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The Effects of Subcellular Nanograting Geometry on the Formation and Growth of Bacterial Biofilms

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Abstract— Biofilm formation by bacteria protects them against environmental stresses such as desiccation, shear forces and antimicrobial agents, making them much harder to remove and increasing their virulence and persistence in industrial water systems and biomedical equipment. One promising method of disrupting biofilm formation and growth is to employ passive surface structures to inhibit bacterial adhesion and aggregation. However, most studies thus far have mainly focused on the early stages of biofilm formation and it is unclear if the influence of surface topography in the early phase will propagate to later stages. Here, we attempt to address this with an investigation into the biofilm formation of *Pseudomonas aeruginosa* on 25 different nanograting geometries, with dimensions that were systematically varied from subcellular to cellular sizes. The biofilms were characterized from the exponential growth phase to the decline phase, in intervals of 24 H over 4 days, using confocal scanning laser microscopy. Comparing the maximum volume of biofilm formed on each surface over 96 H, it was found that approximately 1/3 of the nanograting geometries exhibited 72 ± 16 % lower biovolume density than a flat surface. Bacteria on these nanogratings were also observed to form 40 ± 11 % smaller microcolonies that were 17 ± 6 % less compact than that found on the control surface. The majority of these nanogratings had deep trenches (*i.e.* depth $\geq 70\%$ of the cell diameter). Furthermore, *P. aeruginosa* cells were observed to multiply at approximately twice the rate on almost all the nanogratings compared to flat surfaces, but these cell populations also began to decline 24 H earlier than those on a flat surface. Using available literature on *P. aeruginosa*, a qualitative model was put forth, attributing the results to increased cell motility, decreased exopolysaccharide formation and disrupted *psl* adhesin/ signal trails on nanogratings. These factors, together, led to the net effects of reduced attachment, increased scattering of cells and rapid decline of the biofilms on nanogratings. The insights derived from this study suggest that passive surface geometries can be designed and optimized to successfully control/ inhibit long-term biofilm formation and growth.

Bacteria, Biofilm, Aggregation, Subcellular, Nanostructures, Interference Lithography, Nanotrenches, Nanogratings, Nanochannels, Nanogrooves

I. INTRODUCTION

BIOFILMS, which consist of bacterial communities encased in a matrix of exopolysaccharides, exist in a state that is

distinct from planktonic bacterial cells, with altered growth rates, gene expression and protein production [1]. The biofilm architectures provide cells with access to nutrients through a complex network of channels, while protecting them from unfavorable environmental conditions such as desiccation, shear stress and antimicrobial agents [2], [3]. Since they confer antibiotic resistance to bacterial pathogens [1], [2], [4], therefore, biofilm formation on the surfaces of medical tools and implants is particularly concerning.

To counter the formation of microbial biofilms, numerous approaches have been proposed, including the development of therapies that disrupt the quorum sensing network [5] and/ or signaling pathways in bacterial cells [6]. Thus far, the majority of such proposed treatments have been focused on modifying the chemical environment of the bacteria (*e.g.* nutrient depletion, oxygen concentration etc.) [5], although recent studies suggest that it may be possible to induce similar biofilm resistance through the use of passive surface topographies as well [7]– [9].

For instance, polydimethylsiloxane (PDMS) surfaces decorated with micro-sized supracellular grooves have been shown to decrease the proliferation and microcolony formation of marine microbial communities and *Staphylococcus aureus*, by physically segregating bacterial aggregates using their microstructures [10], [11]. Similarly, bacterial adhesion and agglomeration have been observed to be drastically reduced on nanogratings with sizes that range from subcellular to cellular sizes [7], [12], [13]. A detailed examination of bacterial activities on these nanogratings suggest that the various dimensions of a surface structure (*e.g.* width, depth, period) play different roles in attracting, aggregating and retaining bacterial cells [7]. The design rules that were put forth based on these insights successfully predicted that an array of subcellular holes would greatly inhibit bacterial attachment [14], [15]. In contrast, subcellular nanospikes, which can be naturally found on cicada wings, do not prevent bacterial adhesion but instead, exhibit bactericidal properties by stretching or piercing bacterial cell membranes through capillary action [16]– [20].

