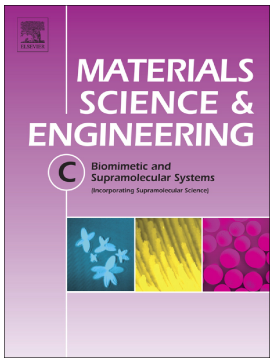

Bactericidal performance of nanostructured surfaces by fluorocarbon plasma

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PII: S0928-4931(16)30995-X
DOI: doi: 10.1016/j.msec.2017.05.111
Reference: MSC 8093

To appear in: *Materials Science & Engineering C*

Received date: 29 August 2016
Revised date: 8 May 2017
Accepted date: 16 May 2017

Please cite this article as: E. Vassallo, M. Pedroni, T. Silvetti, S. Morandi, S. Toffolatti, G. Angella, M. Brasca , Bactericidal performance of nanostructured surfaces by fluorocarbon plasma, *Materials Science & Engineering C* (2017), doi: 10.1016/j.msec.2017.05.111

Bactericidal performance of nanostructured surfaces by fluorocarbon plasma

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Abstract

This study presents the characterization and antibacterial activity of nanostructured Si by plasma treatment method using a tetrafluoromethane (CF_4) and hydrogen (H_2) mixture. Nanostructured-Si is a synthetic nanomaterial that contains high aspect ratio nanoprotrusions on its surface, produced through a reactive-ion etching process. We have shown that the nanoprotrusions on the surfaces produce a mechanical bactericidal effect. Nanostructured-Si exhibited notable activity against three different microorganisms: Gram-negative (*Escherichia coli*), Gram-positive (*Staphylococcus aureus*) and spore-forming bacteria (*Bacillus cereus*) producing a > 4 $\log_{10}$ reduction after 24 h of incubation.

Scanning electron microscopy was used to analysis the structure and morphology character of different surfaces evidencing the physical bactericidal activity of the Nanostructured-Si. These results provide excellent prospects for the development of a new generation of antibacterial surfaces.

Keywords: Nanopattern, Bactericidal, Antibiofouling, *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus.*

1 Introduction

Microbial infection remains one of the most serious complications in several areas, particularly in medical devices, drugs, hygienic applications, water purification systems, textiles, food packaging and storage, and filters used in air-conditioning systems [1]. Both antibiotic-resistant Gram-negative and Gram-positive bacteria are reported to be important causes of bacterial infections [2-3]. Recent years have seen increased development of nanoparticle designs as treatments for various diseases and infections [1]. The antibacterial activity of inorganic materials is of significant interest due to the need for infection control and rising antibiotic resistance. Bacterial adhesion depends on the solid surface properties (roughness, solid surface, chemical structure, hydrophobicity, surface charge, etc.). Ivanova et al. [4] have shown that a suitably nanostructured surface can kill the bacteria based on texture alone. They had previously observed that cicada wings possessed a strong bactericidal activity against *Pseudomonas aeruginosa* (human pathogen) and showed that surface of the wings was covered by an array of regularly spaced nanopillar structures. Studies have demonstrated that these structures can be replicated in the laboratory, with engineering techniques [5]. Based upon this understanding, Ivanova et al. have reproduced a similar texturing on different materials demonstrating that the bactericidal nature was independent of the biochemical functionality of the wing. They showed that bactericidal nature of the wing was due to the mechanical rapture of bacterial cells. On the basis of this, materials with similar surface topologies have the same bactericidal effect. In literature [6], and also in Ivanova et al. research, the Si texturization has been studied mainly in sulfur hexafluoride (SF_6) and oxygen (O_2) mixture. Although this mixture is very used for processes in microelectronics and for biomedical applications, actually it is virtually impossible to be used because of the sulfur that stinks the treated material. Therefore, in addition to the texturing process by plasma, a cleaning process is necessary, resulting in additional costs and longer time of industrial production. To overcome this problem, in this paper, we report a method which utilizes tetrafluoromethane (CF_4) gas diluted with hydrogen (H_2). The process is realized in a low-density capacitively-coupled plasma RIE reactor [7]. The antibacterial activity of the nanostructured-Si against Gram-negative (*Escherichia coli* ATCC 8739), Gram-positive (*Staphylococcus aureus* ATCC 19095) and spore-forming bacteria (*Bacillus cereus* ATCC 14579) is also investigated. This study aims to evaluate the antibacterial property of silicon nano-structures in the formulation of new types of bactericidal materials.

