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Bacterial Attachment, Aggregation and Alignment on Subcellular Nanogratings

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ABSTRACT

Recent investigations on the interactions of bacteria with micro-/nano- structures have revealed a wide range of prokaryotic responses that were previously unknown. Despite these advances, however, it remains unclear how collective bacterial behavior on a surface would be influenced by the presence of anisotropic nanostructures with subcellular dimensions. To clarify this, the attachment, aggregation and alignment of *Pseudomonas aeruginosa* on orderly subcellular nanogratings with systematically varied geometries were investigated. Compared to a flat surface, attachment and aggregation of bacteria on the nanogratings were reduced by up to 83% and 84% respectively, while alignment increased by a maximum of 850%. Using a semi-empirical quantitative model, these results were shown to be caused by a lowering of physicochemical attraction between the substrate and bacteria, possible disruption to cell communication, and physical isolation of bacteria that were entrenched in the nanogratings by capillary action. Furthermore, bacterial attachment level was generally found to be exponentially related to the contact area between the substrate and bacterial cells, except when there were significant deficits in the available contact area, which prompted the bacterial cells to employ their appendages to maintain a minimum attachment rate. Since the contact area for adhesion is strongly dependent on the geometry of the surface features and orientation of the bacterial cells, these results indicate that the conventional practice of using roughness parameters to draw quantitative relationships between surface topographies and bacterial attachment could suffer from inaccuracies, due to the lack of shape and orientation information provided by these parameters. Based on these insights, design principles for generating maximal and minimal bacterial attachment on a surface were also proposed and verified with results reported in the literature.

Introduction

The adhesion and aggregation of bacteria on surfaces is a ubiquitous phenomenon that has many important implications for medical and engineering applications. In certain cases, such as those involving medical implants and instruments, it has to be actively avoided as the subsequent buildup of a protective polysaccharide matrix and biofilm formation can render antibiotic treatments useless against bacterial pathogens¹. On the other hand, the efficiency of microbial fuel cells depends directly on the ability of bacterial cells to proliferate and establish tight physical contact with the electrodes through surface attachment^{2,3}.

For these reasons, much effort has been directed towards understanding and developing surfaces that can influence bacterial adhesion and aggregation. In recent years, nanostructured topographies have generated considerable interest due to the advance in nanofabrication and characterization tools⁴, which have allowed researchers to manipulate individual bacterial cells and bring about a remarkable range of bacterial behavior that were not known before.

For instance, by varying the gap between nanoposts such that it becomes commensurate with the physical dimensions of the rod-shaped *E. coli* bacteria, it was shown that the arrangement and orientation of such bacterial cells can be controlled in both in-plane and out-of-plane directions^{5,6}. As the nanoposts are brought closer together, to slightly below cellular dimensions, bacterial cells can be observed to extend their appendages and take hold of the sides of the nanoposts to facilitate surface attachment⁷. Decreasing the inter-post spacings even further, to approximately an order of magnitude below the size of bacterial cells, cell death can be induced when bacteria settle onto the nanoposts⁸⁻¹¹. This can be caused either by the direct penetration and rupture of the cell membranes by the nanoposts¹⁰, or by capillary action along the sides of the nanoposts, which forces the membranes to bend and stretch so much that they break down mechanically^{12,13}.

Thus far, prior investigations have focused only on isotropic nanostructures^{5,9,14} (*e.g.* nanoposts/ nanoholes) and anisotropic microgratings¹⁵. The effect of anisotropic nanostructures with subcellular dimensions on bacterial attachment and aggregation, on the other hand, remains unclear, and hinders the development of an overarching understanding of bacterial-nanostructure interactions. The objective of the present study, therefore, is to investigate the levels of bacteria alignment, attachment and aggregation on subcellular nanogratings with systematic variations in dimensions. This was done by inoculating the nanogratings with the gram-negative bacteria, *Pseudomonas aeruginosa*, for 24 hours and characterizing them using Scanning Electron Microscopy (SEM) and epifluorescence microscopy. Analysis of the results was then carried out with a semi-empirical quantitative model that separated the effects of physicochemical surface interactions and biological responses.

Methods

Fabrication

Figure 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 . (c) Example of the green bar overlay that was used to indicate the length and orientation of a bacterium. The orientation of the bacteria was taken with reference to the nanograting, which was assigned a value of $\theta = 0^\circ$. Each colour on the axis legend

represents 1 unique axis with a range of 10° . Note that $85^\circ \leq \theta \leq 95^\circ$ lies in the same axis as $-85^\circ \leq \theta \leq -95^\circ$. Scale bar represents 1 μm .

