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Black Silicon Spacing Effect on Bactericidal Efficacy Against Gram-Positive Bacteria

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Abstract. The morphology of regular and uniform arrays of black silicon structures were evaluated for bactericidal efficacy against gram-positive, nonmotile *Staphylococcus epidermidis* (*S. epidermidis*). In this study, uniform and regular arrays of black silicon structures were fabricated using nanosphere lithography. A systematic evaluation of nanomorphology effects on bacterial killing were studied with 300 to 1400 nm pitch silicon nanostructures on spherical cocci of about 500 to 1000 nm in diameter. Our results show that morphology factors such as height and roughness do not directly determine bactericidal efficacy. Instead, the spacing between nanostructures is key in determining how bacteria are stretched and lysed. Smaller pitch nanostructures are better for killing bacteria and an 82 ± 3 % enhancement in the killing was observed for 300 nm pitch nanoneedles surface compared to the flat control substrates.

Keywords: Bactericidal, Nanoneedle, Gram-positive Bacteria, Black Silicon, Spacing Effect, Nanosphere Lithography

Introduction

There is a need for antibacterial surfaces that may reduce infections and illness in a wide variety of applications such as medical devices, sutures, contact lens cases, dental implants, and catheters [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11] as well as everyday household items such as kitchen and bathroom surfaces, appliances, consumer products, health club equipment, and food packaging [12, 13, 14]. Surfaces that are antiviral are also of much interest for these applications [15, 16, 17]. The most widely used antibacterial strategy has been the use of antimicrobials such as organic compounds like triclosan and zinc pyrithione [18, 19] or metallic ions such as Ag^+ [20]. However, this approach suffers from drawbacks such as high-cost, short-term efficacy, and biological and/or environmental toxicity [21, 22, 23]. Bacteria have also evolved resistance and can sustain growth in the presence of low concentrations of antibiotics [24].

Recent nanofabrication approaches such as 3D printing [25], nanosphere lithography [26], and reactive ion etching [27, 28], have been able to demonstrate bio-inspired antibacterial surfaces that either (1) reduce bacteria adhesion [29, 30] or (2) mechanically kill bacteria cells. The first antibacterial approach uses a combination of surface nano topography and surface functionalization to reduce bacteria adhesion [31, 32]. The second antibacterial approach was inspired by cicada *(Psaltoda claripennis)* wing surfaces, which have been shown to be mechanically bactericidal to gram-negative *Pseudomonas aeruginosa* cells [33, 34, 35, 36, 37, 38]. However, cicada wing structures have demonstrated limited effect on rigid bacteria cells such as gram-positive bacteria [39]. Biophysical models of bacterial cell/surface interactions have suggested that this is due to the cell rigidity of gram-positive bacteria [40]. The cell walls of gram-negative bacteria are generally only 5-10 nm thick, consisting of a single peptidoglycan layer [41]. In contrast, the thickness of the cell walls of gram-positive bacteria is around 20-80 nm, which consists of several layers of peptidoglycan. Black silicon and dragonfly wings *(Diplacodes bipunctata)* have both been demonstrated to be bactericidal against gram-negative *Pseudomonas aeruginosa*, gram-positive *Staphylococcus aureus*, and the vegetative cells and spores of *Bacillus subtilis* [39, 42, 43, 44, 45].

Hazell *et. al* used black silicon and diamond-coated black silicon substrates with various surface morphologies and tested those surfaces against both gram-negative *Escherichia coli* and gram-positive *Streptococcus gordonii* bacteria cells [46]. However, killing was only observed for gram-negative *E. coli.* In addition, other studies have also suggested that nanopatterned arrays may be selectively bactericidal towards bacteria that are motile as opposed to those that are non-motile. Diu *et. al* showed that nanopatterned surfaces were effectively bactericidal against motile bacteria (gram-negative *P. aeruginosa*, gram-negative *E. coli*, and gram-positive *Bacillus subtilis*), while ineffective against non-motile bacteria (gram-positive *S. aureus*, gram-positive *Enterococcus faecalis*, and gram-negative *Klebsiella pneumoniae)* [47].