2 Experimental

2.1 The plasma reactor

An RF plasma system [8] has been used to produce a physical structuring of Silicon (type P, dopant B, <100>, 0.01-0.02 Ohm-cm, 1 × 1 cm^2, thickness = 400 µm). The experimental apparatus consists of a parallel-plate, capacitive-coupled system, consisting of a cylindrical stainless steel vacuum chamber with an asymmetric electrode configuration. A powered electrode (3-in diameter) is connected to an RF (13.56 MHz) power supply, coupled with an automatic impedance matching unit, while the other electrode (3-in diameter), consisting of stainless steel, is grounded. Si substrates are placed on the powered electrode at 6 cm away from the ground electrode. The substrate temperature is monitored by a thermocouple fixed directly on the substrate. Before the process, the substrates are cleaned by chemical etching solutions (alcohol followed by rinse in deionized water) to remove surface contaminants. The Atomic Force Microscopy technique has been used to check the surface roughness of substrates after cleaning. The RMS roughness was in accordance to manufacturer's data (≤ 1 nm). The process chamber is pumped to a base pressure below 1 × 10^{-4} Pa and high-purity reactive gases (CF_4 and H_2) are introduced into the vacuum chamber through a mass flow controller in order to establish the desired working pressure, which is fixed at 9 Pa. The plasma process was performed for 30 min. A power density of 1 W/cm^2 was applied to RF electrode and H_2/CF_4 ratio equal to 0.1 was set in plasma mixture.

2.2 Characterization of the nanostructured surfaces

Nanostructured surfaces in CF_4/H_2 plasma were examined by Scanning Electron Microscope (SEM) analysis through a high resolution SEM Hitachi SU70 with Schottky electron source and secondary electron (SE) in-column upper-detector. The SEM observations of the Nanostructured-surface after cells inoculation were coated with carbon with a Jeol evaporation system JEE 4B, and low acceleration voltage (3 kV) was set in order to reduce the damage of the biological material.

2.3 Bacterial strains and growth conditions

Strains used in this study, *E. coli* ATCC 8739, *S. aureus* ATCC 19095 and *B. cereus* ATCC 14579, came from the American Type Culture Collection (U.S.A.). Prior to each experiments, the strains were propagated twice in Brian Heart Infusion BHI broth (Scharlau Microbiology, Spain) and incubated aerobically, overnight, at 37 °C.

2.4 Antimicrobial activity of the nanostructured surfaces

A standard colony-forming units (CFU) assay was performed to determine the reduction in viability during 24 h incubation. One-centimetre square samples of silicon and nanostructured-Si were disinfected with a suspension in 90% ethanol, placed under UV light for 30 min and transferred into sterile Petri dishes. Control experiments confirmed that the adopted procedure ensured the disinfection of the surfaces. After Si sterilization, overnight cultures of *E. coli*, *S. aureus* and *B. cereus* were diluted 1:100 in quarter-strenght Ringer's solution and a single drop of 100 μL (~ 10^6 CFU/mL) was placed on each Si surfaces. Viable counts using were performed on the inocula to ensure that they contained ~ 10^6 CFU/mL. Serial dilutions in quarter-strength Ringer's solution were plated in Petrifilm Aerobic Count plate (3M Minneapolis, MN, USA) according to manufacturer instructions and incubated at 37-°C for up to 24 h. The antimicrobial effect was evaluated in triplicate and in parallel both in silicon and nanostructured-Si surfaces. The samples were incubated at 37 °C and the petri dishes containing the surfaces were covered with a plastic film to maintain high humidity to prevent excessive evaporation. Samples were removed after 0, 3, 6, 24 and 30 h; the bacteria were picked up from surfaces with sterile cotton swabs. Swabs were immersed for 30 s in quarter-strenght Ringer solution and agitated at maximum speed on a vortex for 20' in order to obtain the release of the bacteria from the bud. The sample was serially diluted in quarter-strength Ringer's solution and appropriate dilutions were used to enumerate the tested bacterial strains on Petrifilm Aerobic Count plate.The viability count was performed in triplicate. The plates were incubated at 37-°C for up to 24 h and the number of CFU determined and results were expressed in Log_{10} CFU/cm^2. The data are expressed as mean ± sd of three independent experiments.

