented in Figure 3. These plots are consistent with the global fluorescence measurements presented in Figure 2 (e.g the largest region of high-intensity fluorescence is seen on the rough hydrophobic surface). The data used to generate these maps are represented in histograms of fluorescence intensity in Figure 4(A), while the variability in fluorescence intensity present in each sample is plotted as the standard deviation for each data set in Figure 4(B). We note that the error bars in Figure 4B depict the confidence interval around the estimate of standard deviation for each sample: this plot enables us to compare the variability of fluorescence intensities and not the mean intensities themselves (which are shown as asterisks in Figure 4A). This analysis reveals that fluorescence intensity on both the flat and rough hydrophobic surfaces is significantly more variable than on either of the hydrophilic surfaces; we observe no significant differences in the variability of fluorescence *within* either hydrophobic or hydrophilic surfaces. The conclusions remain when the intervals are adjusted for multiple comparisons using a Bonferroni correction.

The variability of fluorescence measurements from a given sample type sheds light on the tendency of bacteria in our experiment both to aggregate two-dimensionally on surfaces, and to do so differentially depending on surface characteristics. If bacteria were evenly distributed across the sample, we would expect a very narrow histogram in Figure 4(A) and an associated low value for the standard deviation plotted in Figure 4(B). Conversely, if the bacteria have a strong tendency to aggregate, we would expect a more bimodal distribution reflecting either regions of intense fluorescence or regions of no fluorescence, and an associated high value for the standard deviation. The results in Figure 4(B) suggest that, independent of surface topography, the hydrophobic surface chemistry used in our experiment drives more variable bacterial aggregation during the first six hours of exposure than does the hydrophilic surface chemistry, although both types of chemistry reflect at least partial initial aggregation. Importantly, the comparisons in Figure 4(B) suggest that this tendency to aggregate (or not) is driven by surface chemistry alone and is independent of surface topography for the present system.

The possible reasons for surface-chemistry driven aggregation are complex, in part because

even at 6 hours neither the surface nor the bacteria are static actors. Greater aggregation on the hydrophobic surfaces indicates a relative preference (compared to the hydrophilic surface) for bacteria/bacteria interactions over bacteria/surface interactions at the particular surface/solution interface present during this initial period of biofilm nucleation. As noted earlier, since our silanol-terminated SiO₂ “hydrophilic” surfaces are not only highly wettable but are also negatively charged, electrostatic interactions are likely a key component of bacteria/surface interactions on both the flat and rough hydrophilic surfaces. Furthermore, while all the initial underlying silicon substrates are, by design, chemically well-defined, those substrates, and hence the surface/solution interface, are continually evolving as the surface becomes covered with bacteria and/or associated EPS. While the first bacteria to colonize the surface adsorb directly to the initial substrate, later arrivals will adsorb to a “surface” that is partially or even primarily composed of surface-tethered bacteria. Furthermore, upon attachment the bacteria themselves can be expected to undergo changes in gene expression that will alter their own surface properties.[34,9,35]

While more precise characterization of the changing chemical nature of the surface/solution interface during biofilm nucleation is beyond the scope of this work, we recognize that apparent surface chemistry-driven differences in bacterial aggregation in effect involve a more complex set of interactions than go beyond the initial encounter between *P. aeruginosa* and the pristine alkyl-terminated surface. Additional complexity does, in fact, result from the introduction of different substrate topographies. Although it is true that, as stated above, the differences in the magnitude of variability of fluorescence intensity (and hence two-dimensional bacterial distribution) depend only on surface chemistry, even cursory examination of the histograms in Figure 4(A) reveals that nanoscale topography does affect the spatial distribution of bacteria in our system. In other words, while the flat and rough hydrophobic surfaces result in statistically indistinguishable standard deviations of fluorescence intensity, the overall shape and position of those distributions are in fact easily told apart. To better understand the origin of those distinctions, we turn to higher-resolution analysis of the structural qualities of the 6-hour biofilms.

Figure 5 shows a composite view depicting an array of fifteen images of randomly selected 585 μm x 440 μm fields, captured via fluorescence microscopy after 6 hours of exposure on each of the substrate types. These images reveal the distinct patterns of initial cell attachment and early

biofilm colonization induced by differences in both surface chemistry and topography. While there is significant variation within each substrate type in the total fluorescence and in the two-dimensional distribution of that fluorescence, Figure 5 nonetheless reveals clear qualitative differences among the structures of the nucleating biofilms on the different surfaces.

