Micro/nano-structured TiO$_2$ surface with dual-functional antibacterial effects for biomedical applications

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**ABSTRACT**

Implant-associated infections are generally difficult to cure owing to the bacterial antibiotic resistance which is attributed to the widespread usage of antibiotics. Given the global threat and increasing influence of antibiotic resistance, there is an urgent demand to explore novel antibacterial strategies other than using antibiotics. Recently, using a certain surface topography to provide a more persistent antibacterial solution attracts more and more attention. However, the clinical application of biomimetic nano-pillar array is not satisfactory, mainly because its antibacterial ability against Gram-positive strain is not good enough. Thus, the pillar array should be equipped with other antibacterial agents to fulfill the bacteriostatic and bactericidal requirements of clinical application. Here, we designed a novel model substrate which was a combination of periodic micro/nano-pillar array and TiO$_2$ for basically understanding the topographical bacteriostatic effects of periodic micro/nano-pillar array and the photocatalytic bactericidal activity of TiO$_2$. Such innovation may potentially exert the synergistic effects by integrating the persistent topographical antibacterial activity and the non-invasive X-ray induced photocatalytic activity of TiO$_2$ to combat against antibiotic-resistant implant-associated infections. First, to separately verify the topographical antibacterial activity of TiO$_2$ periodic micro/nano-pillar array and the photocatalytic bactericidal activity of TiO$_2$, we systematically investigated its effects on bacterial adhesion, growth, proliferation, and viability in the dark without involving the photocatalysis of TiO$_2$. Moreover, the pillar array with sub-micron motif size can significantly inhibit the adhesion, growth, and proliferation of \textit{Staphylococcus aureus} (\textit{S. aureus}) and \textit{Escherichia coli} (\textit{E. coli}). Such antibacterial ability is mainly attributed to a spatial confinement size-effect and limited contact area availability generated by the special topography of pillar array. Therefore, the X-ray induced photocatalytic antibacterial property of TiO$_2$ periodic micro/nano-pillar array \textit{in vitro} and \textit{in vivo} will be systematically studied in a future work. This study could shed light on the direction of surface topography design for future medical implants to combat against antibiotic-resistant implant-associated infections without using antibiotics.

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1. Introduction

Medical implants have revolutionized modern medicine, but they also involve a higher risk of implant-associated infection that is a severe and frequent complication related to biomaterial applications [1–3]. In the USA, the implant-associated infections account for 25.6% of all healthcare-associated infections [4]. Notably, the implant-associated infections are generally difficult to cure owing to the bacterial antibiotic resistance [3] which is attributed to the widespread usage of antibiotics [5,6]. Many eventual failures of medical implants are caused by the bacterial infection at healing sites [7–11]. Currently, antibiotic-resistant infections result in 700,000 deaths per year all over the world [5]. If there is still no effective solution to combat against the antibiotic resistance, the number of global victims will rise dramatically to 10,000,000 per year by the middle of the 21st century [5].

Given the global threat and increasing influence of antibiotic resistance, there is an urgent demand to explore novel antibacterial strategies other than using antibiotics [12–20]. Recently, using a certain surface topography to provide a more persistent antibacterial solution attracts more and more attention [5,21–26]. Traditionally, researchers focused on the bacterial responses to the surfaces with different roughness values (Rₐ), but the conclusions were contradictory [27–31], because the surfaces with identical Rₐ values could possess totally different surface topographies and identical randomly textured surfaces can hardly be reproduced [32]. Alternatively, researchers turned to devote efforts in studying the bacterial responses on reproducible regular patterned surfaces which were inspired by natural antifouling surfaces [33]. In our previous studies, we demonstrated that Si periodic pillar arrays with sub-micron motif sizes could significantly inhibit bacterial adhesion, proliferation, and colonization by a spatial confinement size-effect [34,35]. Our findings suggested that nano-pillar array might be the optimal topography design against bacteria, which was coincident with the design of nature for the bactericidal topographies on the surface of cicada wing [36–41], dragonfly wing [38,41,42], and gecko skin [43]. The findings [34] were reported even before the first announcement of the natural bactericidal topography on the surface of cicada wing [36].

However, the clinical application of biomimetic nano-pillar array is not satisfactory [41], mainly because its antibacterial ability against Gram-positive strain including Staphylococcus aureus (S. aureus) is not good enough [37]. Since S. aureus usually causes implant-associated infections [3,44], the biomimetic nano-pillar array should be equipped with other antibacterial agents to fulfill the bacteriostatic and bactericidal requirements of clinical application. Recently, Ye et al. [41] combined a nano-pillar array and ZnO nanoslices to prepare a cicada & catkin inspired structure that was demonstrated to simultaneously have ideal biocompatibility, better bacterial anti-adhesive property, broader antibacterial range, and long-lasting antibacterial activity. ZnO-based nanomaterials possess high antibacterial activity against both Gram-positive and -negative bacterial strains [45–48], which has been mainly attributed to Zn²⁺ ions release, bacterial cell membrane damage, and photocatalytic generation of reactive oxygen species (ROS) [49,50].