In this paper, we systematically study the effect of the morphology of different uniform and regular black silicon arrays on bactericidal efficacy. The black silicon is fabricated using a nanosphere lithography approach, which allows us to create a hexagonal array of regularly spaced nanoneedles. Previous studies on mechanically bactericidal nanostructures have focused on non-uniform and irregular nanostructures in cicada wings and black silicon. This randomness makes it difficult to study how specific changes in surface morphology affect bactericidal efficacy [48, 49, 50]. These nanoneedles are assessed on *Staphylococcus epidermidis*, consisting of a diameter of about 500 to 1000 nm cocci arranged in clusters [51, 52]. *S. epidermidis* is a gram-positive, low-motility bacteria, a common infection source on indwelling medical devices such as catheters. The combination of both thick cell walls as a gram-positive bacteria and low motility suggest that *S. epidermidis* is challenging to mechanically kill and no surfaces in the literature have demonstrated a mechanical bactericidal effect against *S. epidermidis*.

The approach of studying uniform black silicon arrays on spherical bacteria enables a more systematic study. Regular and uniformly arrayed black silicon with pitches from 300 to 1400 nm is studied, which spans the range of *S. epidermidis* diameters.

Our results reveal that surface parameters such as height and surface roughness do not directly affect bactericidal efficacy. Instead, pitch is a key parameter. Scanning electron microscope images provide insight into how the bacteria interact with various surfaces and why smaller pitch nanostructures lead to higher bactericidal efficacy. Larger pitch black silicon arrays of 800 and 1400 nm do not demonstrate any killing of bacteria. This is because the *S. epidermidis* can deform and fit into the spaces between nanoneedle structures. In contrast, 500 and 300 nm pitch black silicon samples demonstrate bactericidal efficacy, where the bacteria cells sit on top of the nanoneedle structures. At the three-hour time point, the 500 nm pitch black silicon demonstrates 30 ± 3 % bactericidal efficacy. In contrast, the 300 nm pitch black silicon demonstrates the best bactericidal efficacy of *S. epidermidis* compared to the control. These small pitch nanoneedle arrays provide the highest killing because bacteria cells are deformed the most. This stretching of the cell wall results in tearing where cytoplasmic materials discharge out of the cells and are lysed.

Experimental Procedure

Fabrication of Black Silicon Substrates

P-type boron-doped (100) silicon wafers were used as substrates. The substrates were cleaned with acetone, methanol, and isopropanol and then dried with nitrogen gas. Then, polystyrene (PS) nanospheres were patterned on the substrate by self-assembly [53, 54, 26, 55]. PS nanospheres were self-assembled at the air-water interface and then the monolayer was transferred to the substrate. After that, the substrate was dried in the air at room temperature. Reactive ion etching (RIE) with oxygen was used to reduce the diameter of the PS nanospheres. The pressure was set at 25 mTorr and the RF power was set at 25 W. The flow rate of oxygen was 25 sccm, which yielded an etch rate of 80 nm per minute. By varying the etch duration, it is possible to vary the diameters of the nanosphere mask. After that, we used inductively coupled plasma reactive ion etching (ICP-RIE) to etch the silicon, where the PS nanospheres serve as the mask for the silicon. SF_6 was used as the etching gas and C_4F_8 was used as the passivation gas. The etching gas ratio to passivation gas was 33 sccm:82 sccm. The etch duration was varied to obtain nanoneedles of different heights. We observed tapering effects on the nanoneedles; all structures had different tip and base diameters. Finally, we removed the PS nanospheres by ultrasonication in acetone for 5 minutes.

Bacterial Culture Preparation

Single colonies of *S. epidermidis* were isolated by streaking from frozen glycerol stocks onto Petri dishes with peptone yeast (PY) agar. This agar comprised 15 g/L peptone