The procedure for the fabrication of Si nanogratings in this study is similar to that previously reported for the synthesis of nanostructures on silicon (Si) and polymeric substrates¹⁶⁻¹⁹. Briefly, P-type (100) Si wafers were first cleaned with acetone and dipped in HF for 60 s to remove the native oxide. 400 nm of Ultra-i-123 positive photoresist was then spin coated onto the surface and baked at 110°C for 90 s. The wafers were diced into 1 cm x 1 cm samples and exposed to interference lithography using a He-Cd laser with a wavelength of 365 nm. After a post-exposure bake at 110°C for 90 s, the photoresist was developed in Microposit MF CD-26 for 45 s to obtain photoresist nanogratings (Fig. 1ai). 100 nm of Al was then deposited onto the samples at a pressure of 10^{-5} Torr using Edwards Auto 306 thermal evaporator (Fig. 1a ii). Following this, ultrasonication in acetone was carried out on the samples for 10 min to remove the photoresist nanogratings and Al film on top of them, leaving behind an inverse pattern of Al nanogratings (Fig. 1a iii). The samples were then subjected to CF₄ plasma etching at rf power of 300 W, flow rate of 10 sccm and pressure of 70 mTorr in order to etch nanogratings into the Si substrate (Fig. 1a iv). Finally, the samples were dipped in HF for 60s to remove the Al nanogratings before being diced into 4 mm x 4 mm samples (Fig. 1a v).

The width of the mesa, w , was varied from 100 nm to 500 nm in steps of 100 nm (Fig. 1bi) by adjusting the angle of exposure during the interference lithography step. For each w , nanogratings with depth, δ , varying from 50 nm to 650 nm were fabricated in steps of 150 nm (Fig. 1b ii) by controlling the duration of plasma etching. The width of the trench, w_T , was kept constant at 280 ± 90 nm. A total of 25 different nanograting geometries were employed for this study (Supplementary Information).

Bacteria Strains and Preparation

Pseudomonas aeruginosa PAO1 was used as the wild-type strain in the present study. The *pilA* (incapacitated Type IV pili), *filM* (incapacitated flagellum) and *pilA filM* (incapacitated Type IV pili and flagellum) mutants were constructed by allelic displacement previously described by Kaulsen *et. al.*^{20,21}. All strains were fluorescently-tagged through the insertion of a mini-Tn7-enhanced green fluorescent protein (eGFP) – Gm^r cassette²². *P. aeruginosa* cultures were grown overnight in Luria-Bertani (Miller) broth at 37°C while being shaken at 200 rpm, so that an optical density at 600 nm (OD₆₀₀) of 0.4 was obtained.

Bioadhesion Experiments

The nanograting samples were cleaned thoroughly with 70% ethanol and exposed to UV light in a biosafety cabinet at least a day before the experiments. Duplicates were used for each nanograting geometry, as well as the control, which was a nano-smooth Si surface. Each sample was placed at the base of an individual well in a 96-well plate before being inoculated with 15 µl of bacterial solution and 300 µl of 10% LB (Miller) broth. They were then left at ambient conditions (20°C, relative humidity = 30%).

After 24 hours, each sample was gently rinsed with fresh LB (Miller) broth and the attached bacteria were fixed in 10% formalin for at least 30 min. 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^{23,24}. For viability characterization, the bacterial cells were subjected to propidium iodide (1.5 x 10⁻³ %) staining for 20 min with no fixing.

Characterization

Epifluorescence microscopy images were obtained using Olympus BX-53 upright microscope, while Scanning Electron Microscopy (SEM) was performed on the fixed bacteria 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 at a pressure of 10^{-4} Torr using Edwards Auto 306 thermal evaporator. For quantification of attached cell density, the images were captured at 5000 X (imaging area of $60\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$), except for the nanogratings with $w = 400\text{ nm}$, $\delta = 650\text{ nm}$ and $w = 500\text{ nm}$, $\delta = 650\text{ nm}$, as the deeply entrenched bacteria required a magnification of 10 000 X (imaging area of $30\text{ }\mu\text{m} \times 25\text{ }\mu\text{m}$) to be observed clearly.

To characterize the bacterial cells captured in the SEM images, a custom MATLAB code was used to register the length and orientation of each rod-shaped *P. aeruginosa* bacterium with respect to the nanogratings when the user clicked on its ends (Fig. 1c). A green bar overlay was then used to mark the bacterium to prevent repeated counting of the same cell (Fig. 1c). All possible orientations that a bacterium can lie in were divided into 17 unique axes, each having a range of 10° . This is visually presented in the axis legend in Fig. 1c, where each color represents an axis. In this study, bacterial cells were considered aligned to the nanogratings ($\theta = 0^\circ$) if they had an orientation of $-5^\circ \leq \theta \leq 5^\circ$. For the control surface which had no nanogratings, the aligned axis, $\theta = 0^\circ$, was arbitrarily chosen.

After all the cells in the SEM image had been marked, the total number of bacteria in that SEM image was tallied and divided by the area to obtain the cell densities. Bacterial cells were taken to be in an aggregate if 3 or more cells were in physical contact with each other. At least 5 SEM images were processed for each nanograting geometry and the information from a total of approximately 30,000 bacterial cells were obtained for this study. 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.

Results

Figure 2: SEM images of (a) an aligned cell on nanograting with $\delta = 350$ nm (b) an aligned cell on nanograting with $\delta = 500$ nm (c) non-aligned cells and (d) aggregated cells. Scale bars represent 1 μ m. (e) Epifluorescence microscopy images using GFP and DAPI filters. Green indicates viability while red indicates non-viability. White arrows point to examples of aligned viable cells. Scale bars represent 10 μ m. (f) SEM images of aligned cells for wild type and the different mutant strains on nanogratings with $\delta = 200$ nm (top) and $\delta = 500$ nm (bottom). Scale bars represent 1 μ m.