Similar to ZnO, TiO₂ is another popular biocompatible and bioactive biomaterial [51–54] with photocatalytic antibacterial activity which has been ascribed to the generation of ROS that can oxidize organic materials up to complete mineralization [55,56]. Briefly, the organic material oxidization affects the bacterial membrane permeability first (Scheme 1a and 1b), then gradually damages all cell wall layers to make the bacteria leak small molecules and ions (like K⁺) (Scheme 1c) followed by higher molecular weight components (e.g., RNA and protein) (Scheme 1d) [56]. Eventually, the degradation of bacterial internal components occurs (Scheme 1e) followed by complete mineralization (Scheme 1f) [56]. UV induced photocatalysis on TiO₂ is well known [56], while the X-ray induced photocatalytic activity of TiO₂ has attracted much more attention for biomedical applications since it has a prominent characteristic that a non-invasive treatment can be achieved by adjusting the X-ray penetration depth through the human body [57]. This implies the X-ray irradiation may be used to intentionally trigger the photocatalysis of TiO₂ in vivo to kill the bacteria near or on the TiO₂ surface of a medical implant, which could inhibit the implant-associated biofilm formation or eliminate the already-formed biofilm without surgical or invasive treatments in a controllable manner.

Here, we aimed to design a novel model substrate which was a combination of periodic micro/nano-pillar array and TiO₂ for basically understanding the topographical bacteriostatic effects of periodic micro/nano-pillar array and the photocatalytic bactericidal activity of
TiO₂. Such innovation may potentially exert the synergistic effects by integrating the persistent topographical antibacterial activity and the non-invasive X-ray induced photocatalytic antibacterial property of TiO₂ to combat against antibiotic-resistant implant-associated infections. A high-throughput screening idea was introduced to fabricate one flat area as control and nine patterned areas (motif size 0.6μm–20μm) on a single chip for efficiently discovering which size of TiO₂ periodic pillar array possessed higher antibacterial activity. Sphere-shaped \textit{S. aureus} and rod-shaped \textit{Escherichia coli} (\textit{E. coli}) were employed in this work since they are common pathogens for medical implant infections \cite{3,44} and they have distinct morphologies. First, to separately verify the topographical antibacterial activity of TiO₂ periodic micro/nano-pillar array, we systematically investigated its effects on bacterial adhesion, growth, proliferation, and viability in the dark without involving the photocatalysis of TiO₂. Then, the X-ray induced photocatalytic antibacterial property of TiO₂ periodic micro/nano-pillar array \textit{in vitro} and \textit{in vivo} will be systematically studied in a future work.

2. Materials and methods

2.1. Preparation of TiO₂ periodic micro/nano-pillar arrays

2.1.1. Photomask design

A photomask design plays a vital role in the photolithography, since the final geometric shape of the micro/nano-scale topography is directly determined by the photomask design which should be placed at the very beginning in the micro/nano-scale topography fabrication process. In this study, we designed to locate one flat area and nine patterned areas on a single Si chip. Every two areas were insulated by a 4-μm-wide fence. As shown in Fig. S1, the pillar array motif size was defined as below:

\[ \text{pillar array motif size} = \text{pillar width} (W) = \text{pillar length} (L) = \text{spacing} (S). \]

There were nine different motif sizes corresponding to nine patterned areas (Fig. S1). Since some motif sizes were shorter than 1μm, a high-resolution mask aligner (ASML 5000 Stepper, Resolution = 0.5μm, Reduction ratio = 5:1) was used for photolithography in this study. The design of stepper photomask is schematically shown in Fig. S1. Owing to the reduction ratio (5:1) of the stepper, the original dimension of the pattern on the stepper photomask should be designed 5 times longer than the actual dimension of the motif.

2.1.2. Thermal SiO₂ growth

A SiO₂ layer which works as a mask when etches the Si substrate is needed to grow on the Si substrate surface before photolithography (Fig. 1a). In this step, the SiO₂ layer was generated by Si thermal oxidation other than SiO₂ deposition. A SiO₂ layer with a thickness around 100nm was obtained by a wet oxidation method using a diffusion furnace (ASM LB 45).

