Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2016-0463.R2
Manuscript Type:	Article
Date Submitted by the Author:	09-Mar-2017
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Keyword:	Biofilm, <i>B. subtilis</i> , Micro-patterned surfaces, Bacteria settlement

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Using micro-patterned surfaces to inhibit settlement and biofilm formation by *Bacillus subtilis*

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Abstract: Biofilm is a biological complex caused by bacteria settlement to the substrates, their subsequent reproduction and secretion. This phenomena reduce heat transfer efficiency and cause significant losses in treated sewage heat recovering systems. This paper describes a physical approach to inhibit the bacteria settlement and biofilm formation by *Bacillus subtilis*, which is the dominant genera in the treated sewage. Here, the micro-patterned surfaces with different characteristics (stripe and cube) and dimensions (1 μm to 100 μm) were fabricated as surfaces of interest. Model sewage was prepared and a rotating coupon device was used to form the biofilms. Precision balance, scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM) were employed to investigate the inhibitory effects and the mechanisms of the biofilm-surface interactions. The results have shown that surfaces with small pattern sizes (1 μm and 2 μm) all reduced biofilm formation significantly. Interestingly, the CLSM images showed that the surfaces do not play a role in 'killing' the bacteria. These findings are useful for future development of new process surfaces on which bacteria settlement and biofilm formation can be inhibited or minimized.

Keywords: Biofilm / *B. subtilis* / Micro-patterned surfaces / Bacteria settlement

Introduction

Treated sewage is in huge quantity in modern society. It is an important carrier of urban waste heat energy (Chan et al 2009). Its temperature (20-30°C) is ideal for maintaining high efficiency for heat pump operations (Meggers and Leibundgut 2011). Treated sewage source heat pump systems (TSSHPS) reuse the low-rank energy in the treated sewage to provide heat and cold for buildings (Yang et al 2013), and practical projects such as the 2008 Beijing Olympic Village TSSHPS have been proven to be efficient (Tian et al 2012a). However, the treated sewage contains “impurities” such as bacteria, which can form biofilms in the plate heat exchangers in the TSSHPS (Yang et al 2015). Previous studies have also shown that considerable biofilms were formed in the heat exchangers within 40 days of operation and reduced the heat transfer coefficient by 60% (Murthy et al 2004; Tian et al 2012b).

Biofilm, a complex community of microbes attached to a solid surface and encased in extracellular polymeric substance (EPS), could be used for root protection or pollutant removal (Haggag and Timmusk, 2008; Chug et al 2016). However, in some other cases, biofilms could lead to efficiency decrease of heat-exchanging systems, and compromise safety in food processing and medical instruments (Steinhagen et al 1993; Cartwright and Spurr 1998; Dahlström et al 2004; Vinh and Embil 2005). Biofilms are formed by bacteria and/or other microorganisms attaching to biotic or abiotic surfaces and then forming microbial colonies and encased in the EPS. (Galván et al 2016). Studies have shown that the biofilm structures protect indwelling organisms from physical and chemical ‘attacks’, including oligotrophic environment, flow erosion and high concentration of bactericides (Stoodley et al 2001; Dolan and Costerton 2002).

In the interest of reducing biofilms, innovative inhibition and eradication strategies are constantly being sought (Mah and O’Toole 2001; Joshi et al 2009). Among these strategies, micro-patterned surfaces have been found to reduce bacteria settlement and cause no harm to the environment (Callow et al 2000; Baier 2006; Scardino and de Nys 2011). Some biomimetic surfaces with micro-patterns have been developed and their ability to inhibit the microorganism settlement

explored (Vejborg and Klemm 2008). Metal surfaces with nano-scale topographies have been processed and found to mitigate biofilm formation through the bactericidal effects (Sapkota et al. 2011). Several commercial micro-patterned polydimethyl siloxane (PDMS) surfaces have also been reported to reduce bacteria coverage (Ling et al. 2014). However, it has been reported that micro-patterned surfaces, with certain pattern sizes and under certain flowing conditions, might aggravate biofilm formation and, filamentous structures, also known as streamers, might form on such surfaces and act as precursors to matured biofilms (Valiei et al. 2012; Vucko et al. 2014; Hassanpourfard et al. 2016). Even though the body of research on micro-patterned surfaces has increased in recent years, there remains a need for studying the biofilm inhibitory effect of these surfaces when considering the operating conditions of the TSSHPS, which is a practical system already in use, e.g. the flowing conditions in the heat exchangers and the bacteria specific to the treated sewage.

For this work, micro-patterned surfaces with different pattern shapes and sizes were fabricated for biofilm inhibition. Model sewage was prepared and a rotating coupon device was used to simulate the operating conditions in the TSSHPS. Different testing methods were used to analyze the biofilms on the micro-patterned surfaces. Results at both macro- and micro-levels have been obtained to investigate the biofilm inhibitory effect and possible inhibitory mechanisms of the micro-patterned surfaces.