P. aeruginosa cells attached on the nanogratings could be aligned (Fig. 2a and 2b) or non-aligned (Fig. 2c) with respect to the nanogratings, and isolated (Fig. 2a and 2b) or aggregated (Fig. 2c and Fig. 2d). Aligned cells were mostly found entrenched in the nanogratings, rather than on the mesas. Furthermore, the extent of entrenchment was limited only by the depth of the nanogratings, δ , *i.e.* the entrenched bacteria always descend to the bottom of the trenches. Non-aligned cells, on the other hand, were observed to lie across the nanogratings, contacting only the top of the mesas. Aggregated cells were, in general, made up of both aligned and non-aligned cells.

The embedding of aligned bacterial cells in the nanogratings is particularly remarkable, considering that the width of each nanotrench ($\approx 280 \pm 90$ nm) was only about half the cross-sectional diameter ($\approx 540 \pm 150$ nm) of the rod-shaped *P. aeruginosa* cells. In spite of this, it was found that the entrenched bacteria remained viable throughout the duration of the experiments (Fig. 2e).

To ascertain the driving force behind this entrenchment, the experiments were repeated using mutant strains with varying degrees of motility. As can be seen from Fig. 2f, the entrenchment of *P. aeruginosa* bacteria did not require the use of either the flagellum or Type IV pili, suggesting that the bacterial cells were not actively lodging themselves into the nanogratings, but rather, they were embedded through the passive intermolecular forces of attraction between the bacterial membrane and the sides of the nanogratings. This capillary action was also previously proposed to be behind the bactericidal activity of cicada wings, which work by drawing attached bacterial cells into a bed of nanospikes to rupture their cell membranes^{11,12}.

Figure 3: Plot of (a) total cell density (b) aggregation fraction (c) alignment fraction (d) (i) density of cells that were aligned and isolated (ii) density of cells in aggregates that were aligned (iii) density of cells that were non-aligned and isolated and (iv) density of cells in aggregates that were non-aligned. Note that aggregates were generally made up of both aligned and non-aligned cells. The cell densities were plotted against the depth of the nanotrenches, δ , for different mesa widths, w . The data points for nanogratings with the same w were joined with linear lines to enhance the clarity of the trends.

Fig. 3a shows the total density of bacterial cells found on the different nanograting geometries, as well as on the flat control surfaces ($\delta = 0$ nm). It can be observed that bacterial attachment on the nanogratings ($\delta > 0$ nm) was 29 % - 83 % ($p < 0.05$) lower than that for a flat surface, except in 4 cases — $w = 400$ nm, $\delta = 650$ nm (attachment = - 7 %, $p > 0.05$), $w = 500$ nm, $\delta = 350$ nm (attachment = + 0.3 %, $p > 0.05$), $w = 500$ nm, $\delta = 500$ nm (attachment = - 13 %, $p > 0.05$) and $w = 500$ nm, $\delta = 650$ nm (attachment = + 68 %, $p < 0.05$).

Similarly, the aggregation fraction (ratio of aggregated cells to total cells), shown in Fig. 3b, decreased in the presence of nanogratings, with 88% of the nanograting geometries exhibiting values that were significantly lower ($p < 0.05$) than that of the control surface. For $\delta = 50 - 350$ nm, the aggregation fraction fell and remained approximately constant at an average of $73\% \pm 8\%$ of the control value. As δ increased to 650 nm, the aggregation fraction decreased further to a minimum of 15.3%. In contrast to its dependence on δ , however, the aggregation fraction did not vary significantly with changes in w .

On the other hand, the alignment fraction (ratio of aligned cells to total cells), shown in Fig. 3c, was observed to increase steadily with δ , up to a maximum of 8.5 times that found on the flat control surface. The improvement in alignment fraction on the nanogratings over the control surface was statistically significant for all of the nanograting geometries ($p < 0.05$).

For the control surface, which had no nanogratings, the probability of the bacteria landing on any one of the 17 axes, including the one arbitrarily chosen to be $\theta = 0^\circ$, was $1/17$ ($= 0.059$). The good agreement between this expected value and the alignment fraction measured for the control surface, 0.063 ± 0.013 , implies that our method of quantifying the cell orientation and density is reliable. Further support for this can be found in the histogram analysis included in the Supplementary Information.

To investigate the reasons behind the trends observed in Fig. 3a - 3c, the cell densities for isolated aligned (Fig. 3d(i)), aggregated aligned (Fig. 3d(ii)), isolated non-aligned (Fig. 3d(iii)) and aggregated non-aligned (Fig. 3d(iv)) cells were plotted. Note that the summation of cell densities in Fig. 3d(i) – 3d(iv) would give the total attached cell density shown in Fig. 3a.

The plots in Fig. 3d indicate that the isolated aligned (Fig. 3d(i)), aggregated aligned (Fig. 3d(ii)) and isolated non-aligned cell densities (Fig. 3d(iii)) found on the nanograting geometries were generally similar to that found on the control surface ($p > 0.05$). There is, however, a substantial reduction (52 % - 92 %, $p < 0.05$) of aggregated non-aligned cells (Fig. 3d(iv)), which was the main cause behind the decrease in bacterial attachment levels and aggregation fraction, as well as the increase in alignment fraction on the nanogratings.