2.5 Cell viability analysis

Bacteria viability was assessed by using using LIVE/DEAD BacLight Bacterial Viability Kit, L7012 following the manufacture's instructions (Molecular Probes Europe BV, The Netherlands). Alive cells were observed under a video-confocal microscope (Nikon Eclipse 80i equipped with a video-confocal system; Nikon Instruments S.p.a., Calenzano, FI, Italy) with a excitation and emission wavelenghts of 480 and 505 nm, respectively.

3 Results and discussion

3.1 Surface texturization of silicon by plasma

As previously reported by our group [9], the Si surface was textured by means of a mask-less plasma process employing tetrafluoromethane and hydrogen as reaction gases. The method employs CF_4 and H_2 gases to generate F* and H* radicals. When F* radicals react with Si, a volatile compound SiF_4 is formed, resulting in the surface etching. At the same time a passivation layer of CF_x is deposited randomly on the silicon substrate, obtaining an auto masking effect towards the etching by F*. However, the passivation layer is partly removed by the simultaneous ions bombardment. Thus the passivating layer thickness is minimized and the etching by F* can proceed. The competition between passivation layer deposition and its removal by energetic ions bombardment cause local variation of etching rate on the Si surface, leading to the growth of random high-aspect-ratio silicon nanostructures [10] over the process time. Due to the high aspect ratio, the textured silicon surface shows antireflective properties and appears black in color after the process and is therefore termed as 'black silicon'. Black silicon has been used for solar cell applications [11].

The surface morphology of Si specimen processed by plasma is shown in the SEM images in Fig. 1. Top view (Fig. 1a) shows disordered nanopillars, and looking at the cross-sectional view (Fig. 1b) it resembles a pattern of spaced vertical nanopillars of 150-200 nm diameters separated by 100-250 nm. As expected, the produced texturing is similar to that of the wings of different insects [12] with surface multifunctional properties such as hydrophobicity and anti-bacterial action [13].