Materials and methods

Micro-patterned surface processing

The micro-patterned surfaces in the experiments were prepared using the wafer photolithography technology described by Lopez et al. (2010). Briefly, 4-inch N-type wafers (Topvondor Tech., Beijing, China), which were polished on both sides, 1mm thick and with a Si purity of 99.9999%, were cleaned with a mixture of sulfuric acid and hydrogen peroxide for 10 min and then washed in an ultrasonic cleaner for 30 min. Then EPG-590, a positive photoresist, was spin-coated on the wafers at a speed of 5,000 rpm for 30 seconds for a desired thickness. The photoresist-coated wafers were

exposed through a photomask (Institution of Microelectronics of Chinese Academy of Sciences, Beijing, China), and the light-transmitting zones in the photomasks were designed with different shapes (stripe and cube) and sizes (1, 2, 5, 10, 20, 50, and 100 μm). The wafers were transferred to a high resolution mask aligner (ABM, Beijing, China) with an exposure time of 5 s. Finally the wafers were etched and immersed in photoresist developer to form micro-patterns, all with a height of 1 μm , as the diameter of the bacteria used in this study was close to 1 μm . Some of the accordingly developed micro-patterned surfaces were shown in Fig. 1.

Then the wafers were laser cut (Foxconn Nano Center, Beijing, China) into square coupons with the size of 20 mm $\times$ 20 mm $\times$ 1 mm to fit the rotating coupon device. Prior to use, the coupons were washed in ultrafiltered water and then a mixture of ethanol and acetone, and ultrasonic cleaned at 50°C for 120 min, to remove impurities such as micro silicon debris, unwanted bacteria, and chemical residuals on the surfaces.

Bacteria culture and model sewage preparation

A previous study by Tian et al. (2012a) has monitored the water quality of the treated sewage in six sewage plants in Beijing, and the results were used to determine the water quality parameters, e.g. temperature, inorganic ion concentrations, biological oxygen demand (BOD) and pH, for model sewage preparation under laboratory conditions in this study.

Polymerase chain reaction and 16S rRNA analysis were performed on the treated sewage and the results showed that *Bacillus* sp. was the dominant bacteria in the treated sewage ($\geq 62\%$). *B. subtilis* was commonly found in soil and organic sewage and was identified in the sewage in Beijing (Tian et al. 2012a). Studies have shown that glucose, fructose, sucrose and protein were the main EPS components of *B. subtilis* and these components produced in biofilms were significantly more than those produced by bacteria in culture solutions (Harmawan and Ting 2016). In this study, *B. subtilis* was used for model sewage preparation and was kindly provided by the Biomedical Engineering Department in Tsinghua University.

B. subtilis solution was mixed with 100% sterilized glycerol at a 1:1 ratio, then precooled in a -20°C refrigerator, and stored in a -80°C refrigerator. For each trial, the stored samples were first plate streaked on LB agars (Thermo Fisher, MA, USA), which were then incubated at 30°C for 24 h. A single colony formed on the plates was picked and inoculated to the culture solution, which consisted of 1 wt% glucose, 1wt% peptone, 0.5 wt% beef extract, and 0.5 wt% NaCl (Sinopharm, Beijing, China). Prior to inoculation, the culture solution was autoclaved at 121°C for 30 min. The inoculated culture solution was then incubated in a shaker at 30°C, 200 rpm for 24 h to reach a final bacterial concentration of approximately 1.0×10^9 CFU/ml.

Then the bacterium solution was diluted to ultra-filtered water to reach a concentration of 1×10^5 CFU/ml, which was the average value of bacteria concentration of the treated sewage during summer operating conditions in the TSSHPS.

Solutions of glucose, sodium hydroxide and calcium chloride were added to the bacterium solution to adjust the BOD, pH and the concentration of Ca^{2+} to 15 mg/l, 7.2 and 65 mg/l, which were the average values of the treated sewage during summer operating conditions.

Thus, the model sewage with certain bacteria concentration and water quality parameters was prepared and then used for biofilm formation.

The rotating coupon device and experimental procedure

In this study, a rotating coupon device (Motian Tech., Suzhou, China) was used to form biofilms on the micro-patterned surfaces. The schematic diagram of the setup is shown in Fig. 2.

First, three wafer coupons with micro-patterned surfaces were fixed using screw nuts onto the rotating shaft. The angular velocity was set at 96 rpm, and the linear velocity at the micro-patterned surfaces was 0.4 m/s, which was a common flow velocity in plate heat exchangers. Then the prepared model sewage was contained in a polyethylene beaker and the coupons were immersed and rotated in the model sewage. The beaker was placed in the thermal controlled

water bath of the rotating coupon device and the temperature was set at 25°C, which was the average temperature of the treated sewage during summer operating conditions of the TSSHPS. A T thermal couple (Omega, Connecticut, USA) was placed in the beaker to monitor the temperature of the model sewage. The results showed that the temperature reached 25°C in less than 30 min after the beaker was placed in the water bath, and remained at $25 \pm 0.2^\circ\text{C}$ during the whole process.