For the 4 outlier geometries that displayed bacterial attachment levels close to that of the control surface, however, it can be observed that the rise in aggregation and alignment fraction for these surfaces was mainly due to a disproportionate increase in attached isolated aligned cells (1700% - 11200%) (Fig. 3d(i)) and aggregated aligned cells (25% - 430%) (Fig. 3d(ii)). Therefore, the increase in aligned cell attachment on these 4 nanograting geometries had the same effect as a decrease in aggregated non-aligned cell attachment for the other nanograting geometries.

Discussion

Quantitative Model

To derive further insights from the results with regards to the various factors influencing the alignment, adhesion and aggregation of *P. aeruginosa* on nanogratings, a semi-empirical quantitative analysis that describes the surface attachment of the bacteria in distinct stages was employed (Fig. 4). In the first stage, the bacterial cells are drawn close to the substrate by physicochemical forces derived from van der Waals' (VDW), acid-base (AB) and electrostatic (EL) interactions between the bacteria and substrate. According to the extended DLVO (XDLVO) model, which had previously been shown to give good estimates of attached cell densities^{14,25–28}, the density of bacterial cells initially attracted to the surface, ρ' , is exponentially related to the physicochemical interaction energy²⁸, and can be expressed quantitatively as

$$\rho' = NA \exp(-B\Phi) \quad (1)$$

where

$$\Phi = \frac{w}{w + w_T} \phi_{M,min} + \left(1 - \frac{w}{w + w_T}\right) \phi_{T,min} \quad (2)$$

A and B are constants and N refers the the number of orientations the cells can have. For the present study, the full spectrum of possible orientations was grouped into 17 axes (*i.e.* $N = 17$), with each axis covering a 10° range. $\phi_{M,min}$ and $\phi_{T,min}$ refer to the interaction potential at the secondary minimum for the mesa and trench respectively²⁸ (Supplementary Information). Φ , the net surface interaction energy, is obtained through the weighted average of $\phi_{M,min}$ and $\phi_{T,min}$ in Eq. (2). Following convention, a negative value for Φ is used to indicate an attractive potential²⁸. Since $w/(w + w_T) < 1$ and $\phi_{M,min} > \phi_{T,min}$, it can be deduced from Eq. (2) that the surface attraction of bacterial cells is always reduced on nanogratings compared to a flat surface. Consequently, the density of bacteria drawn to nanogratings is lower.

The attracted bacterial cells have an equal probability ($= 1/N$) of lying in any one axis, and therefore, the likelihood of a cell aligning itself with the nanogratings is $1/N$. Out of these aligned cells, only a fraction, $\Gamma^{//}$, will eventually attach (*i.e.* make contact) and be retained on the nanogratings (Fig. 4). Similarly, the probability of the attracted bacteria being misaligned with respect to the nanogratings is $1 - 1/N$, and the fraction of non-aligned bacterial cells that will eventually attach irreversibly to the nanogratings is Γ^+ (Fig. 4). Furthermore, the proportion of bacterial cells that is able to form aggregates before attachment is f and F for aligned and non-aligned cells respectively. Therefore, the various attached cell densities can be given as

$$\text{Isolated Aligned:} \quad \rho_{iso}^{//} = \Gamma^{//} (1 - f) A \exp(-B\phi) \quad (3)$$

$$\text{Aggregated Aligned:} \quad \rho_{agg}^{//} = \Gamma^{//} f A \exp(-B\phi) \quad (4)$$

$$\text{Isolated Non-Aligned:} \quad \rho_{iso}^+ = (N - 1)(1 - F) \Gamma^+ A \exp(-B\phi) \quad (5)$$

$$\text{Aggregated Non-Aligned:} \quad \rho_{agg}^+ = (N - 1) F \Gamma^+ A \exp(-B\phi) \quad (6)$$

$$\text{Total Attached Cell Density:} \quad \rho = \rho_{iso}^{//} + \rho_{agg}^{//} + \rho_{iso}^+ + \rho_{agg}^+ = \rho^{//} + \rho^+ = [\Gamma^{//} + (N - 1) \Gamma^+] A \exp(-B\phi) \quad (7)$$

where the superscripts, $//$ and $+$, and the subscripts, *iso* and *agg*, represent aligned, non-aligned, isolated and aggregated respectively. Eq. (1) – (7) are also applicable to the control surface without nanogratings (*i.e.* $w_T = 0$), but since there is no geometrical difference between the arbitrarily chosen “aligned” axis and the “non-aligned” axes on the flat surface, its attachment fraction, Γ_c , is the same for the “aligned” and “non-aligned” cells on the control surface. Note that the current model does not take into account bacterial reproduction after attachment, as Zhao *et. al.* had previously shown that there is a 90 % chance that one or both of the parent and daughter bacterial cells would leave the surface after cell division²⁹.

Figure 4: Schematic diagram illustrating the attraction, attachment, alignment and aggregation of bacteria on nanogratings.