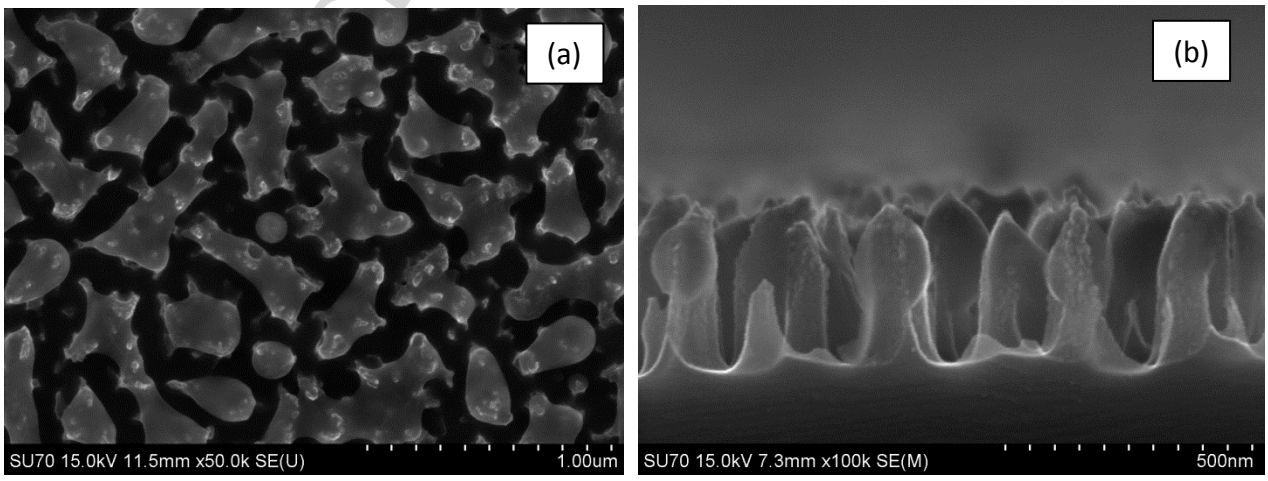

Fig. 1. SEM pictures of Si wafers processed at 200 W of RF Power: (a) top view and (b) cross-sectional view SE imaging with upper detector.

3.2 Antimicrobial activity

To determine the bactericidal performance of nanostructured surfaces, bacterial cells of different morphologies and structure were cultured simultaneously on Si wafers processed and not processed by plasma. Confocal microscopy (Figure 2) and bacterial plate counts (Table 1) show the antimicrobial efficacy of nanostructured-Si surfaces against *E. coli* ATCC 8739, *S. aureus* ATCC 19095 and *B. cereus* ATCC 14579.

Cell viability was evaluated using a mixtures of green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. In accordance with the manufacturer's instructions, all green cells were considered viable. Microscopy images showed that after 24 h on nanostructured-Si, only dead or injured cells with compromised membranes were present (Figure 2). All the strains tested were completely killed (Log_{10} reduction factor of > 5) after 24 h of incubation. Considering these data, the killing efficiency of the nanostructured-Si surfaces during the first 6 h of incubation was quantified and the number of cells killed by the Si samples was determined by subtracting the number of surviving cells from the number of cells remaining in controls at the corresponding incubation time interval, in order to account for the low levels of natural cell death that occurs in low-nutrient conditions [14].

E. coli strain was reduced of 1.57 and 1.76 Log_{10} CFU/cm^2 after 3 and 6 h respectively, while *S. aureus* had a Log_{10} reduction factor of 0.42 after 3 h increasing to 2.33 after 6 h of incubation.

B. cereus ATCC 14579 was more resistant than the other strains tested showing a Log_{10} reduction factor of 0.33 after 6 h. Taking in account the results obtained in this work and the minimum infective dose of *E. coli* (10^2-10^8 cells), *S. aureus* (10^5 cells) and *B. cereus* (10^5 cells) [15-17] the nanostructured-Si samples could be an efficient antibacterial surfaces, in fact 1 cm^2 of this material reduce $3.3*10^5$ cell/h, $2.2*10^5$ cell/h and $1.8*10^5$ cell/h of *E. coli*, *S. aureus* and *B. cereus,* respectively. These findings suggested that over 1 h nanostructurated-Si could be capable of killing the minimum infective doses of *S. aureus* and *B. cereus* and indicated the potential for this surfaces to decrease bacterial loads in aqueous environments.

The antibacterial activity against *S. aureus* and *B. cereus* indicates that the nanostructured surfaces exert a compromising effect also on Gram-positive bacteria. This represents an interesting result since in Gram-positive bacteria peptidoglycan cell wall provides great strength and rigidity while maintaining elasticity and flexibility, thus a greater impact is required to disrupt it, damage the

inner membrane and cause the death of the cell. Moreover, recent studies demonstrated that the antimicrobial activity of engineered surfaces is influenced by the size, shape, motility, adhesivity and structural process of cell division of the bacterial strain. In particular, *S. aureus* resulted resistant to the bactericidal effect of different nanoscale surfaces [18-19]. However, the bacterial adhesion on nanostructured surfaces is strictly correlated to the nanoscale morphological features of the surface, thus the bactericidal effect is morphology-dependent [20].