(BD Difco), 1 g/L yeast extract (BD Difco), 15 g/L bacto agar (BD Difco), and 0.25% dextrose (Fisher Scientific). After streaking, the dishes were incubated at 37 °C overnight. From these colonies, a single colony was selected to inoculate 5 ml of PY broth, which has the same constituents as the PY agar but without the agar. This culture was incubated overnight at 37 °C with agitation at 250 rpm. Subsequently, a 50 mL culture was initiated using 2 mL of the overnight culture, combined with 48 mL of peptone yeast-extract dextrose (PYD) in a 250 mL baffled flask. Following an 18-hour incubation at 37 °C with 250 rpm agitation, the culture achieved an Optical Density at 560 nm (OD560) of 1.49 absorbance units. The 50 mL culture was then transferred to a 50 mL conical tube and centrifuged at 5000 × g. The supernatant was discarded, and the bacterial pellet was resuspended and diluted 1:10 in 0.01 M Phosphate-Buffered Saline (PBS), resulting in a culture with an OD of 0.1 absorbance units. PBS was chosen not as a growth medium but as a buffer, given its lack of nutrients essential for bacterial proliferation. However, it offers suitable conditions for preserving the structural integrity of the bacterial cells for subsequent experimental procedures.

Bactericidal Efficiency Experiments

For all silicon samples, 10 μL of the 1:10 *S. epidermidis*:PBS culture was aliquoted onto each substrate of 1.5 cm by 1.5 cm size. Four replicates of each of the five different samples were used for each of the three-time points (0, 1, and 3 hours) evaluated. 60 samples were evaluated in total. Matlab 2021a was used for conducting statistical analyses and generating corresponding plots. For the 0-hour time, the bacteria culture was dropped and immediately withdrawn from the substrates. For the one-hour and three-hour time points, the substrates were kept inside a humidity-controlled environment after the culture was aliquoted for the specified amount of time. The relative humidity was maintained at nearly 100% to avoid evaporation. A complete recovery of the 10 μL culture is difficult, 90 μL PBS was added and mixed well by pipetting before 100 μL was withdrawn. Then the bacterial culture was serially diluted 10-fold in 270 μL of 1x PBS. Of the E-4 dilution, 100 μL was plated on PYD agar and spread with sterile glass beads. The bactericidal effect was evaluated in triplicate for both the bare silicon control and black silicon arrays. The plates were incubated at 37 °C for 24 hours. Afterward, the plates were counted and CFU/mL counts were obtained. Scanning electron microscopy (SEM; Zeiss Sigma 500 VP) was used to evaluate the surface of the used black silicon samples after the experiment.

Results and Discussion

Four different black silicon surfaces were fabricated and evaluated and compared to a flat control sample of silicon. Figure 1 shows the tilted view SEM images of varying pitch: (a) 1400, (b) 800, (c) 500, and (d) 300 nm where the view is 70° from overhead. The physical morphologies of four different black silicon surfaces were characterized by

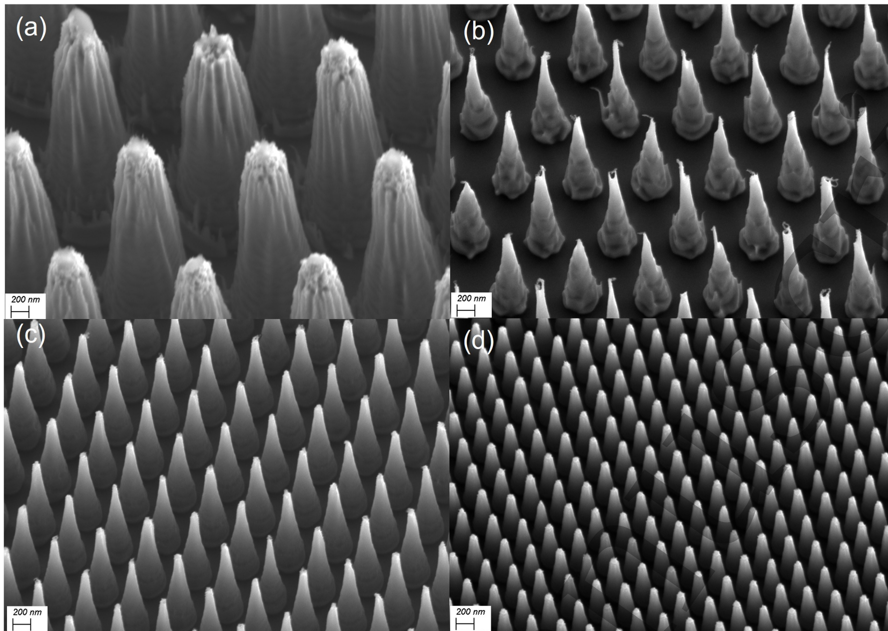

Figure 1. SEM images of fabricated black silicon substrates Pitches of a) 1400, b) 800, c) 500, and d) 300 nm pitch. Scale bar: 200 nm

SEM. The different pitches were created by using PS nanospheres of varying diameters, where the pitch is determined by the diameter of the close-packed nanospheres. Thus, the range of pitches studied span the size of the bacteria the surface interacts with.