While much progress has been made towards understanding bacterial interaction with different surface topographies, most

studies, to date, have only focused on the early stages of biofilm formation, particularly for geometries with cellular and subcellular dimensions. Because bacteria can build biofilms through clonal growth of attached cells [21], it is unclear if an initial reduction in bacterial adhesion to a surface will be sufficient to prevent long-term biofilm buildup.

The aim of the present study, therefore, is to examine the effects of nanostructures on the later stages of biofilm formation and development. 25 different nanogratings with systematically varied geometries were used in this study. Rod-shaped *P. aeruginosa* was cultivated on these surfaces up to 4 days (96 H) and the evolution of biofilm formation was examined by means of 3D confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Here, the primary focus is on characterizing the overall bacterial growth kinetics and microcolony morphologies on different nanograting geometries, although a qualitative model, describing the signaling and sensing pathways that could have led to the observed results, has been suggested as well. This model was assembled with considerable literature review and may be useful as a guide for future investigations into the molecular interactions in biofilms on nanostructures.

II. METHODS

A. Fabrication

Fig. 1. (a) Schematic diagram showing the procedure for the fabrication of Si nanogratings. (b) SEM images of nanogratings with different (i) mesa width, w , and (ii) depth, δ . Scale bar represents 1 μm .

The fabrication of silicon (Si) nanogratings had been reported previously (Fig. 1a) [7]. Briefly, 400 nm of Ultra-i-123 positive photoresist was first spin-coated onto the surface of P-type (100) Si wafers that had been cleaned with acetone and hydrofluoric acid (HF). Exposure to a laser interference lithography setup [22]–[25] (laser wavelength = 325 nm) and a subsequent development step, using Microposit MF CD-26 solution, generated photoresist nanogratings. 100nm of Al was deposited onto the samples using Edwards Auto 306 thermal evaporator, followed by a lift-off procedure that involved 10 min of ultrasonication in acetone. This dissolved the photoresist, leaving only an inverse pattern of Al nanogratings on the Si substrate. The samples were then subjected to CF_4

plasma etching at rf power of 300 W, flow rate of 10 sccm and pressure of 70 mTorr to remove Si that was not covered by the Al gratings. Lastly, the samples were dipped in HF for 60s to remove the Al nanogratings before being diced into $\sim 3 \text{ mm} \times 3 \text{ mm}$ samples.

The width of the mesa, w , was varied from 100 nm to 500 nm (Fig. 1bi) by adjusting the angle of exposure in the laser interference lithography setup. For each w , nanogratings with different depths, δ , from 50 nm to 650 nm were fabricated (Fig. 1bii) by varying the duration of plasma etching. The width of the trench, w_T , was kept constant at $\sim 300 \text{ nm}$. A total of 25 different nanograting geometries were employed for this study.

B. Bacteria Preparation and Cultivation

Pseudomonas aeruginosa PAO1, which had been fluorescently-tagged through the insertion of a mini-Tn7-enhanced green fluorescent protein (eGFP) – Gmr cassette [26], was used for the present study. *P. aeruginosa* cultures were grown overnight in Luria-Bertani (Miller) broth at 37°C while being shaken at 200 rpm, to obtain an optical density of 0.4 at wavelength of 600 nm (OD_{600}).

The nanograting samples were cleaned with 70% ethanol and exposed to UV light for at least a day in a biosafety cabinet before the experiments. A total of 156 samples were prepared and split into 3 sets, with each set consisting of duplicates of each nanograting geometry and a nano-smooth Si surface which acted as control *i.e.* 26 geometries (including control) $\times$ 2 (duplicates) $\times$ 3 sets = 156 samples. Each set of samples was placed flat in a different 96-well plate, one sample in each well. Every individual sample was then inoculated with 15 μl of bacterial solution and 300 μl of 10% LB (Miller) broth, before being left at ambient conditions (20°C, relative humidity = 30%). The medium in each well was removed and replaced with 300 μl of sterile LB (Miller) broth every 24 H. This minimizes the influence of planktonic cell division on the results, so that any growth observed on the substrates over time came mainly from the attached cells.