Bacterial Attachment

To verify the quantitative model, the total attached cell density, ρ , was plotted against the interaction energy, Φ , in Fig. 5a (see Supplementary Information for calculations of Φ). It can be observed that ρ exhibits an exponential dependence on Φ for most of the nanograting geometries, as expected from Eq. (7). Evaluating the slopes of the trendlines for $\delta = 200 - 650$ nm in Fig. 5a yields $B = 2.19 \pm 0.69 \times 10^{19}$ /J, which agrees well with the value of $B = 1.9 \times 10^{19}$ /J obtained in prior reports²⁸. The only exception to this result was found on nanogratings with $\delta = 50$ nm, for which ρ remained relatively large and independent of Φ , similar to the control surface. This could be because the nanotrenches were too shallow ($\sim 10\%$ of bacterial cell diameter), and thus, the bacterial cells were not as sensitive to the decrease in surface attraction for these nanogratings.

Going further, the normalized attachment fractions, I/I_c , for aligned (/) and non-aligned (+) cells were computed and plotted against χ in Fig. 5b and 5c respectively, where χ represents the normalized contact area between the bacterial cells and surface. Since nanotrenches provide additional surface for attachment through its side walls, and non-aligned cells contact only the mesas of the nanogratings, χ can be expressed as

$$\text{Aligned Cells:} \quad \chi = 1 + \frac{2\delta}{w_T} \quad (8)$$

$$\text{Non-Aligned Cells:} \quad \chi = \frac{w}{w + w_T} \quad (9)$$

From Fig. 5b, it can be observed that I'/I_c is approximately an order of magnitude greater than I_c , and generally exhibits an exponential relationship with χ , especially for $w = 400$ nm and $w = 500$ nm. This result is expected, as an increasing trench depth (*i.e.* higher χ) offered more

area for capillary force to act on the bacterial cells, which reduced the likelihood of voluntary²⁹ and involuntary (*e.g.* shear-induced³⁰) detachment of attached cells (Fig. 5d).

However, when χ is reduced below 1 (*i.e.* less area than a flat surface), which was the case for non-aligned cells attached across the nanogratings, it was found that the attachment fraction, I^+ , remained approximately constant at the value of I_c (*i.e.* $I^+/I_c \sim 1$) for all the nanograting geometries (Fig. 5c). A closer inspection of the SEM images suggests that this is most likely because non-aligned cells were using cellular appendages to facilitate attachment (Fig. 5e). Furthermore, it can be observed from Fig. 5e that as w decreased (*i.e.* decreasing χ), the bacterial cells extended longer appendages to mitigate the increasing lack of surface area for adhesion. In contrast, bacteria that were attached to a flat surface ($\chi = 1$) and entrenched in the nanogratings ($\chi \geq 1$) did not exhibit any cellular appendages other than the flagellum. These observations are consistent with previous reports of bacteria employing more appendages and extending them further when the surface porosity increases (*i.e.* χ decreases)³¹.

The above results suggest that the *P. aeruginosa* bacterial cells were actively sensing and responding to mechanical cues in the environment, most likely through their mechanosensitive Type IV pili³², in order to maintain a constant rate of successful attachment. Despite the efforts of the non-aligned bacteria, however, I^+ values remain an order of magnitude smaller than I'' values, indicating that capillary forces are much more effective at retaining bacterial cells on a surface than extensive appendage usage.

Figure 5: (a) Plot of total attached cell density, ρ , against the net surface interaction energy, ϕ , for the different nanograting geometries. Dotted lines indicate best fit trendlines. Note that the outlier data point for ($w = 100$ nm, $\delta = 200$ nm), highlighted with a black

background, was not included in the trend fitting. (b) Plot of normalized attachment fraction, Γ'/Γ_c vs. normalized contact area, χ , for aligned bacteria. (c) Plot of attachment fraction, Γ^+/Γ_c , vs. normalized contact area, χ , for non-aligned bacteria. Since the attachment fraction for the control surface, Γ_c , is constant in (b) and (c), any trend observed in the plots originate from variations of Γ' and Γ^+ with χ . (d) Representative SEM images of aligned bacterial cells being entrenched in nanogratings with different δ . Scale bar represents 1 μm . (e) Representative SEM images of non-aligned cells showing the extended cellular appendages (white arrows) used in surface attachment. Scale bar represents 1 μm .

Bacterial Aggregation

The aggregation fractions of aligned and non-aligned cells, f and F , were empirically determined through the ratios, $\rho''_{agg}/(\rho''_{iso} + \rho''_{agg})$ and $\rho^+_{agg}/(\rho^+_{iso} + \rho^+_{agg})$, respectively. From Fig. 6a and 6b, it can be seen that f and F exhibit the same general trend as the overall aggregation fraction shown in Fig. 3b, where the aggregation fractions fell and remained constant for $\delta = 50 - 350$ nm, before steadily declining further for $\delta = 350$ nm – 650 nm.

To account for the fall in f and F for $\delta = 50$ nm - 350 nm, we note from previous studies that *P. aeruginosa* cells produce trails of *psl*, an exopolysaccharide, as they move across a surface before irreversibly attaching to it²⁹. These *psl* trails allow cells that approach the surface later to locate attached cells and form micro-colonies with them²⁹. On a flat surface, these *psl* trails would be well-laid and aggregation can be achieved relatively easily. However, for nanogratings, the *psl* trails would be formed over an uneven surface, causing them to be disrupted and rendering them less effective in guiding planktonic cells to attached cells.