Table 1. Morphology parameters of black silicon substrates, as characterized under SEM

Black Silicon Type	Pitch(nm)	Tip(nm)	Base(nm)	Height(μm)	Roughness(μm)
1	1400	460	1000	6.0	8.8
2	800	110	400	2.3	4.1
3	500	80	360	2.0	7.0
4	300	80	220	1.0	6.6

Table 1 provides a summary of the morphology of the four black silicon samples fabricated and evaluated for bactericidal efficacy. Different ICP RIE times were used to vary the height of the samples. The four black silicon samples were etched for 32, 17, 11, and 7 min, respectively, to achieve heights of 6.0, 2.3, 2.0, and 1.0 μm, respectively. The roughness is calculated by the ratio of the surface area of the substrate after etching compared to the flat control surface.

The four black silicon and the control samples were then subject to bactericidal efficiency experiments. Figure 2a shows the bacteria counts at different time points on the five different silicon samples (control and four black silicon samples). The bacterial culture was aliquoted and retrieved immediately to get the data for zero hour time point. In these experiments, the bacteria did not have sufficient time to interact with the black

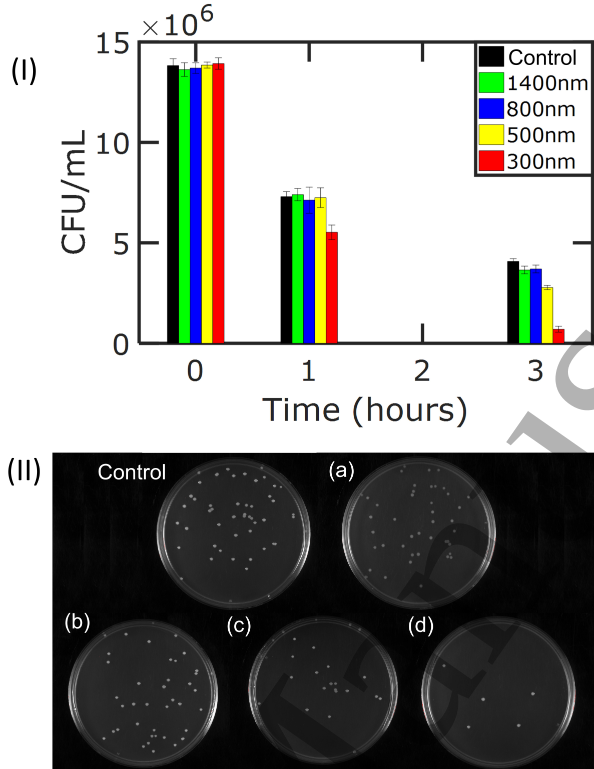

Figure 2. Colony forming unit studies of bacteria on different pitch nanoneedle surfaces compared to control. (I) CFU/mL vs Time (hours). Error bars indicate standard error. (II) Representative optical images of plates of the 3-hour time point after the 24-hour incubation period: control, and black silicon with pitches of (a) 1400, (b) 800, (c) 500, and (d) 300 nm.

silicon surface and were thus utilized to evaluate the effect of aliquoting and withdrawal of the bacteria from the surface. No statistically significant difference in *S. epidermidis* has been observed at the 0-hour time point in any of the samples evaluated.

The bactericidal effect for a particular type of sample was quantified by subtracting the number of surviving cells on the substrate from the number of cells remaining in the bare silicon sample at that same corresponding time point. At the one-hour time point, the bactericidal effect was observed only for the 300 nm pitch samples. 20 ± 6 % killing was observed for these samples. Two sample t-tests with the control samples produce a p-value of 0.006. There was some variation amongst the other types of samples, but there was no statistically significant killing of bacteria. For two sample t-tests with the control samples, the p-values were 0.35, 0.43, 0.66 respectively for the 1400, 800, and 500 nm pitch black silicon substrates.