At the time intervals of 48 H, 72 H and 96 H, 1 set of samples was gently rinsed with fresh LB (Miller) broth before fixation was performed with 4% formaldehyde for at least 30 min. Previous studies have indicated that the GFP fluorescence of the bacteria is not affected by this concentration of formaldehyde [27], [28]. Following this, the samples were dehydrated through a graded series of ethanol (25%, 50%, 75%, 90% and 100%), with an immersion time of at least 10 min for each grade. The samples were then immersed in HMDS (hexamethyldisilazane) and air-dried to minimize surface tension damage during the drying process [29], [30].

Here, fixation was performed before confocal microscopy examination because live imaging was not possible – the design of the microscope and opaque nature of the silicon substrates required the substrates to be upside down for imaging. Using a transparent substrate would likely not have helped, as the dimensions of the nanogratings used were similar to the wavelength of the confocal laser, leading to diffraction of any transmitted laser light. Furthermore, because of the sheer

number of samples, if the cells were to be imaged live, the confocal images taken for the 1st sample and last (*i.e.* 52nd) sample can be easily 24 hours apart, even with continuous equipment usage. Rather than have the data for the different nanograting geometries spread out across a long duration, it was elected to have the samples fixed at given time intervals, so that the process of biofilm formation can be compared fairly across the different nanograting geometries.

Assays were not used in the current study for high throughput characterization of cell viability as they do not provide information on the morphology of the biofilms and reliable comparisons between nanogratings require very tight tolerances on the sample size, which was not easily achievable here, as the Si substrates were manually diced.

C. Characterization

3D Confocal Laser Scanning Microscopy (CLSM) images were obtained using Olympus Fluoview FV1000 at 20X magnification and an excitation wavelength of 473 nm. Settings for image capture were fixed across all samples. At least 2 images, with a scan area of 0.64 mm x 0.64 mm, were taken for each sample *i.e.* at least 4 images for each surface topography. The total number of confocal images taken for this study is, therefore, greater than 2 (no. of images) x 156 (no. of samples) = 312. Quantification of the biovolume, surface area and count (*i.e.* number of separate bodies) of viable cells in the 3D reconstructed microscopy images was performed using the Imaris software package (Bitplane, UK). Statistical analysis between data sets was conducted using two-tailed, paired Student's t-test and a result of $p < 0.05$ was taken to indicate statistical significance.

Scanning Electron Microscopy (SEM) was performed using FEI Inspect F50 at an accelerating voltage of 5 kV. Prior to SEM examination, the sample surface was coated with 40 nm of Al to increase the electrical conductivity of the surface which improved the imaging resolution.

III. RESULTS

A. Microscopy Images

Fig. 2 shows the CLSM images for the maximum bacterial density found in biofilms formed on the various surface topographies over the course of the study *i.e.* 96 H (see Supplementary Information for more images). Here, we have elected not to use propidium iodide to characterize the non-viable bacteria in the biofilm as it stains extracellular DNA in the biofilm as well, which could lead to significant over-counting of dead cells [31], [32]. Besides, it had already been demonstrated that GFP fluorescence deteriorates rapidly upon cell death and therefore, GFP fluorescence intensity has often been used as a measure of the concentration of viable cells in a biofilm [27], [33].

Fig. 2. Top view CLSM images of *P. aeruginosa* biofilms found on each surface topography. The images included here depict the maximum biofilm formed on each nanograting geometry over the course of 96 H. Images with red borders indicate that the biovolume on that nanograting geometry was statistically lower ($p < 0.05$) than that of the flat control surface. The white dotted box in “ $w = 500$ nm, $\delta = 650$ nm” image highlights the proliferation of bacterial cells in aligned rows. The schematic diagram illustrates the dimensions of w , w_T and δ for the nanogratings.