In addition, for nanogratings with $\delta \geq 500$ nm, capillary entrenchment would also have completely concealed the aligned bacterial cells and physically isolated them (Fig. 6c). This prevented other cells from establishing contact with the entrenched bacteria easily (Fig. 6c), and as a result, the aggregation fractions fell rapidly for $\delta = 350 - 650$ nm.

Figure 6: Plot of aggregation fraction for (a) aligned cells and (b) non-aligned cells for the different nanograting geometries. Data points for the same w are given the same color. (c) Top view SEM images, accompanied by side view schematic diagrams, illustrating the increasing difficulty for non-aligned cells to aggregate with aligned cells as δ increases. Scale bars represent $1 \mu\text{m}$.

Bacterial Alignment

Based on the analysis presented thus far, it is clear that the alignment fraction of the attached bacteria increased with δ because a greater trench depth improved the attachment fraction of aligned bacteria, $I^{\prime\prime}$, while I^+ remained constant at an order of magnitude below $I^{\prime\prime}$. This is because the trenches offered more area for the capillary force to act on the entrenched bacteria, in comparison to the non-aligned bacteria, which had to actively mitigate a lack of adhesive contact area in order to stay attached to the mesas of the nanogratings.

To account for the observation that aligned bacteria were always found in the nanotrenches, the potential energy of an aligned bacterium approaching nanogratings from the mesa (Fig. 7a), ϕ_M , and from the trench (Fig. 7b), ϕ_T , were computed based on the XDLVO model (Supplementary Information), and then plotted with respect to the separation distance from the mesa, d , in Fig. 7c. Note that positive values for ϕ indicate a repulsion between the bacterium and substrate surface while negative values indicate an attraction.

From the plot of ϕ_M vs. d (Fig. 7c), it can be seen that an aligned bacterium approaching the nanogratings would initially hover above the mesa, which was a position of lower potential energy, as compared to the position above the trenches. This carried on until $d \approx 2.5$ nm, when the position of lower potential energy shifted to the space above the trench (i.e. $\phi_T < \phi_M$), and the bacterium began experiencing repulsive forces from the mesa. As a result, the bacterium was driven from the mesa to the trench (Fig. 7d). Because the bacterium would always maintain a minimum distance of δ from the trench floor, there was no repulsion barrier for it to overcome before attaching to the top of the nanotrench, unlike the case for the mesa (Fig. 7c). Once it settled over the nanotrench, capillary action between the cell membrane and the nanograting walls would then cause the bacterium to “wick” into the channel, leading to entrenchment.

Figure 7: Schematic diagram illustrating (a) a bacterium approaching the mesa of a nanograting and (b) a bacterium approaching the trench of a nanograting. (c) Plot of ϕ_M and ϕ_T computed for different δ . (d) Schematic diagram illustrating the approach and guidance of an aligned bacterium into the nanotrench. Red arrow represents repulsive force while green arrow represents attractive force.

Overall Effects of Surface Topography

In summary, the level of bacterial attachment on a surface is dependent on 2 topographical factors, namely the surface area that exerts a meaningful physicochemical attraction on the bacterial cells, and the adhesive contact area between the substrate and bacterial cell. Based on the above results and computations, tall subcellular surface features with a height of 50 nm and above tend to attract bacteria with only the top of the structures while shielding them from valley floor. Therefore, compared to supracellular features or flat surfaces, tall subcellular features ensure that bacterial cells will be subjected to a reduced attraction, and consequently, a smaller bacterial population will approach the surface.

As the bacteria settle onto the surface, however, capillary action from subcellular structures will draw the cells into tight crevices and trap them, and the resultant increase in attachment fraction can offset the decrease in attracted cell population. If the bacterial cells cannot enter the crevices as a result of mismatched size and/ or orientation, however, they will employ appendages in an attempt to attach more firmly to the surface, suggesting that there will always be a minimum level of bacterial attachment that cannot be eliminated through topographical design.

Similarly, bacterial aggregation was found to be reduced on subcellular surface asperities. It was posited that this is because the corrugated topography of dense arrays of subcellular surface structures disrupted the *psl* trails laid by *P. aeruginosa* for cell-cell communication. Furthermore, for nanostructures taller than the cell diameter, it was found that capillary entrenchment physically isolated the aligned bacteria, hindering their ability to form aggregates with other cells.

It had previously been suggested that the alignment effect of bacteria observed on microgratings and supracellular nanogratings^{15,33} could have been the result of dewetting³⁴. This

is clearly not the case here, since surface tension effects had been minimized through the use of HMDS for chemical drying in this study. Instead, the large alignment fractions observed on the nanogratings stemmed mainly from the improved attachment fraction of entrenched aligned cells over non-aligned cells.

In addition, it was also determined that aligned cells were found in the nanotrenches because the bacterial cells were repelled from the mesas of the nanogratings by physicochemical interactions, before being embedded into the nanogratings through capillary action. Interestingly, although the width of the nanotrenches had only half the width of the bacterial cells, the entrenched bacteria remained viable for the duration of the study (24 hours). The pliability that *P. aeruginosa* cells demonstrated here is comparable to previous observations made for *E. coli*³⁵.