The biofilm-formation experiments lasted for 5 days. Initial trials by Yang et al. (2013), which lasted over 30 days, indicated that 5 days were sufficient for the biofilm growth to reach the stable phase.

In this study, as the model sewage was contained in a closed system, the nutrient would be consumed and the bacteria viability might decrease over time. So during the experiments, every 12 h fresh model sewage was prepared to replace the old model sewage in the beaker. LB agars were used to monitor the bacteria concentration in the beaker during the 12 h period (Alavi and Hansen 2013). Every 2 h since the beginning of the 12 h period, 1 ml model sewage in the beaker was diluted and spread on the agars. The number of colonies was counted to calculate the bacteria concentration in the model sewage.

Biofilm analysis

Biofilm wet weight

As the biofilm thermal resistance, which is positively correlated with the wet weight of the biofilms (Tian et al 2012a; Yang et al 2013), is the main interest in this study, the biofilm weight wet was weighed every 24 h during the experiments. Prior to being weighed, the coupons were taken off from the shaft and suspended vertically in air for 5 min to drain off the water droplets of the biofilms. A previous study by Yang et al (2015) showed that 5 min was long enough for all the extraneous water to be eliminated. The remaining water was stabilized water in the biofilms, and such static water led to thermal resistance increase in the heat exchangers and should not be eliminated. The coupons were then weighed using a

XS105 balance (Mettler Toledo, Zurich, Switzerland) with a precision of ± 0.01 mg. The biofilm wet weight per unit area, m_f , was defined as:

$$m_f = \frac{M_f - M_c}{S} \quad (1)$$

where M_f is the weight of the coupon with biofilm (g), M_c is the weight of the clean coupon (g), S is the base area of the coupon without the influence of the patterns (m^2) and m_f is the biofilm wet weight per unit area (g/m^2).

Scanning electron microscopy

An SEM (FEI, Oregon, USA) was used to visualize the bacteria settlement and the biofilm structures. Prior to being visualized, the biofilms should be pretreated. Briefly, the 24 h, 72 h and 120 h biofilms on the coupon surfaces were rinsed in phosphate buffer solution (PBS) for 10 min to remove the loosely attached cells. Then the biofilms were fixed using 2.5% (w/v) glutaraldehyde at 4°C for 2 h. Samples were rinsed in PBS for three times, 5 min each time. Then the biofilms were dehydrated using ethanol solutions of 50% (v/v), 70%, 90%, 100%, 100% and 100% for 10 min each, followed by overnight drying in a freeze drier (Alpha1-2LDplus, CHRIST, Beijing, China). Dried samples were mounted to SEM stubs and coated with evaporated gold for 60 s. A voltage of 15 kV was used for SEM photography of these samples.

Confocal laser scanning microscopy

CLSM was used to assess the cell viability, 3D structure, biovolume and thickness of the biofilms. First, the 24 h, 72 h and 120 h biofilms were dyed with BacLight® bacterial viability kit (Invitrogen, California, USA) according to the manufacturer's instructions. The kit included two fluorescent nucleic acid stains, the red-fluorescent propidium iodide and the green-fluorescent SYTO® 9. Briefly, the two stains were mixed at a 1:1 ratio, and 6 μl of the mixed stain was added to 1 ml of deionized water. The biofilms on the coupons were then covered with 500 μl of the solution and stained for 25 min in the dark, followed by washing with deionized water for 5 min to remove the stain residuals.

The stained coupons were then put on the objective stage of a LSM-710 CLSM (Carl Zeiss, Oberkochen, Germany) and

the biofilms were pictured with a 40× objective lens. Five random fields of view were imaged for each sample for quantitative analysis (biovolume and thickness). Each field covered a surface area of $212.3 \times 212.3 \mu\text{m}^2$ and Z-stack images were obtained using the ZENS2010 software. Shooting parameters were set at: pinhole 46.7 μm ; laser intensity 1.5%; master gain 550 for PI and SYTO® 9 and 8 bit image resolution with 1024×1024 image size.

The image stacks were imported to the software IMARIS (Bitplane, Zurich, Switzerland), and the “Smart 3D” function in the software was used for 3D reconstruction of the biofilm images. The “Surpass” function was used to show the biofilm thickness. The “Surfaces” function, a sub-function of the “Surpass” function, with a surfaces area detail level of 0.1 μm was used to calculate the biovolume of the live and dead cells.

Statistical analysis

In this study, the results are reported as mean value $\pm$ standard deviation, and analyzed with one-way analysis of variance (ANOVA). All statistical analyses were performed with MATLAB (version 8.3, Mathworks, MA, USA).

Results

Bacteria concentration of the model sewage

In this study, plate counting was used to monitor the bacteria concentration of the model sewage in every 12 h period, and the result was shown in Fig. 3. It could be seen that the bacteria concentration of the model sewage during the 12 h period was constant at $(1.0 \pm 0.2) \times 10^5$ CFU/ml and did not change significantly ($F=0.53$, $p=0.75$).