From Fig. 2, it can be observed that the orientation of the rod-shaped *P. aeruginosa* cells on the nanogratings was generally random, with only sporadic locations showing directional spreading of the bacterial cells (dotted white box for “ $w = 500$ nm, $\delta = 650$ nm” image in Fig. 2). These orderly lines of cells were formed by bacteria that were aligned to and entrenched in the nanogratings (indicated by white arrow in Fig. 3a). A previous study had determined that this entrenchment happened because physicochemical repulsion from the nanograting mesa directed approaching bacteria towards the gaps between the nanogratings, where capillary forces subsequently acted to entrench the cells [7]. It is also worth noting that the presence of part or whole of the bacterial cell in the trench indicates that the nanogratings were not trapping air at these spots (*i.e.* the nanograting surface was in a Wenzel [34], [35], not Cassie-Baxter state [36], [37]).

In addition, the maximal surface coverage of viable *P. aeruginosa* cells on about 2/3 of the nanograting geometries do not seem to be significantly different from that of the flat control surface. However, the bacterial cells on nanogratings generally appear more scattered (*i.e.* fluorescence appears more diffused) than those on the control surface, which concentrated together to form intense spots of fluorescence. SEM examination confirms that biofilms on nanogratings tend to be flatter and consist of dispersed bacterial cells (Fig. 3b-c), while those on the control surface were more agglomerated and had compact 3D architectures where cells bind tightly to one another (Fig. 3d-e) (see Supplementary Information for more images).

Fig. 3. (a) Top view SEM image of nanogratings ($w = 300$ nm, $\delta = 650$ nm) after 72 H of bacterial cell culture, showing *P. aeruginosa* cells that were aligned (white arrow) and non-aligned to the nanogratings. SEM images of a bacterial biofilm on nanogratings ($w = 300$ nm, $\delta = 350$ nm) after 72 H at (b) low and (c) high magnifications. SEM images of different bacterial biofilms on a flat, nano-smooth surface after 72 H at (d) low and (e) high magnifications.

B. Nanogratings can Reduce Bacterial Biovolume

Fig. 4. Biovolume per unit scan area found on the various surface topographies at different time intervals. Each data point represents the average obtained from at least 4 CLSM images. Error bars indicate standard deviation. * indicates that the maximal biovolume found on the nanograting geometry over 96 H was statistically lower than that found on the flat control surface (*i.e.* $p < 0.05$). The time interval that this statistically different biovolume was found is indicated next to *. See Supplementary Information for more details.

Fig. 4 shows the quantified biovolume per unit scan area for the different surface topographies over time. Each data point was obtained by averaging the biovolume density of 4 CLSM images. The biovolume in each CLSM image was quantified through the addition of the fluorescent voxel volumes, which was automatically processed by the Imaris software package. The results for 24 H was obtained from a previous study that

followed the same experimental procedure [7].

From Fig. 4, it can be observed that the biovolume on all surface geometries increased until 48 H – 72 H, after which they began to decline. Comparing the peak biovolumes observed on the nanogratings with that of the control surface, it was found that 28% of the nanograting geometries exhibited statistically lower biovolumes (indicated with * in the legend in Fig. 4), and the average biovolume density amongst these topographies was about 27.7 ± 15.6 % of that found on the control surface. These nanogratings generally had depths similar to cellular dimensions ($\delta \geq 350$ nm) but exhibited no clear trend with respect to the width of the mesas, $100 \text{ nm} \leq w \leq 500$ nm. These results indicate that during biofilm formation and maturation, *P. aeruginosa* bacterial cells do not differentiate between surface geometries with depths shallower than 70 % of the cell diameter, or separations smaller than ~ 33% of their cell lengths (assuming each *P. aeruginosa* cell was approximately 1.5 μm long [7]).

C. Nanogratings can Speed Up Bacterial Growth and Decline

Fig. 5. (a) Full plot and (b) magnified plot of the generation time, G , computed for the various nanograting geometries over the different time periods. See Supplementary Information for more details. No error bars are indicated as G was calculated based on the time-based trends in Figure 4 using Equation (1), and only 1 trend was available for each nanograting geometry.