The distribution of fluorescence on the two flat surfaces (panels A and B) underscores the relatively even attachment of *Pseudomonas* colonies on the surfaces after 6 hours. In certain panels on the flat hydrophobic surface, the initial nucleation has begun to give rise to more extended colony formation, reflected in patches of high fluorescence. In contrast, the flat hydrophilic surface shows a more even distribution of initial biofilm colonists.

The introduction of nanotopography appears to enhance biofilm formation on both types of surface chemistry. On the rough hydrophilic surface (Figure 5C) bacteria are intensely localized into microcolonies that are distributed relatively evenly across the surface. On the rough hydrophobic surface the enhancement of biofilm formation is characterized by an elevated amount of EPS production, visible as a diffuse green background between the brighter localized fluorescence emitted by individual bacteria. The bacterial film on this surface is also distinguished from those on the other three substrates by the clear development of bacterial multilayer structures, as this is the only substrate with fluorescence arising from multiple focal planes.

An uneven distribution of biofilm across the substratum can be observed on all surface types, as reflected by different amounts of fluorescence captured across the randomly selected areas in each group. When comparing total fluorescence emission from the 15 fields studied for each surface type (Table 2), flat hydrophilic samples exhibit the least fluorescence at both 6 and 12 hours, suggesting that this combination of nanotopography and chemistry is least conducive to biofilm formation. Conversely, at the early biofilm formation stages, rough hydrophobic surfaces have the highest substratum coverage, followed by rough hydrophilic and flat hydrophobic surfaces. Even after 12 hours—when we can expect that newly-arriving bacteria will likely no longer encounter the surface directly—the rough surfaces still appear most conducive to biofilm growth.

We investigated the architecture of these surface-dependent biofilms in greater detail by using LSCM to study their 3D structural features. Figure 6 shows 3D projections of representative biofilm formed on each substrate after 6 hours and 12 hours of growth. As shown in Figure 6A, after 6

hours of growth the biofilm attached to rough hydrophobic substrates is thicker and more robust than the biofilms developing on the other substrates. The thinnest biofilm is seen on the flat hydrophilic surface. Figure 6B, reflecting the structure of the biofilms after 12 hours, reveals more significant surface-associated differences in the resulting biofilms. The biofilm on the rough hydrophobic surface appears thicker and denser than any of the others, while the biofilm developing on the flat hydrophilic surface is significantly thinner. We quantified the overall biofilm thicknesses after 6 hours and summarize those data in Table 2.

This study was performed in order to elucidate how nanotopography and chemistry of the underlying surface affects biofilm attachment and colonization. Our sample preparation method allows us to decouple the respective influence of surface chemistry and surface topography on biofilm adhesion. The results show that both nanoscale surface roughness and surface chemistry affect bacterial adhesion. Broadly speaking, while increased topography (roughness) and increased hydrophobicity appear to promote early stage biofilm development, important interactions between these two factors influence bacterial attachment. It is also important to note that the switch from hydrophilic to hydrophobic surface chemistry is, in our system, accompanied by a switch from negatively-charged to electrically neutral. While in this work our results indicate that the two surface chemistries studied influence biofilm nucleation differently, attributing this influence to surface wettability vs. surface charge would require study of additional surface chemistries.

The initial events of attachment clearly depend on the physicochemical characteristics of both the surface and the bacteria, as well as on the initial and subsequent response of the *Pseudomonas* cell to first contact. The hydrophobicity of substrate surface, for example, affects biofilm adhesion mediated by the thermodynamically predicted preference of hydrophilic cells for hydrophilic substrata and of hydrophobic cells for hydrophobic substrata.[36,37] In addition, *Pseudomonas aeruginosa* cells respond, individually and collectively, following the initial events of biofilm formation. Those directed responses, in turn, modify the surface itself, primarily through the secretion of EPS, forming a conditioning layer that alters the chemistry and the architecture of the surface for subsequent colonists. Less obviously, the transition from a planktonic to a sessile state alters the surface of the bacterial cells in ways that affect subsequent biofilm formation. Attachment to surfaces with different physical or chemical profiles may affect the production of EPSs[38] or influence the dynamic motion of

the outer surface proteins, in turn modifying the apparent polarity or charge of the cell.