Biofilm wet weight

During the experiments, the coupons were weighed every 24 h. The biofilm wet weight development and the 120 h final biofilm wet weight on various surfaces were shown in Fig. 4.

As can be seen in Fig. 4, the biofilm wet weight on the control group surfaces (plain surfaces without any patterns) increased over time, reaching a final value of 350~450 g/m² at 120 h. In addition, micro-patterned surfaces with small

pattern sizes (1 μm and 2 μm) inhibited biofilm wet weight growth significantly when compared to the control group surfaces, while there was no significant biofilm wet weight decrease on other micro-patterned surfaces.

To get a further understanding of the influence of the pattern shape and size, the biofilm wet weight of the 120 h biofilms on various surfaces was shown in Fig. 4C and Fig. 4D. The results showed that, for the stripe surfaces, 1, 2 and 5 μm surfaces decreased biofilm wet weight significantly ($p < 0.01$), and for the cube surfaces, 1 and 2 μm surfaces decreased biofilm wet weight significantly ($p < 0.01$). For both patterns, surfaces with pattern sizes larger than 10 μm hardly showed any inhibitory effect ($p > 0.05$).

The statistics and the ANOVA results of the 120 h biofilm wet weight were shown in Table 1. The results indicated that, for both patterns, 1 μm and 2 μm surfaces inhibited biofilm formation significantly ($p < 0.01$ and $p < 0.01$), and the 5 μm surfaces inhibited biofilm formation as well, but less significantly ($p < 0.01$ and $p < 0.05$). Most surfaces with larger micro-patterns showed no inhibitory effect ($p > 0.05$). For the two micro-pattern shapes, there was no significant difference between each other with p in most groups larger than 0.05.

SEM imaging of the biofilms

Bacteria attached to the surfaces and the structure of the biofilms developed over time. SEM was used to observe the topographies of the 24 h, 72 h and 120 h biofilms on various surfaces. Fig. 5 showed the SEM pictures of biofilms on 1 μm , 20 μm micro-patterned surfaces and the control group surfaces. Topographies of the biofilms on other micro-patterned surfaces (2, 5, 10, 50, 100 μm , data not shown) followed a similar trend noted in Fig. 5.

As can be seen in Fig. 5-A1 and B1, for the 24 h biofilms, single rod-shaped cells attached to the 1 μm micro-patterned surfaces and thin layers of bacterial colony were formed. Besides, there were streamers on the 1 μm stripe surface, which was consistent with the study by Valiei et al (2012). However, for the 24 h biofilms on the 20 μm micro-patterned surfaces and control group surfaces, a large amount of bacteria settled and biofilms with three-dimensional structures

were formed (Fig. 5-C1, Fig. 5-D1 and Fig. 5-E1)..

For the 72 h biofilms, more cells settled to the 1 μm surfaces and more streamers were formed. These streamers created a web-like structures and thin layers of biofilm were formed (Fig. 5-A2 and Fig. 5-B2). On the 20 μm and control group surfaces, large bulks of biofilm with three-dimensional porous structures were formed and covered the majority of the solid substrates (Fig. 5-C2, Fig. 5-D2 and Fig. 5-E2).

For the 120 h biofilms on the 1 μm surfaces, thin EPS layers covered the whole substrates and formed several small protuberances (Fig. 5-A3 and Fig. 5-B3). On the 20 μm and control group surfaces, matured biofilms with certain thickness covered the whole substrates and the original micro-patterns could not been seen anymore (Fig. 5-C3, Fig. 5-D3 and Fig. 5-E3).

CLSM imaging of the biofilms and quantitative analysis

24 h, 72 h and 120 h biofilms were dyed with vital stains and imaged with a CLSM. The CLSM pictures of biofilms on 1 μm , 20 μm and control group surfaces were shown in Fig. 6. As could be seen in Fig. 6, the structural development of biofilms on these surfaces was consistent with that indicated by the SEM pictures.

At 24 h, on the 1 μm surfaces, only several single cells attached to the surfaces. Furthermore, it could be seen that on these surfaces, most of the rod-shaped bacteria settled along the bottom of the micro-patterns and the outlines of the patterns could be seen (Fig. 6-A1 and Fig. 6-B1). On the 20 μm and control group surfaces, clusters of bacteria and layers of biofilm were observed (Fig. 6-C1, Fig. 6-D1 and Fig. 6-E1).

At 72 h, on the 1 μm surfaces, both single cells and thin biofilms could be observed (Fig. 6-A2 and Fig. 6-B2). On the 20 μm and control group surfaces, the biofilms developed into a thick and porous structure (Fig. 6-C2, Fig. 6-D2 and Fig. 6-E2).

At 120 h, on the 1 μm surfaces, thin biofilms with filament EPS and small protuberances were formed (Fig. 6-A3 and Fig. 6-B3). On the 20 μm and control group surfaces, matured biofilms developed into a dense structure (Fig. 6-C3, Fig. 6-

D3 and Fig. 6-E3).