Design Principles

The results of the XDLVO calculations above (Fig. 7c) indicate that, for maximum bacterial adhesion on a surface, subcellular features with a height of 10 nm or less should be employed, so that both the top and bottom surfaces of the nanostructures would exert physicochemical attraction on the planktonic bacteria population (Fig. 8a). In addition, these subcellular features should be densely packed to ensure that a large capillary retentive force acts on the attached bacteria by wicking the cells into the interstitial spaces. There is, however, an upper limit to the packing density of the subcellular features, which depends on the elasticity of the bacterial cell membrane. Gram-positive bacteria, for instance, have thicker and stiffer cell membranes than Gram-negative bacteria¹², and therefore, a lower packing density with bigger interstitial spaces are required to allow such bacteria to mechanically deform and wick into the subcellular features. These insights are consistent with the results previously obtained by Mitik-

Dineva *et. al.*³⁶, which showed that reducing the height of surface asperities from 12 nm to 5 nm can triple the level of bacterial attachment.

Conversely, for a surface to minimize bacterial adhesion, it should be perforated with cavities that are at least 200 nm deep to eliminate the physicochemical attraction arising from surfaces at the bottom of the cavities (Fig. 8b). This is because the XDLVO forces decrease rapidly with increasing distance (Fig. 7c), and thus, with tall subcellular features (> 200 nm), physicochemical attraction from the valley floors would be negligible, effectively reducing the overall attraction exerted by the surface on the bacteria. Furthermore, the diameters of the cavities should be approximately an order of magnitude smaller than the size of the bacteria, so that the bacterial cells cannot wick into the holes without damaging their cell membranes. The exact size of the cavities required depends, again, on the elasticity of the bacterial cell wall – stiff Gram-positive bacteria can attach across a larger cavity than soft Gram-negative bacteria without being drawn in by capillary forces. These insights are in good agreement with Feng *et. al.*'s work^{14,25}, which demonstrated highly reduced bacterial attachment and biofilm formation on alumina membranes perforated with holes of diameters 25 nm and less.

The work presented here clearly shows that bacterial attachment and aggregation on a surface are highly dependent on the shape and size of subcellular features, as well as the orientation of the bacterial cells with respect to these features. These attributes are not always taken into account by surface roughness measurements, which are extensively used to quantitatively relate surface topography with bacterial attachment rates^{1,36}. For instance, nanogratings with $w = w_T = 300$ nm, $\delta = 500$ nm and $w = 500$ nm, $w_T = 300$ nm, $\delta = 650$ nm have the same arithmetic mean deviation of the roughness profile, R_a , but highly dissimilar bacterial attachment densities (Fig. 3a). This imprecision in topographical characterization may have led

to long standing contradictions about the effect of surface roughness on bacterial adhesion^{37–40}, which the insights derived from the current study may help resolve.

Figure 8: Examples of surfaces designed to (a) enhance and (b) reduce bacterial attachment.

Green arrows represent physicochemical attraction of the bacteria to the surface.

Conclusions

In conclusion, the activities of *P. aeruginosa* on anisotropic nanogratings with subcellular dimensions were investigated, and it was found that attachment and aggregation of the bacteria were generally reduced on the nanogratings. Using a quantitative semi-empirical model, it was demonstrated that these results were mainly caused by a decrease in the physicochemical attraction on the planktonic bacteria population, physical isolation of entrenched bacterial cells and possible disruption of cell communication. In addition, it was observed that there was a statistically significant alignment of attached bacterial cells on the nanogratings. This disproportionate level of aligned and non-aligned cell attachment was determined to be due to the disparity between the adhesive contact areas available for aligned cells and non-aligned cells. Based on these insights, general design principles for constructing surfaces that aim to maximize or minimize bacterial adhesion were presented, which agree well with results reported in previous studies.

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Author Information

CQL designed and carried out the experiments, performed the analysis, and wrote the manuscript.

Supporting Information Available

The supporting information provides derivations of the equations in the text, additional results, and information on the surface characteristics of the materials used.

TOC Image

Nanogratings

Flat Surface

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Supplementary Information

Bacterial Attachment, Aggregation and Alignment on Subcellular Nanogratings

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Geometric Parameters of Nanogratings

Table S1: Geometric parameters of the 25 types of nanogratings investigated.

Sample No.	w (nm)	δ (nm)
1	100	50
2		200
3		350
4		500
5		650
6	200	50
7		200
8		350
9		500
10		650
11	300	50
12		200
13		350
14		500
15		650
16	400	50
17		200
18		350
19		500
20		650
21	500	50
22		200
23		350
24		500
25		650

SEM Images

Figure S1: SEM images of *P. Aeruginosa* on the flat control surface and nanogratings with different w and δ . All images were taken at 5000 X except for those shown for $\delta = 650$ nm, which were at a magnification of 10 000X.

Histogram Analysis

Figure S2: Histograms (orange) showing cell densities, ρ ($\times 10^9 /m^2$), of *P. aeruginosa* for each orientation axis, θ , on each nanograting geometry. Histograms (blue) showing cell densities on the flat control surface are included as well. Asterisks indicate that the cell densities between the control surface and nanogratings is statistically different for that particular orientation (*i.e.* $p < 0.05$). Error bars represent standard deviations. It can be observed that there is no preferential orientation for the attached bacteria on the control surface, and for the non-aligned bacteria on the nanogratings (*i.e.* $\theta \neq 0^\circ$).