By taking the gradients of the plots in Fig. 4 and using the equation for binary fission [38],

$$B_t = B_0 \cdot 2^{t/G} \quad (1)$$

where B_t refers to bacterial population (proportional to biovolume because of cell size homeostasis [39]) after t H of culturing and B_0 refers to the initial bacterial population, the

generation time, G , for *P. aeruginosa* was computed and plotted in Fig. 5. Negative values indicate that viable cell populations were declining.

From the plots, it can be observed that exponential cell growth, indicated by positive values of G , occurred between 24 H – 48 H for most of the nanogratings, while that for the flat control surface was extended up to 72 H (Fig. 5b). Furthermore, G for bacteria found on the nanogratings was generally about half that for the control surface within this period (highlighted by red dashed lines in Fig. 5b) *i.e.* cells were multiplying faster on nanogratings than flat surface. Bacterial populations for these nanogratings were also generally found to decline after 48 H, compared to 72 H on the control surface.

Fig. 6. (a) Average biovolume per disconnected body (*i.e.* per microcolony) (b) Average surface area to volume ratio of biofilm found on the various surface topographies at different time intervals. Each data point represents the average obtained from at least 4 CLSM images. Error bars indicate standard deviation. * indicates that the maximal biovolume found on the nanograting geometry over 96 H was statistically lower than that found on the flat control surface (*i.e.* $p < 0.05$). ^ indicates statistical significance between nanograting geometry and control surface for (a) maximum biovolume/count found over 96 H or (b) minimum surface area/ biovolume found over 96 H. The time interval that the maximum/ minimum values were found is indicated next to * or ^. See Supplementary Information for more details.

D. Nanogratings can Reduce Microcolony Formation in Biofilms

To examine the level of bacterial aggregation in the biofilms, 2 figures of merit were utilized – biovolume/count, which quantifies the average biovolume per disconnected body in a CLSM image (*i.e.* average no. of cells in each microcolony) (Fig. 6a) and surface area/ biovolume, which quantifies the average surface area to volume ratio of the biofilms (*i.e.* measures how tightly the cells adhere to each other) (Fig. 6b). A biofilm that consists of many bacterial cells strongly attached to each other in big colonies would exhibit high biovolume/ count and low surface area/ biovolume values.

It was found that 40 % of the nanograting geometries exhibited a statistically lower value ($p < 0.05$) than the control surface for peak biovolume/ count (the geometries are indicated with ^ in Fig. 6a). The average value for these nanogratings was 60.1 ± 10.8 % of that found on the control surface.

In addition, 32 % of the nanograting geometries exhibited a statistically higher value ($p < 0.05$) for surface area/ biovolume compared to the control (the geometries are indicated with ^ in Fig. 6b). The average value for these nanogratings was 120.2 ± 6.7 % of the value for the control surface. It can also be observed that, generally, most of the nanogratings that exhibited statistically lower biovolume density (* in Fig. 6) had smaller microcolonies (^ in Fig. 6a) and more dispersed cell populations (^ in Fig. 6b), indicating a strong correlation between the mechanisms behind these effects.

IV. DISCUSSION

Based on the results presented above and the literature data available for *P. aeruginosa*, we propose, in the following, one possible mechanism that may have led to reduced bacterial cell population and microcolony formation on some of the nanograting geometries.

For the flat control surface, daughter cells spawned from attached cells can stay on the surface (Fig. 7ai) or escape to join the planktonic population [40] (Fig. 7aii). For cells that remain on the surface, the ample friction between the cell body and surface causes an increase in intracellular cyclic-*di-GMP* (*c-di-GMP*) level [8] which transforms the daughter cells from a motile planktonic state to a sessile state, initiating biofilm formation through the production of adhesins and exopolysaccharides [41]– [43] (Fig. 7ai). At sufficient cell densities and autoinducer concentrations, quorum-sensing-regulated genes were expressed through the assistance of the *las/rhl* quorum-sensing system to produce thick, mature biofilm architectures with intricate channel networks for nutrient passage [3], [44]. Daughter cells that became planktonic may re-join these aggregates or settle down elsewhere on the surface to form their own aggregates, laying down *psl* trails that served to guide other planktonic cells to their location for biofilm formation [40] (Fig. 7aiii).