2.1.3. Photolithography

The photolithography consists of 8 sub-steps which are hexamethyldisilazane (HMDS) prime, spinning photoresist (Fig. 1a), soft baking, UV exposure (Fig. 1b), postexposure baking, development (Fig. 1b), hard baking, and de-scumming. All these sub-steps can be completed within a wafer track (SVG WaferTrack) automatically except for the UV exposure which should be conducted using the high-resolution mask aligner (ASML 5000 Stepper). The details of the 8 sub-steps are described in the Supplementary Data.

2.1.4. SiO₂ layer etching

The pattern was transferred from the photoresist layer to the SiO₂ layer (Fig. 1c) using an advanced oxide etching (AOE) machine (STS AOE Etcher, Surface Technology Systems, Coventry, UK). Since the plasma reactant has a much higher reaction rate with SiO₂ than with the photoresist, the SiO₂ layer covered by the photoresist pattern will be retained, while other uncovered regions of the SiO₂ layer will be etched by the plasma etchant. Here, the wafers were etched for 1 min to make sure that the uncovered SiO₂ layer (~100 nm thick) could be removed.
completely, since the SiO₂ etching rate was around 200 nm/min in the AOE machine.

2.1.5. Photoresist pattern striping

So far, the photoresist pattern had finished its mission as a mask during the SiO₂ layer etching and should be removed (Fig. 1d). Firstly, the photoresist pattern on the Si wafer was dry stripped for 20 min in a photoresist asher (PS210 PhotoResist Asher) after the step of SiO₂ layer etching. Secondly, the photoresist pattern on the Si wafer was wet stripped for 10 min in a H₂SO₄ + H₂O₂ bath at 120 °C to further remove any possible remaining photoresist.

2.1.6. Si substrate etching

We employed a deep reactive-ion etching (DRIE) machine (STS ICP DRIE Silicon Etcher) to etch the Si substrate for transferring the pattern from the SiO₂ layer to the Si substrate (Fig. 1e), which was a crucial step in the whole micro/nano-scale topography fabrication process since it directly and finally determined the geometric dimensions of the micro/nano-scale topography on the Si wafer. We used an etching program of which Si etching rate was around 0.143 μm/cycle to etch the Si wafer nano-scale topography on the Si wafer. We used a deep reactive-ion etching (DRIE) machine (STS ICP DRIE Silicon Etcher) to etch the Si substrate for transferring the pattern from the SiO₂ layer to the Si substrate (Fig. 1f), which was a crucial step in the whole micro/nano-scale topography fabrication process since it directly and finally determined the geometric dimensions of the micro/nano-scale topography on the Si wafer. We used an etching program of which Si etching rate was around 0.143 μm/cycle to etch the Si wafer for 21 cycles aiming at obtaining a final etching depth of around 3 μm.

2.1.7. SiO₂ pattern striping

We immersed the Si wafers into a buffered oxide etching (BOE) solution for 5 min to thoroughly remove the SiO₂ pattern which had finished its mission as a mask during the Si substrate etching. So far, the Si wafers with well-defined geometric Si periodic micro/nano-pillar arrays (Si scaffold) had been obtained (Fig. 1f).

2.1.8. Ti thin film deposition and thermal oxidation

The experimental procedure was conducted according to that in our previous study [58]. Briefly, a RF magnetron sputtering system (Model Explorer 14, Denton Vacuum, Moorestown, NJ, USA) equipped with a target of pure titanium disk (purity = 99.99%) was employed to deposit a pure titanium thin film onto the Si scaffold (Fig. 1g) [58]. The thin film deposition was conducted under an argon gas pressure of 5 × 10⁻³ Torr at a constant RF power of 100 W at 25°C for 300 s [58]. Then, the thin film was transformed from pure titanium to TiO₂ by a thermal oxidation at 700 °C with air flow for 1 h (Fig. 1h) [58].

2.2. Characterization of TiO₂ periodic micro/nano-pillar arrays

The morphologies of samples were examined using a field emission scanning electron microscope (FESEM; JSM-6700F, JEOL, Japan). The samples were pasted onto copper stubs using silver glue and double-sided carbon tapes [35,59,60] and coated with a gold thin film. To further decrease the surface charging effect during SEM observation, a low accelerating voltage (5 kV) was employed in this work [61–64].

Surface chemical compositions of samples were analyzed by an X-ray photoelectron spectrometer (XPS; Axis Ultra DLD, Kratos, Japan). The broad range XPS spectra were obtained with an Al Kα excitation source (hν = 1486.6 eV) and a take-off angle of 90° at a passing energy of 160 eV. The high-resolution spectra of the Ti 2p region were obtained with a passing energy of 40 eV.