The nanoscale topography of our surfaces places it at roughly the same scale as certain cell-surface features, including flagella and cell-surface receptors. Patterning of this sort has been seen as creating unfavorable geometric and electrostatic conditions for attachment.[39-41] These simple predictions would suggest that we should see the most robust biofilm growth on our flat, hydrophilic surfaces—the exact opposite of what our data suggest. This apparent contradiction underscores two important points: one methodological, and one mechanistic. Methodologically, investigating the effects of surface characteristics on bacterial adhesion requires strict reproducibility of the relevant characteristics, and an experimental system that allows the independent manipulation of surface geometry and surface chemistry. Our system, we contend, is a step in the right direction. Second, investigations of bacterial attachment must acknowledge that neither the bacteria nor the surfaces remain static and constant throughout these experiments. The interactions between surfaces and bacteria are dynamic and self-reinforcing, and will require careful dissecting. It is also important to note that while simple surface area effects can be expected to result in greater total numbers of bacterial attachment to the rough surfaces in this study, those effects (which we have not quantified in the present work) cannot account for the qualitative structural differences observed in biofilm nucleation on flat vs. rough surfaces of both types of surface chemistry.

In virtually every important interaction between humans and bacteria—from bacterial infections to food-borne outbreaks, from antibiotic-resistant pathogens to commensal microbiomes—the relevant bacteria are embedded in a biofilm. In most settings biofilms, once established, are remarkably difficult to dislodge. As a result we have for some time been interested in approaches that might retard or altogether prevent the establishment of biofilms. The rational development of new biomaterials and surfaces that affect biofilm formation depends on an increasingly detailed and nuanced understanding of the initial events of biofilm formation. As these data suggest, those interactions are not likely to be straightforward nor simple to characterize, but this study represents an important step in separating out the effects of topography and chemistry on biofilm nucleation and growth.

Conclusions

Using a previously characterized process to generate well-defined Si surfaces with nanoscale features [26-28], we have demonstrated the ability to systematically and independently vary surface chemistry and surface topography on the nanoscale and to observe the effects of these surface characteristics on the nucleation and early growth of biofilms of *Pseudomonas aeruginosa*. While numerous previous studies have investigated the role of specific types of nanoscale surface topography on bacterial attachment, the current work is distinguished by our ability to examine two distinct surface chemistries in both the presence and absence of nanoscale features while holding the surface chemistry constant. Nanotopography in the current study promotes more extensive biofilm growth, consistent with previous studies that found a similar effect for different types of nanoscale features (though with less well-characterized surface chemistries) both for *P. aeruginosa* [14, 18] and for other bacteria [13-15] and in contrast with studies that found the opposite effect for some specific surface topographies [16, 18]. While surface area effects may account for some of this increase in our and other studies that found nanoscale topography enhances bacterial attachment, structural differences in both the 2-D and 3-D distribution of bacteria in the current work suggest that nanotopography has a more profound influence on bacterial behavior than simply providing more sites for surface attachment.

Careful analysis of our results on multiple length scales and in both lateral and vertical dimensions provides strong evidence that there are characteristic biofilm structures associated with each of the four surface types we investigated. For example, while the introduction of nanoscale topography enhances biofilm growth on both the hydrophilic and hydrophobic surfaces in the current work, it does so differently in each case—through intense microcolony formation on the hydrophilic surface and with a significant increase in associated EPS on the hydrophobic surface. This apparent increase in EPS adsorption in the combined presence of nanotopography and hydrophobic surface chemistry is especially interesting as enhanced EPS adsorption has previously been proposed both to enhance [18] and inhibit [16] biofilm formation. These results and the reproducibility of our surfaces suggest that our substrate of flat and water-etched Si(100) provides a useful model system [12] for further elucidation of the combined role of surface chemistry and topography, potentially not only for bacterial biofilm proliferation but for eukaryotic cell attachment as well. Expansion of the types of surface chemistry used could allow us, for instance, to separate out the effects of surface wettability

and surface charge, something that is confounded in this study by the use of hydroxylated SiO₂ as our hydrophilic substrate. The reproducibility of both the chemistry and topography of our surfaces may also allow us to delve more deeply into evaluation of the proposed underlying mechanisms for bacterial response to different substrates, in particular the role of EPS conditioning films as described above. Finally, expansion of both the surfaces and bacteria used in these types of studies, maintaining the high degree of surface reproducibility achieved herein, will allow improved understanding of whether these types of bacteria/surface interactions are as individualized as the variety of published results suggest, or whether there are in fact a consistent set of underlying principles that govern these complex and evolving interactions.