Similarly, daughter cells produced on the nanogratings may escape the surface (Fig. 7bi) or stay on it. For daughter cells divided from entrenched bacteria, strong attractive capillary forces from the sidewalls and base of the trench makes the latter choice more likely (Fig. 7bii). However, the rough sidewalls may have discouraged the rate of cell division or reduced the viability of daughter cells through membrane damage, as no more than 3 generations of entrenched bacteria were observed in the SEM images.

For daughter cells divided from bacteria lying across the gratings, the gaps in the nanogratings reduce the friction sensed by the cells, leading to reduced *c-di-GMP* levels and a lack of motivation for the cells to attach across the nanogratings. However, the sidewalls and base of the trenches provide copious surface area for Type IV pili attachment, and through the tension and retraction of these pili, the *Chp* chemosensory system can be activated to produce *cAMP*, a cyclic nucleotide

secondary messenger, which, in turn, leads to more Type IV pili activity. A positive feedback loop between twitching motility and *cAMP* production is thus formed [9] (Fig. 7biii), causing the daughter cells to become highly mobile across the surface of the nanogratings, so that flatter and more dispersed biofilms are formed. This is consistent with previous results reported by Klausen *et. al.*, which showed that cells with higher twitching motility led to flatter biofilms [21].

Fig. 7. Schematic diagrams illustrating the proposed sequence of events leading to biofilm formation on (a) flat surfaces (b) nanograting surfaces. Note that *P. aeruginosa* also produces other exopolysaccharides such as *pel* [8] and alginate [3] (not illustrated here), other than *psl*.

Because of the gaps in the nanogratings, any *psl* trails laid by exploring cells will be disrupted [7] and cannot form a positive feedback loop to increase *c-di-GMP* levels for biofilm formation [45], [46] (Fig. 7biv). In addition, the gaps may also cause the bacterial cells to prefer to lie down flat and maximize

adhesive contact with the surface [47], rather than attach to the surface at an inclined angle, which would have been necessary to build 3D biofilms. The planktonic daughter cells will also have difficulty re-joining the surface, as any *psl* trails laid by prior bacteria would be disrupted by the nanograting topography, preventing any effective guidance to attached bacterial cells [40] (Fig. 7bv). These factors, together, reduces cell attachment and microcolony formation on nanogratings.

Because the biofilms formed by the bacteria on the nanogratings are so flat and dispersed, nutrient access will not be hindered and therefore, the cells can multiply faster than their flat surface counterparts [48], which may explain the shorter generation time observed (Fig. 7bvi). Compared to the flat surface, however, the intracellular *c-di-GMP* levels for bacteria attached across the nanogratings are likely lower, due to a lack of shear sensing [8]. This is known to induce Pseudomonas Quinolone Signal (*PQS*) molecule formation, which, in turn, promotes cell autolysis [49]– [51] (Fig. 7biv). This may explain the earlier decline of viable cell populations found on nanogratings. Table 1 sums up the overall effects of nanogratings on *P. aeruginosa* biofilm formation.

It is worth noting that the interconnection and complexity of various signaling and sensing pathways in *P. aeruginosa* means that the proposed model above is only one possible explanation for the bacterial behavior observed. There may be other processes taking place simultaneously that enforce or reduce the effects of the mechanism proposed in this model. For instance, we note that Ma *et. al.* had previously identified a putative holin - anti-holin system in *P. aeruginosa*, *cid/lrg*, and demonstrated that an imbalance between the holin and anti-holin production leads to over-multiplication of cells and early cell death [31], which is similar to our observations here. Therefore, nanogratings may also regulate the production of *cidA* and *lrgA* proteins in a way that has not been identified yet.