Furthermore, as can be seen in Fig. 6, the majority of the fluorescence is green and very little red fluorescence could be observed, suggesting the majority of the cells in the biofilms were live cells.

Imaris was used for quantitative analysis of the CLSM pictures. The calculated biovolume and thickness of the biofilms on various surfaces were shown in Fig. 7. It can be seen that the 1 μm and 2 μm surfaces inhibited biofilm formation, with both the biovolume and biofilm thickness significantly less than the control group ($p < 0.01$). However, surfaces with micro-pattern sizes larger than 5 μm showed no significant inhibitory effect ($p > 0.05$), and the biovolume and biofilm thickness on some of these surfaces were significantly larger than those on the control group. When comparing the two patterns, it could be observed that there was no significant difference found between the biofilms on stripe surfaces and cube surfaces ($p > 0.05$). This is in line with the results of the biofilm wet weight.

Discussion

Numerous studies have been carried out to investigate the formation and inhibition of biofilms. In some of these studies, open circulation systems were used to provide biofilm-forming fluids with steady bacteria concentrations (Percival et al. 1999; Sanchez et al. 2013). However, more chose to place the substrates in closed systems, such as Petri dishes or 96-well plates (Martino et al. 2003; Machado et al. 2015; Brambilla et al. 2016). As the biofilm-formation experiments in such studies did not last long (usually less than 48 h), not many has considered the decline of bacteria concentration that might happen in such closed systems.

Studies have shown that bacteria concentration and nutrient level had significant influences on biofilm formation (Cerca et al. 2004). In this study, as the biofilm-formation experiments lasted for 120 h and the model sewage was contained in a closed system, the possible decline of bacteria concentration must be considered. The bacteria concentration of the model sewage was monitored, and the results showed that the bacteria concentration did not change significantly over time

during the 12 h period experiments, so it could be considered that the model sewage had a stable biofilm-forming ability. The bacteria concentration not decreasing significantly over the 12 h period might be due to the fact that the bacteria solution was diluted and some original culture solution was added to the model sewage. Besides, glucose was added to the model sewage to adjust the BOD to 15 mg/l, so with the low bacteria concentration and extra nutrient in the model sewage, the bacteria would not die during the 12 h period and the bacteria concentration could remain at a constant level.

In nature, the majority of bacteria exist in biofilms and live on surfaces or at interfaces (Branda et al. 2005). Bacteria in the liquids prefer to settle on surfaces and then secrete EPS to form biofilms. In this study, biofilms were formed on the coupon surfaces as the coupons were immersed and rotated in the model sewage. The biofilm wet weight on the control group surfaces increased over time and the final biofilm wet weight at 120 h was consistent with the stable biofilm wet weight in the 30-day trials (Yang et al. 2013).

From the results of biofilm wet weight development described in Fig. 4, it is evident that surfaces with small pattern sizes inhibited biofilm formation. Similar trends have been observed in previous studies (Vucko et al. 2014). Micro-patterned PDMS surfaces with different pattern sizes have been developed and immersed in seawater and solutions containing a wide range of marine biofilm-forming organisms. It was found that the settlement was inhibited on surfaces with pattern sizes close to the sizes of the organisms. The author explained it with the attachment point theory, i.e. surfaces with pattern size close to the size of the bacterium provide less attachment points than surfaces with large or small pattern size. Fig. 8 is a schematic diagram illustrating the attachment point theory. When the attachment points are decreased, the substrate and the bacteria become less connected and the settlement is considered more unstable.

In this study, surfaces with pattern size ranging from 1 to 100 μm were designed to testify the attachment point theory. The results showed that, *B. subtilis*, a rod-shaped bacterium with 1 μm in diameter and 3 μm in length, settled significantly less on the 1 μm and 2 μm surfaces, and surfaces with pattern sizes larger than 5 μm barely showed any inhibition. So this

result could be explained with the attachment theory, i.e. the inhibition of bacteria settlement and subsequent biofilm formation was due to that 1 μm and 2 μm surfaces provided less attachment points and the bacteria was less steadily settled on these surfaces.

However, in addition to attachment point theory, other researchers have explained the biofilm inhibitory effect of micro-patterned surfaces with the change of wettability, surface free energy, or thermodynamical state (Kananeh et al 2010; Decker et al 2013; Vasudevan et al 2014). Most of these experiments were carried out under static immersion conditions. Some has considered the influence of flowing conditions (Halder et al 2014), and the results showed that the velocity fields and shear rate fields on the patterned surfaces were more non-uniform and the bacteria settlement was disturbed. It has been reported that the shear stress on surfaces with smaller microstructures varied more rapidly (Zussman et al 2002). In this study, the results showed that surfaces with small pattern sizes inhibited biofilm formation significantly. As the experiments were performed under flowing conditions, it is possible that the flow field was more disrupted on the surfaces with small patterns, thus inhibiting the bacteria settlement. So the flow field disturbance is a potential explanation for the inhibition of biofilm formation of these surfaces. Further study is being carried out using CFD simulations to understand how the flow field changed on such surfaces and will be presented in the near future.