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Surface type	H ₂ O contact angle (°)		RMS surface roughness (nm)
	θ_A	θ_R	
Flat hydrophobic	100 ± 3	92 ± 3	0.2
Flat hydrophilic	--	--	0.2
Rough hydrophobic	108 ± 5	92 ± 5	4.8 ± 0.5
Rough hydrophilic	--	--	3.9 ± 0.6

Table 1: Characterization of surface roughness by dynamic contact angle and atomic force microscopy. Uncertainty is reported as twice the standard error of each measurement. Contact angle values are averaged from 12 separate samples of each type with 5 drops measured per sample. Reported rms roughness values are averaged from 6 different images of one type of each sample for the rough surfaces; measurements for the flat surfaces are calculated from 1 image of each surface type.

Substrate type	Substratum coverage (%)		Estimated biofilm thickness (μm)
	6 hours	12 hours	6 hours
Flat hydrophobic	4.5±0.3	19.2±4.9	12.8±1.7
Flat hydrophilic	3.5±0.4	12.6±5.2	11.7±1.5
Rough hydrophobic	5.5±0.5	25.2±10.0	15.4±2.6
Rough hydrophilic	4.9±0.5	25.1±3.7	11.3±1.4

Table 2. Quantitative analysis of biofilm formation: Percent substratum coverage is calculated from 20 randomly-selected fluorescence microscopy images of each substrate type/exposure time. Estimated biofilm thickness is calculated from 6 different confocal microscopy images of each substrate type. No data are included for estimated thickness of the 12-hour exposures, because the increase in total fluorescence from both bacteria and their associated EPS results in a much higher sensitivity to thresholding value used to determine the upper limit of the biofilm.

Figure 1: Tapping mode AFM images of the surfaces used in this study: (A) flat hydrophobic, (B) flat hydrophilic, (C) rough hydrophobic, and (D) rough hydrophilic. All images are $1\ \mu\text{m}^2$; the vertical scale is 10 nm.

Figure 2: Quantification of *P.aeruginosa* biofilm initiation and colonization via total fluorescence intensity. Fluorescence intensity of the resulting arabinose-induced eGFP expression after (A) 6 h and (B) 12 h of growth on the substrates indicated. Average values and error bars representing the standard deviation are based on four samples for each surface type. The results presented here are derived from one of three experiments with the same conditions and set of surfaces.

Figure 3. Representative biofilm density map after 6 hours of. Density maps were recreated using fluorescence intensity data (a.u.) acquired by a TECAN Infinite M1000 microplate reader. Fluorescence intensity data were from 44 reads in an 8x8 mm filled circle on a surface of interest.

Figure 4: Statistical analysis of low-resolution spatial distribution of fluorescence from 6-hour biofilms, presented as (A) histograms of the intensity distribution and (B) standard deviation of the intensity for each surface type. Note that the number of bins in (A) is constant across surface type; the bin size therefore varies, with narrower bins indicating smaller variability in intensity. The error bars in (B) represent 95% confidence intervals calculated using a BCa bootstrap.[42][156 Efron 1993;}}

Figure 5. Top-view of biofilm attached on (top to bottom) (A) flat hydrophobic (B) flat hydrophilic (C) rough hydrophobic (D) rough hydrophilic surface substratum at 6 hours of

growth. 20 images were acquired at x20 magnification using a fluorescence microscopy, with each area representing a dimension of 585 μm x 440 μm on the surface. Each group of images presented above consists of 15 images chosen randomly from the 20 available images for each surface type. All images in this figure were acquired from one experiment, e.g. each surface type was exposed to an aliquot from the same bacterial culture.

Figure 6: 3D projections of representative *P. aeruginosa* biofilm attached on Si-based substratum at 6 and 12 hours of growth. Image stacks of adhered biofilm were taken by confocal microscopy at x40 magnification with a step-size of 2 μm, and 3D projections of biofilm were obtained using LAS AF software.