Furthermore, we note that approximately 2/3 of the nanograting geometries investigated here did not show changes

TABLE I
SUMMARY OF THE EFFECTS OF NANOGRATINGS ON *P. AERUGINOSA* BIOFILM FORMATION

Nanograting Characteristic	Cells Affected	Behavior Observed	Reason	Secondary Effect	Reason
Reduced horizontal area	Unaligned cells	Reduced surface attachment	Insufficient shear force to induce increase in <i>c-di-GMP</i> levels for production of adhesins and exopolysaccharides [8].	Early autolysis.	Low <i>c-di-GMP</i> level leads to overproduction of <i>PQS</i> , resulting in autolysis [49]–[51].
Uneven surface topography	All cells		<i>psl</i> trails are disrupted on surface, unable to form positive feedback loop between <i>psl</i> production (an adhesin) and <i>c-di-GMP</i> levels [45].	Less biofilm matrix formation.	Low <i>c-di-GMP</i> level discourages exopolysaccharide production [8], [45].
Increased surface area	Unaligned cells	Reduced aggregation	<i>psl</i> trails are disrupted on surface, unable to guide planktonic cells to attached cells on surface [40].	Higher cell division rate.	Dispersed cells have better access to nutrients and multiplies faster [48].
			Mechanosensing of Type IV pili increases <i>cAMP</i> production and forms positive feedback loop, increasing motility of cells [9].	Less biofilm matrix formation.	Dispersed cells unable to concentrate autoinducers to induce biofilm matrix formation [3], [52].
	Aligned cells	Physical separation by gratings			
		Reduced growth rate	Rough sidewalls may slow down growth or cause membrane lysis.	-	-

in biovolume and/ or aggregation levels that were statistically different from those found on the control surface. Most of these geometries had shallow gratings ($\delta = 50$ nm and $\delta = 200$ nm), which suggests that the influence of nanogratings on bacterial behavior detailed in Fig. 7 and Table 1 is only observable beyond a threshold depth.

Similarly, nanograting geometries with $\delta = 500$ nm did not display significant changes to the biovolume density and microcolony formation with respect to the control as well. Changing the criteria for statistical significance from $p \leq 0.05$ to $p \leq 0.1$ did not change these results, suggesting that the factors for this anomaly were systematic. Therefore, there may be competing mechanisms that allow the formation of thick biofilms on nanogratings with $\delta = 500$ nm, rather than the flat, dispersed ones observed on most of the $\delta = 350$ nm and $\delta = 650$ nm geometries. Further studies, possibly using the proposed model in Fig. 7 as an initial guide, would be required to identify the actual mechanisms.

Regardless of the specific mechanisms, however, it is clear that surface nanostructures have the potential to disrupt *P. aeruginosa* biofilm formation. The biofilms formed on nanogratings exhibited a flat and highly dispersed nature, and cell growth and death were accelerated. By reducing the duration that these bacteria spend in a thick, compact biofilm, surface topography may help lower the antibiotic resistance of bacterial pathogens.

V. CONCLUSIONS

Pseudomonas aeruginosa biofilm formation on nanogratings with dimensions systematically varied from cellular to subcellular sizes was investigated over 96 H by means of confocal scanning laser microscopy and scanning electron microscopy. The results indicate that, generally, peak biovolume density was reduced and bacterial cells were more dispersed and isolated on nanogratings with depths greater than or equal to 350 nm. The growth and decline of bacterial cells were also accelerated on nanogratings when compared to flat control surfaces.

Using literature data, a model was proposed to show that these results possibly came about because nanogratings reduced the amount of area available for shear sensing by the bacterial cells but increased the surface area available for Type IV pili attachment. In addition, adhesins that would have promoted thick biofilm formation was disrupted on the corrugated surface of the nanogratings. These led to increased motility of the cells over the nanograting surfaces and reduced surface attachment, resulting in flatter and more dispersed biofilms. The scattering of cells across the nanograting surface allowed for better nutrient access and promoted cell multiplication, but the lack of surface attachment may have induced early autolysis, resulting in a steep decline of viable cell populations at an earlier time point than the flat control surface. These results suggest that specific surface geometries can potentially be used to control biofilm formation and growth, so as to increase bacterial susceptibility to antimicrobial agents such as antibiotics.

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