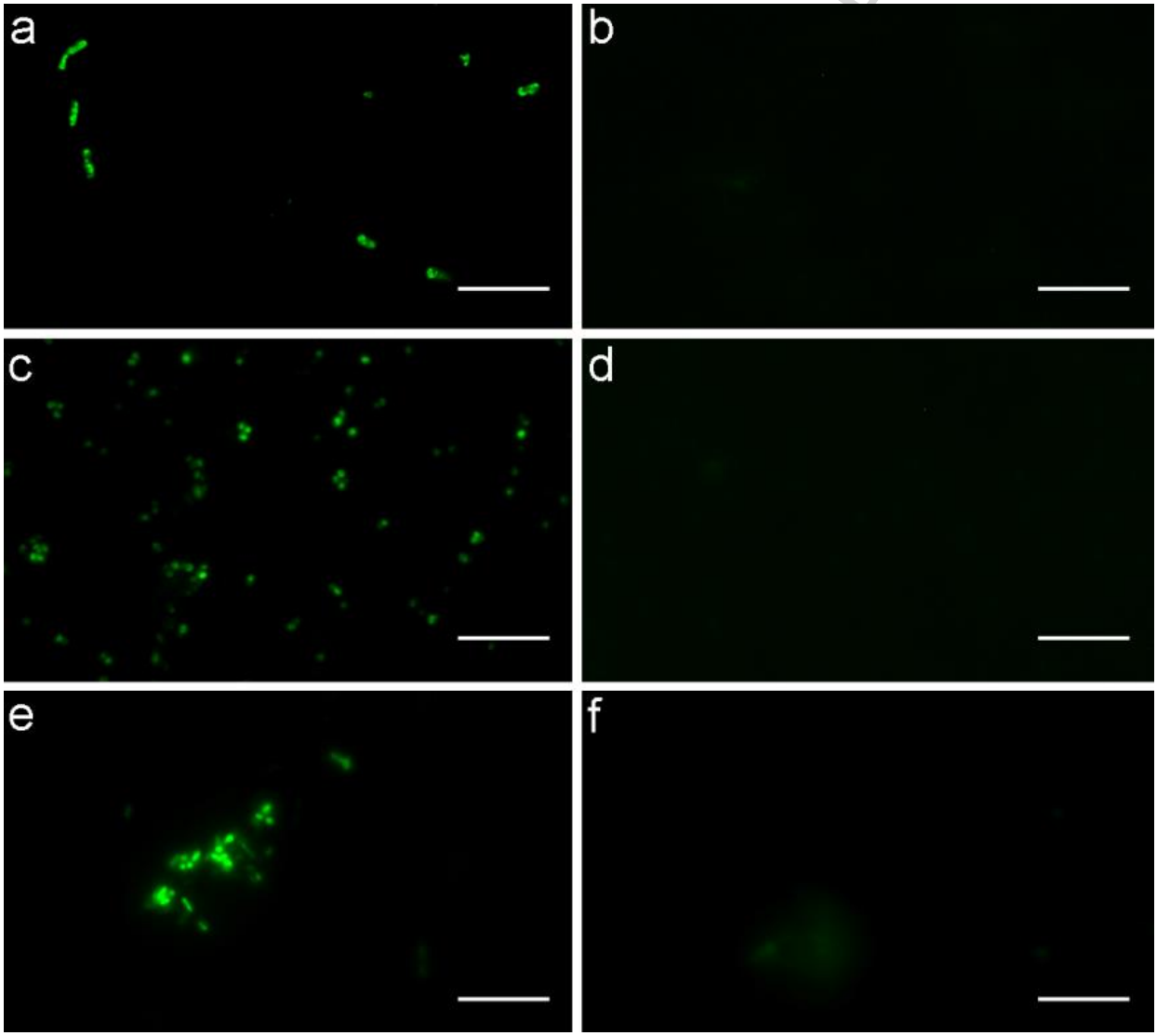

Fig. 2 Video-confocal micrographs showing in green alive *Bacillus cereus* (a, b), *Staphylococcus aureus* (c, d) and *Escherichia coli* (e, f) cells before (a, c, e) and after incubation for 24 hours (b, d, f) on nanostructured-Si surfaces. Scale bar: 10 µm