At the three-hour time point, the 1400 and 800 nm pitch samples exhibit no statistically significant killing with two sample t-test p-values of 0.12 and 0.16, respectively. The 500 nm pitch samples demonstrate killing (p-value of 0.003), where 30 ± 3 % bacteria are killed. The best performance was observed for the 300 nm pitch samples with a p-value $< .00001$. These samples significantly reduced the number of viable cells and the killing was measured to be 82 ± 3 %.

Figure 2(ii) shows representative agar plates at the three-hour time point. We observed the maximum bactericidal effect on the 300 nm pitch samples with nanoneedles with a minimum height and spacing. Among different reports regarding the bactericidal effects of nanostructured surfaces, some reports suggested that the pitch of the nanostructures plays an important role in the bactericidal performance and an increase in bactericidal performance is observed associated with the variation of pitch. Theoretical studies have suggested that a larger pitch can enhance bactericidal efficiency [56]. However, this theoretical study modeled bacteria as a semi-infinite thin elastic layer. This model assumes that the bacteria are much larger than the nanostructures and their spacing. Dickson *et. al* studied *E. coli* on the uniform, nanopatterned PMMA surfaces [57]. These studies report that more tightly packed nanopillars are more effective at killing bacteria. However, *E.coli* is a gram-negative bacteria. Linklater *et. al* [58] also studied various black silicon surfaces on gram-positive *S. aureus* and gram-negative *P. aeruginosa*, but these surfaces were non-uniform. They found that surfaces with denser nanoneedles were more effective at killing bacteria. Hazell *et. al* studied various black silicon surfaces on *E. coli* and *S. gordonii* [46]. The black silicon surfaces studied were nonuniform, but higher areal density was found to result in the greater killing of *E. coli*. However, the surfaces were ineffective against gram-positive *S. gordonii*.

SEM images were taken of *S. epidermidis* on various black silicon samples at the 3-hour timepoint to provide some mechanistic insight into the differences in bactericidal efficiency. Figure 3 shows these results. Figure 3(a) and (b) show results for the 1400 and 800 nm pitch black silicon samples. These samples demonstrate statistically insignificant killing of bacteria. *S. epidermidis* has a diameter of 0.5-1 μm. The two larger pitch-black silicon surfaces are ineffective at killing *S. epidermidis* as the pitch is too large and bacteria can simply settle in spaces between the nanoneedles. Even when the pitch is 800 nm, which should be smaller than some *S. epidermidis* cocci, the bacteria can deform and nevertheless, squeeze into areas between the nanoneedles, such that there is no statistically significant killing.

Figure 3(c) shows SEM images of *S.epidermidis* on top of the 500 nm pitch black silicon samples. Some deformation can be seen in the bacteria cells, though they largely remain spherical. As discussed above, 30 ± 3 % bacteria are killed. In contrast, the 300 nm pitch black silicon samples have a nanoneedle areal density of 2.8 times that of the 500 nm pitch black silicon samples. Instead of just 1 or 2 nanoneedles in the 500 nm pitch case, several nanoneedles are present under each bacteria cell. SEM images in Figure 3(d) shows severely deformed lysed *S. epidermidis* cells observed on the 300 nm sample. The significant stretching of the cell wall results in tearing where the cytoplasmic materials discharge out of the cell. The top surface of the spherical cell is flattened and lysis is observed.