Bacteria settlement is the initial step of biofilm formation, and many previous studies have proposed that the biofilm mitigation could be attributed to the bacteria settlement inhibition (Vucko et al 2014; Moreira et al 2015). With less bacteria cells attached to the surfaces, the subsequent cell reproduction and EPS secretion would be reduced and the biofilm formation would be inhibited. A similar phenomenon was observed in this study. It could be seen in Fig. 5 that at 24 h, surfaces with small micro-patterns inhibited bacteria settlement significantly, so for the 120 h biofilms, when matured and dense biofilms were formed on the control group surfaces, only thin layers of biofilms were observed on the 1 μm surfaces. The inhibition of the initial bacteria settlement on the 1 μm and 2 μm surfaces might be due to the fact that

these surfaces, whose pattern sizes were close to the bacterium, provided less attachment points (as illustrated in Fig. 8).

As the experiments were performed under flowing conditions, these less steadily settled bacteria might be swept away from the surfaces.

However, it has been reported that the micro-patterned surfaces might lose the biofilm inhibitory effect after being immersed in seawater for a long period of time, mainly due to the fact that the surfaces were totally covered by biofilms (Ling et al. 2014). The micro-patterned surfaces used in this study might have the same problem, so further studies with long-period experiments are needed to investigate the biofilm inhibitory effect of the surfaces after being totally covered.

In this study, CLSM was used to investigate the structural development and bacteria viability in the biofilms. The results suggested that the bacteria preferred to settle to the gaps instead of the top of the micro-patterns (Fig. 6-A1 and Fig. 6-B1). Studies explained this with the relatively static flowing conditions in the recessed regions (Hou et al. 2011).

In addition, the results suggested that, in the biofilms formed on the micro-patterned surfaces, the majority of the cells were viable (Fig. 6-A to Fig. 6-D). In previous studies (Sapkota et al. 2011; Al-Fori et al. 2014), surfaces with zinc oxide (ZnO) nanorods have been developed to inhibit biofilm formation, and it was found that the mortality of the settled organisms increased significantly and the biofilm inhibition was mainly due to the bactericidal effect of the ZnO nanorods. In this study, however, the micro-patterned surfaces did not kill the bacteria, so the biofilm inhibition was most likely due to certain physical processes, as described by the attachment point theory and the flow field disturbance, instead of any bactericidal effect.

Furthermore, it was interesting to notice that in Fig. 7A, for most surfaces, the biovolume decreased at 120 h, which did not match the biofilm wet weight development. This might be caused by the block of the fluorescent signal. The matured 120 h biofilms have developed into a certain thickness, and the fluorescent signals of the cells in the bottom of the biofilms were blocked by the bacteria cells, the EPS and the water in the top layer. Thus the bottom signals were very weak and

could not be received by the CLSM, and the accordingly calculated biovolume would be smaller than the true value. In addition to that, this difference in biofilm wet weight and biovolume might be caused by the formation of spores as well. *B. subtilis*, a spore forming organism, could become spores in low nutrient or high metabolite conditions (Setlow et al. 2008). In this study, the bacteria in the bottom of the 120 h biofilms might become spores due to the lack of nutrient and the abundance of accumulated metabolite. Such spores would not be dyed by the fluorescent stains, thus no signal could be received by the CLSM. So the accordingly calculated biovolume might decrease when compared to that of the 72 h biofilms. Besides, using the thickness (μm) shown in Fig. 7B and biofilm density (kg/m^3) to roughly estimate the biofilm wet weight, the estimated value would be significantly lower than the measured value shown in Fig. 4. This is mainly due to that the biofilms were compressed between the coupon and a supporting cover slip as the CLSM worked in an inverted mode. So the biofilm thickness might decrease as the biofilms were compressed and the accordingly estimated biofilm wet weight would be less than the measured value.

In conclusion, from the results described in this study, surfaces with small pattern sizes ($1\ \mu\text{m}$ and $2\ \mu\text{m}$) inhibited biofilm formation significantly and the inhibitory effect showed no significant difference between the two pattern shapes. However, further studies are needed to understand the exact mechanism for biofilm inhibition of these surfaces and to investigate the biofilm inhibitory effect in long-period experiments.

Acknowledgments

This work was supported by The National Natural Science Foundation of China (grant no. 51476090 and grant no. 50976060) and The Science Fund for Creative Research Group (grant no. 51321002).