Strains	Time (h)	Si (control)		Nanostructured Si	
		Log_{10} (CFU/cm^2)	SD	Log_{10} (CFU/cm^2)	SD
E. coli	0	5.99	0.01	5.99	0.01
ATCC8739	3	6.21	0.03	4.64	0.30
	6	6.30	0.03	4.54	0.66
	24	4.15	0.30	< 1	
	30	1.91	0.19	< 1	
S. aureus	0	6.05	0.11	6.05	0.11
ATCC19095	3	6.03	0.08	5.61	0.21
	6	6.39	0.16	4.06	2.21
	24	5.69	0.13	< 1	
	30	5.65	0.07	< 1	
B. cereus	0	5.86	0.09	5.86	0.09
ATCC14579	3	6.02	0.05	5.70	0.21
	6	5.81	0.13	5.48	0.33
	24	4.14	0.27	< 1	
	30	4.08	0.32	< 1	

Tab.1 Tab.1 Bactericidal activity effectiveness of nanostructured-Si surfaces compared to not treated silicon surfaces (control). The data were expressed as mean values ± standard deviation of three independent experiments.

3.3 Pillar effects on cell survival

Morphology studies were performed both on the control non-treated Si and on nanostructured samples using SEM, as shown in Fig. 3-4. On the control surface, cocci and rod-shaped bacteria show typical morphology with intact cell membrane (Fig. 3a; 4a; 4c). Different states of lysis and cell destruction are visible on nanostructured surface, on which the bacteria appear stretched over pillars (Fig. 3b-d). It appears that the cells, due to their motile behaviour, stretch themselves while

parts of the membrane are clutched by pillars, upon reaching the limit of stretching. The cells undergo therefore the ruptured of the cell membrane, leading to collapse (Fig. 3e-f; Fig.4b-d) and death in the end. A similar observation was reported for cicada wings that stretch the membrane of Gram-negative rod shaped cells [14]. The results are also consistent with previous studies highlighting that nanostructured surfaces induce rupturing cell process in bacteria and yeasts [4, 21].