XDLVO Calculations

Table S2: Values of the various parameters used in the calculation of the surface interaction potential.

Symbol	Value	Remarks
λ	0.6 nm	Characteristic decay of acid-base interactions in water. From Ref. 1.
d_0	0.158 nm	Minimum separation distance between two bodies. From Ref. 1.
ψ_b	-0.01 V	From Ref. 2.
ψ_s	-0.02 V*	From Ref. 3.
γ_b^{LW}	41.6 mJ/m ²	From Ref. 3.
γ_b^+	8.52 mJ/m ²	
γ_b^-	38.8 mJ/m ²	
γ_s^{LW}	33.7 mJ/m ²	From Ref. 4.
γ_s^+	6.57 mJ/m ²	
γ_s^-	19.2 mJ/m ²	
γ_w^{LW}	21.8 mJ/m ²	From Ref. 5.
γ_w^+	25.5 mJ/m ²	
γ_w^-	25.5 mJ/m ²	
ϵ	80 x 8.85 x 10 ⁻¹²	-
κ	1.47 x 10 ⁹ m ⁻¹	

- Estimated from glass surface

Note that $\kappa = \frac{0.304}{\sqrt{M}}$ for 1-1 electrolyte, where M (in mol/l) is the ionic strength³. For LB

(Miller) broth, the NaCl concentration is $M = 0.2$ mol/l.

According to the XDLVO theory^{1,2,5-7} bacterial cells can be treated as inanimate objects that adhere to a surface due to the net attraction or repulsion generated by the van der Waals' (VDW), acid-base (AB) and electrostatic (EL) interactions.

The VDW potential energy can be written as⁵

$$\phi_{VDW} = -\frac{Hr}{6D} \quad (S1)$$

where

$$H = -24\pi d_0^2 \left(\sqrt{\gamma_W^{LW}} - \sqrt{\gamma_S^{LW}} \right) \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_W^{LW}} \right) \quad (S2)$$

and r refers to the cross-sectional radius of a bacterium, which for *P. aeruginosa* is approximately 250 nm. D refers to the separation distance between the bacterium and surface, d_0 refers to the minimum separation between two bodies and γ^{LW} refers to the Lifshitz-van der Waals' non-polar component of surface tension. The subscripts B , W and S refers to the bacterium, water and substrate respectively.

The acid-base interaction can be expressed as⁵

$$\phi_{AB} = 2\pi r \lambda \Delta G_{AB} \exp\left(\frac{d_0 - D}{\lambda}\right) \quad (S3)$$

where

$$\Delta G_{AB} = 2\sqrt{\gamma_W^+} \left(\sqrt{\gamma_S^-} + \sqrt{\gamma_B^-} - \sqrt{\gamma_W^-} \right) + 2\sqrt{\gamma_W^-} \left(\sqrt{\gamma_S^+} + \sqrt{\gamma_B^+} - \sqrt{\gamma_W^+} \right) - 2 \left(\sqrt{\gamma_S^+ \gamma_B^-} + \sqrt{\gamma_B^+ \gamma_S^-} \right) \quad (S4)$$

λ refers to the characteristic decay of acid-base interactions in water and the superscripts '+' and '-' refer to the electron-acceptor and electron-donor polar components of the surface tension respectively.

The electrostatic interaction is given by⁵

$$\phi_{EL} = \pi \epsilon r \left\{ 2\psi_B \psi_S \ln \left[\frac{1 + \exp(-\kappa D)}{1 - \exp(-\kappa D)} \right] + (\psi_B^2 + \psi_S^2) \ln [1 - \exp(-2\kappa D)] \right\} \quad (S5)$$

ε refers to the permittivity of the medium, ψ refers to the surface potential (assumed to be equivalent to zeta potential) and κ refers to the reciprocal of the Debye length.

The overall XDLVO potential energy for a bacterial cell is then

$$\text{Mesa:} \quad \phi_M = M \left(\phi_{VDW} + \phi_{AB} + \phi_{EL} \right)_{D=d} \quad (\text{S6})$$

$$\text{Trench:} \quad \phi_T = M \left(\phi_{VDW} + \phi_{AB} + \phi_{EL} \right)_{D=d+\delta} \quad (\text{S7})$$

where N is a multiplier that relates the XDLVO potential energy between a spherical bacterium and surface to that for a rod-shaped bacterium and surface. Here, we have estimated it using the volume ratio of a *P. aeruginosa* bacterium, modelled as a 1.4 μm long cylinder with $r = 250$ nm, to a sphere with the same cross-sectional radius, giving $M \approx 4$. $\phi_{M,min}$ and $\phi_{T,min}$, the interactions energies at the secondary minimum for the mesa and trench respectively, refer to the maximum attractive potential energy between the bacterium and the surface before repulsion occurs. Their values can be found when D satisfies $\frac{d\phi}{dD} = 0$ and

$\frac{d^2\phi}{dD^2} > 0$, which typically occurs at $D \sim 5\text{nm}$.

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