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Tables

Table 1. The 120 h biofilm wet weight (g/m²) on various surfaces and the ANOVA results

	1 μm	2 μm	5 μm	10 μm	20 μm	50 μm	100 μm	control group
Stripe	227.3±17.7	252.5±23.2	269.9±21.8	403.8±6.2	356.8±11.2	333.9±16.5	359.3±25.0	392.1±23.4
surfaces	**	**	**	-	-	*	-	
Cube	214.3±20.3	272.9±19.0	329.4±15.2	342.8±10.5	425.3±25.5	322.7±23.4	382.5±19.8	372.1±14.8
surfaces	**	**	*	-	-	-	-	
Pattern								
difference	-	-	* Stripe	** Cube	* Stripe	-	-	-

Note: The 120 h biofilm wet weight was shown in a way of mean value ± standard deviation, and one-way ANOVA was performed to analyze the significant difference. The 1st line showed the sizes of the patterns. The 2nd and 3rd lines showed the mean value and standard deviation of the final biofilm wet weight and the significant difference when compared to the control groups, on stripe and cube surfaces respectively. The 4th line showed the significant difference between the biofilm wet weight on surfaces with the same pattern size but different pattern shapes, and which exhibited a stronger inhibitory effect. (-, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$. $n=3$).

Figure captions

Figure 1. SEM pictures illustrating the topographies of the micro-patterned surfaces. The micro-patterns were fabricated on 4-inch wafers using the photolithography technology. Scale bar (on the right lower corner of each figure) = 100 μm , the heights of all patterns were 1 μm . Pattern size: A and D, 5 μm ; B and E, 20 μm ; C and F, 100 μm . Pattern shape: A-C, stripe; D-F: cube.

Figure 2. Schematic diagram of the rotating coupon device, which was used to simulate the biofilm formation process in the TSSHPS (A. Front view, B. Top view). The model sewage was contained in a beaker, which was placed in the water bath to maintain the temperature of the model sewage at 25°C. The coupons were fixed onto the shaft and rotated in the model sewage at 96 r/min.

Figure 3. Bacteria concentration in the model sewage during the 12 h periods. The results were shown in a way of mean value $\pm$ standard deviation ($n=10$). Even though the model sewage was contained in a closed system, the bacteria concentration did not change significantly during the 12 h periods ($F=0.53$, $p=0.75$).

Figure 4. The biofilm wet weight development during the 120 h biofilm formation process and the final (120 h) biofilm wet weight. The results were shown in a way of mean value $\pm$ standard deviation ($n=3$). (A) Biofilm wet weight development on stripe pattern surfaces. (B) Biofilm wet weight development on cube pattern surfaces. (C) 120 h biofilm wet weight on stripe pattern surfaces. (D) 120 h biofilm wet weight on cube pattern surfaces. The results indicated that the 1 μm and 2 μm surfaces inhibited biofilm growth significantly, and surfaces with pattern sizes larger than 10 μm showed no significant inhibitory effect. For the two micro-pattern shapes, there was no significant difference between each other.

Figure 5. SEM pictures illustrating the micro-structures of the biofilms. A1-A3, 24 h, 72 h and 120 h biofilms on 1 μm stripe surfaces; B1-B3, 24 h, 72 h and 120 h biofilms on 1 μm cube surfaces; C1-C3, 24 h, 72 h and 120 h biofilms on 20

μm stripe surfaces; D1-D3, 24 h, 72 h and 120 h biofilms on 20 μm cube surfaces; E1-E3, 24 h, 72 h and 120 h biofilms on control group surfaces. At 24 h, 72 h and 120 h, thicker and more inhomogeneous biofilms were observed on the 20 μm and control group surfaces, compared to the biofilms on the 1 μm surfaces. The 1 μm surfaces significantly inhibited bacteria settlement and the subsequent biofilm formation. Scale bar (on the right lower corner of each figure) = 100 μm .

Figure 6. CLSM pictures illustrating the micro-structures and the cell viability of the biofilms. A1-A3, 24 h, 72 h and 120 h biofilms on 1 μm stripe surfaces; B1-B3, 24 h, 72 h and 120 h biofilms on 1 μm cube surfaces; C1-C3, 24 h, 72 h and 120 h biofilms on 20 μm stripe surfaces; D1-D3, 24 h, 72 h and 120 h biofilms on 20 μm cube surfaces; E1-E3, 24 h, 72 h and 120 h biofilms on control group surfaces. Green fluorescence indicated live bacteria cells. Red fluorescence, though could hardly be observed, indicated dead bacteria cells. The structural development of the biofilms showed a similar trend illustrated in Fig. 5. Most of the bacteria cells settled to the bottom of the patterns and the outline of the patterns could be seen in A1, A2, B1 and B2. The majority of cells were live cells (green fluorescence), indicating the micro-patterned surfaces showed no bactericidal effect. Scale bar (on the right lower corner of A1) = 100 μm .

Figure 7. Quantitative analysis of the 24 h, 72 h and 120 h biofilm CLSM pictures. (A) Biovolume (B) Biofilm thickness. The results were shown in a way of mean value $\pm$ standard deviation ($n=5$). The biovolume and biofilm thickness on 1 μm and 2 μm surfaces were significantly decreased. For the two pattern shapes, there was no significant difference between each other.