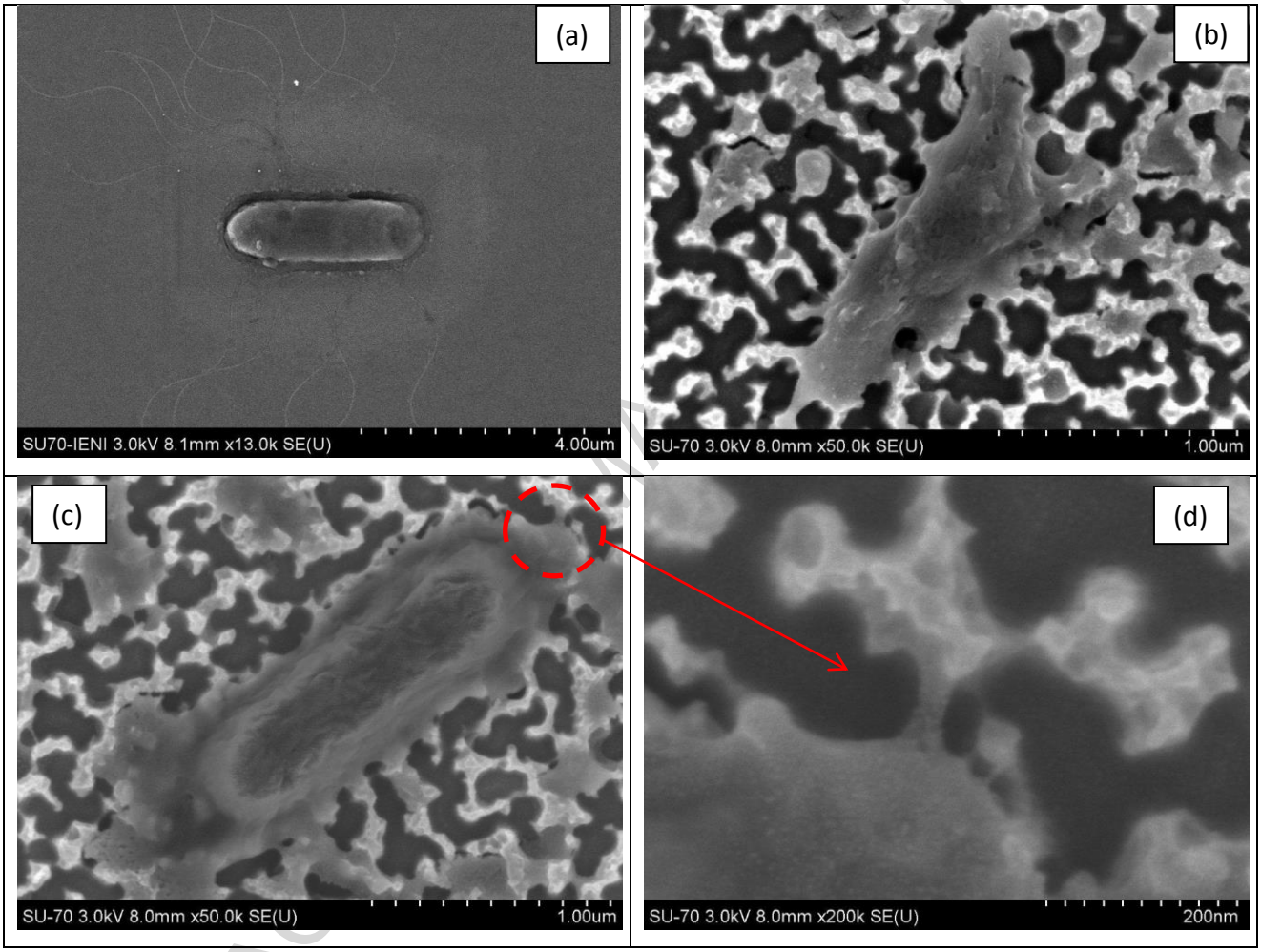

Fig. 3. SEM images of *E. coli* on the control surface (a) and nanostructured surface (b-d) view SE imaging with upper detector.