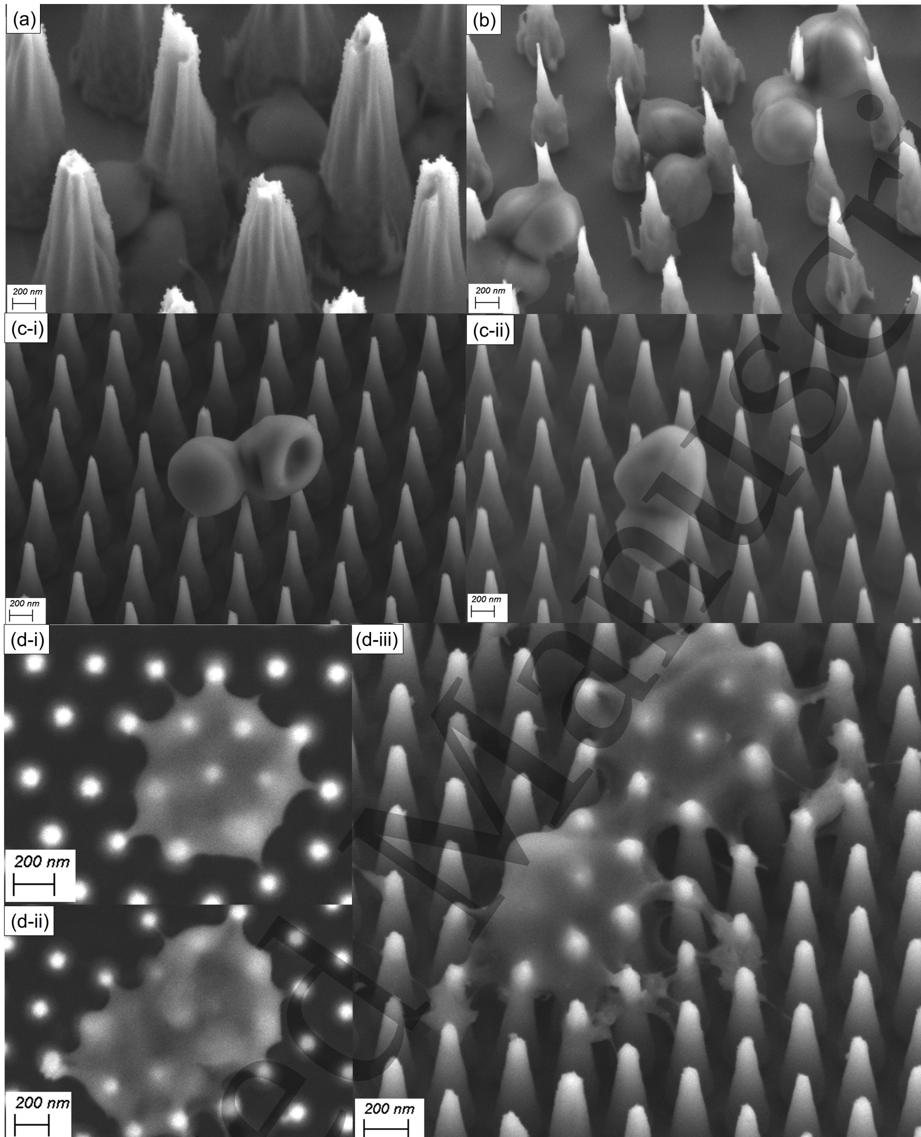

Figure 3. SEM image of bacteria cell on different samples (a) 1400nm pitch, (b) 800 nm pitch (c) 500nm pitch, (d) 300 nm pitch All images are tilted view SEM images except for (d-i), and (d-ii), which are top-view images.

Conclusion

Black silicon substrates, fabricated using nanosphere lithography and deep reactive ion etching, demonstrated notable bactericidal effects on *S. epidermidis*, a gram-positive, non-motile bacterium. By controlling the height, pitch, and top diameter of these substrates, we observed the interaction between the sharp nanoneedles of black silicon and bacterial cells. As the bacterial cells encounter these nanoneedles, their cellular walls and membranes can be damaged. Notably, gram-positive bacteria, characterized by their thicker cell walls, are often more resistant to mechanical destruction. Despite this, our SEM images provided evidence of significant bacterial deformation, particularly for substrates with a smaller pitch. Samples with a 300 nm pitch were particularly effective, exhibiting an 82% bactericidal rate at the three-hour mark. This deformation on the black silicon surface results in compromised cellular integrity, causing the contents to leak and eventually leading to cell death. In an era where bacterial resistance to drugs is escalating, our findings underscore the potential of black silicon substrates as an innovative approach to designing bactericidal surfaces.

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Competing interests

The authors declare that they have no competing interests.

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