Figure 8. Schematic diagram illustrating the attachment point theory. A, surfaces with pattern size much smaller than the bacterium size. B, surfaces with pattern size close the bacterium size. C, surfaces with pattern size much larger than the bacterium size. Dark gray, the settled bacterium. Light gray, the substrate with micro-patterns. It could be seen that surface B provides only two attachment points, while surface A and C provide attachment zones. So the bacteria settlement on surface B is considered less stable.

Figure 1. SEM pictures illustrating the topographies of the micro-patterned surfaces. The micro-patterns were fabricated on 4-inch wafers using the photolithography technology. Scale bar (on the right lower corner of each figure) = 100 μm , the heights of all patterns were 1 μm . Pattern size: A and D, 5 μm ; B and E, 20 μm ; C and F, 100 μm . Pattern shape: A-C, stripe; D-F: cube.

167x94mm (200 x 200 DPI)

Figure 2. Schematic diagram of the rotating coupon device, which was used to simulate the biofilm formation process in the TSSHPS (A. Front view, B. Top view). The model sewage was contained in a beaker, which was placed in the water bath to maintain the temperature of the model sewage at 25°C. The coupons were fixed onto the shaft and rotated in the model sewage at 96 r/min.

80x129mm (200 x 200 DPI)

Figure 3. Bacteria concentration in the model sewage during the 12 h periods. The results were shown in a way of mean value $\pm$ standard deviation ($n=10$). Even though the model sewage was contained in a closed system, the bacteria concentration did not change significantly during the 12 h periods ($F=0.53$, $p=0.75$).

80x55mm (300 x 300 DPI)

Figure 4. The biofilm wet weight development during the 120 h biofilm formation process and the final (120 h) biofilm wet weight. The results were shown in a way of mean value $\pm$ standard deviation ($n=3$). (A) Biofilm wet weight development on stripe pattern surfaces. (B) Biofilm wet weight development on cube pattern surfaces. (C) 120 h biofilm wet weight on stripe pattern surfaces. (D) 120 h biofilm wet weight on cube pattern surfaces. The results indicated that the 1 μm and 2 μm surfaces inhibited biofilm growth significantly, and surfaces with pattern sizes larger than 10 μm showed no significant inhibitory effect. For the two micro-pattern shapes, there was no significant difference between each other.

180x139mm (300 x 300 DPI)

Figure 5. SEM pictures illustrating the micro-structures of the biofilms. A1-A3, 24 h, 72 h and 120 h biofilms on 1 μm stripe surfaces; B1-B3, 24 h, 72 h and 120 h biofilms on 1 μm cube surfaces; C1-C3, 24 h, 72 h and 120 h biofilms on 20 μm stripe surfaces; D1-D3, 24 h, 72 h and 120 h biofilms on 20 μm cube surfaces; E1-E3, 24 h, 72 h and 120 h biofilms on control group surfaces. At 24 h, 72 h and 120 h, thicker and more inhomogeneous biofilms were observed on the 20 μm and control group surfaces, compared to the biofilms on the 1 μm surfaces. The 1 μm surfaces significantly inhibited bacteria settlement and the subsequent biofilm formation. Scale bar (on the right lower corner of each figure) = 100 μm .

131x191mm (300 x 300 DPI)

Figure 6. CLSM pictures illustrating the micro-structures and the cell viability of the biofilms. A1-A3, 24 h, 72 h and 120 h biofilms on 1 μm stripe surfaces; B1-B3, 24 h, 72 h and 120 h biofilms on 1 μm cube surfaces; C1-C3, 24 h, 72 h and 120 h biofilms on 20 μm stripe surfaces; D1-D3, 24 h, 72 h and 120 h biofilms on 20 μm cube surfaces; E1-E3, 24 h, 72 h and 120 h biofilms on control group surfaces. Green fluorescence indicated live bacteria cells. Red fluorescence, though could hardly be observed, indicated dead bacteria cells. The structural development of the biofilms showed a similar trend illustrated in Fig. 5. Most of the bacteria cells settled to the bottom of the patterns and the outline of the patterns could be seen in A1, A2, B1 and B2. The majority of cells were live cells (green fluorescence), indicating the micro-patterned surfaces showed no bactericidal effect. Scale bar (on the right lower corner of A1) = 100 μm.

125x176mm (300 x 300 DPI)

Figure 7. Quantitative analysis of the 24 h, 72 h and 120 h biofilm CLSM pictures. (A) Biovolume (B) Biofilm thickness. The results were shown in a way of mean value $\pm$ standard deviation ($n=5$). The biovolume and biofilm thickness on 1 μm and 2 μm surfaces were significantly decreased. For the two pattern shapes, there was no significant difference between each other.

180x68mm (300 x 300 DPI)

Figure 7. Quantitative analysis of the 24 h, 72 h and 120 h biofilm CLSM pictures. (A) Biovolume (B) Biofilm thickness. The results were shown in a way of mean value $\pm$ standard deviation (n=5). The biovolume and biofilm thickness on 1 μ m and 2 μ m surfaces were significantly decreased. For the two pattern shapes, there was no significant difference between each other.

51x14mm (300 x 300 DPI)

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