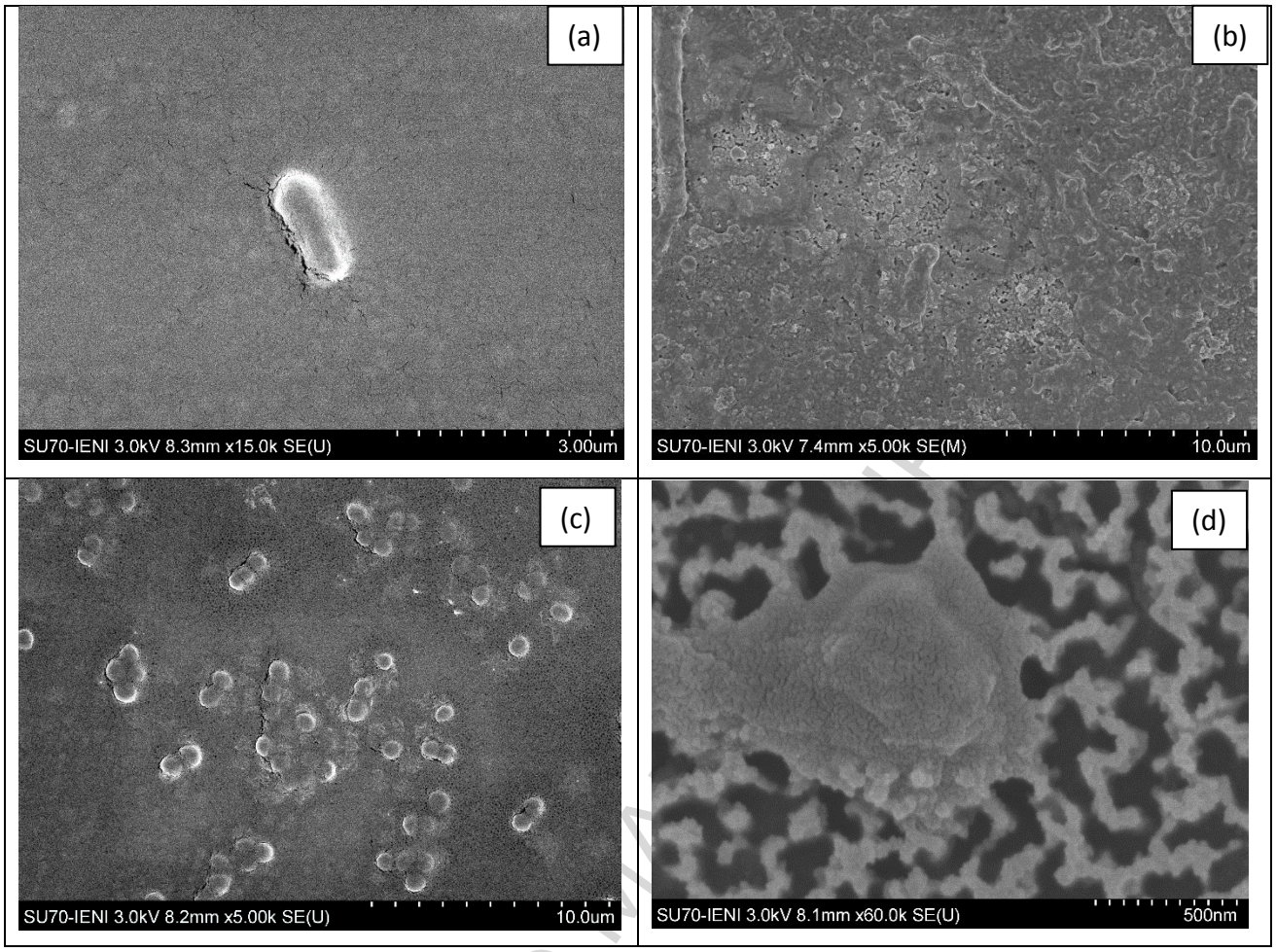

Fig. 4. SEM images of *B. cereus* and *S. aureus* on the control surfaces (respectively, a and c) and nanostructured surfaces (respectively, b and d) view SE imaging with upper detector.

4. Conclusions

A nanostructured surface of Si was prepared in this research inspired by the topography the nanostructures present on insect wing surfaces. Random nanoscale structures were fabricated in a capacitive coupled plasma reactor by using a CF_4/H_2 plasma. Experimental data evidenced that nanostructured-Si surfaces possess a highly bactericidal effect against Gram-negative (*E. coli*), Gram-positive (*B. cereus* and *S. aureus*) and also spore-forming bacteria (*B. cereus*).

These results confirmed the bactericidal mechanism due to the mechanical rupture of the cell membrane. The technique of cold plasma turned out to be very promising as a possible tool for designing an antibacterial surface. Anyway, though cicada wings were chosen as the reference surface in this study, the method of nano-structuring developed has great potential for replicating the surfaces of other plant and animal tissues. This approach will provide an important research

tool for understanding surface−bacteria interactions and facilitating development of technology to enhance inactivation of bacteria pathogens and improve public health.

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Highlights

- A Si surface was textured in a tetrafluoromethane/hydrogen plasma process.
- The plasma treatment has produced a pattern of spaced vertical nanopillars.
- The nanostructured surface showed a mechanical bactericidal effect.