An X-ray diffractometer (X Pert PRO, PANalytical, Netherlands) operating at 40 mA and 40 kV was employed to determine the crystal structure of the thermally oxidized thin film by a thin-film X-ray diffraction (TF-XRD) method [58]. The diffraction patterns were collected using Cu-Kα radiation (wavelength = 1.54056 Å) “in a 2θ range of 20°–80° with a step size of 0.05° and a count time of 2.5 s” [58].

The water contact angles (WCA) of samples were measured by a sessile drop method using a contact angle instrument (DIGHDROP, GBX, France). A motorized-syringe was employed to gently lay a droplet (4 μL) of deionized (DI) water onto a sample surface at room temperature. The instrument recorded the WCAs immediately after laying the droplet onto the sample surface within 30 s. Five measurements of sample WCAs were conducted and the average value corresponding to each sample was presented.

2.3. Bacterial experiments

2.3.1. Bacterial strain culture

As common pathogens for medical implant infections [3,44], S. aureus ATCC 25923 (sphere-shaped, Gram-positive) and E. coli JM109 (rod-shaped, Gram-negative) were involved in this study. The components of agar and liquid Luria-Bertani (LB) medium which were used as the culture media for both S. aureus and E. coli are listed in Table S1. The experimental procedure was conducted according to that in our previous study [35]. Briefly, “frozen S. aureus or E. coli strain was streaked on an agar plate and incubated at 37 °C for a minimum of 12 h” [35]. An aseptic micropipette tip was used to scrape off an as-prepared bacterial colony on the agar plate. Then, the micropipette tip was put into a test tube filled with LB liquid medium. The test tube was incubated in an orbital shaker at 37 °C for one incubation cycle which was determined by bacterial growth curves. Using the bacteria which have entered the exponential phase of growth can ensure the bacteria stay in the same situation for following assays. After incubation, the turbidity (OD₆₀₀) of the bacterial suspension in the test tube was measured using a turbidimeter (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany). The initial bacterial colony forming unit (CFU) density of the bacterial suspension can be calculated via multiplying the OD₆₀₀ value by 10⁶ CFU/mL. Finally, according to the pre-test result, the bacterial suspension was diluted to demanded concentrations for different assays with LB liquid medium based on the initial CFU density.

2.3.2. Bacterial adhesion assay

The S. aureus or E. coli suspension with a CFU density of 7 × 10⁹ CFU/mL was employed. Prior to use, the samples of TiO₂ periodic micro/nano-pillar arrays were “sterilized by steam autoclaving in a high-pressure steam sterilization pot at 121 °C for 20 min” [35]. Then, the samples were immersed into the bacterial suspension and incubated in the dark at 37 °C for 30 min. Before staining, the samples were “gently rinsed with a HEPES Buffered Saline Solution (HBSS)” (Table S2) [35]. All experiments were repeated in triplicate.

2.3.3. Bacterial growth and proliferation assay

The S. aureus or E. coli suspension with a CFU density of 1 × 10⁸ CFU/mL was employed. Prior to use, the samples of TiO₂ periodic micro/nano-pillar arrays were “sterilized by steam autoclaving in a high-pressure steam sterilization pot at 121 °C for 20 min” [35]. Then, the samples were immersed into the bacterial suspension and incubated in the dark at 37 °C for 12 h and 24 h. Before staining, the samples were gently rinsed with the HBSS [35]. All experiments were repeated in triplicate.

2.4. Bacterial characterization

2.4.1. LIVE/DEAD staining

The experimental procedure was conducted according to that in our previous study [35]. Briefly, a LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) was employed to stain the adhered and colonized bacteria on the TiO₂ periodic micro/nano-pillar arrays. The kit contains SYTO® 9 and propidium iodide which are two kinds of nucleic acid stains. “After being cultured for a certain period of time, a chip with the bacteria was gently rinsed by the HBSS, and then incubated with a 0.5 μL: 0.5 μL: 1 mL mixture of SYTO® 9, propidium iodide, and HBSS for 20 min at room temperature in the dark” [35]. Before the following characterizations, the chip was “gently rinsed in HBSS and DI water in sequence and dried in the air at room temperature” [35]. Live bacteria appear green while dead bacteria appear red under a fluorescence microscope.
2.4.2. Confocal laser scanning microscope (CLSM) observation and statistics

The experimental procedure was conducted according to that in our previous study [35]. Briefly, the dried chips were observed under a CLSM (LSM7 DUO (710 + LIVE), Carl Zeiss MicroImaging GmbH, Germany). “The SYTO® 9 green was excited by a 489 nm laser and the emission spectrum from 510 nm to 540 nm was collected in CLSM. The propidium iodide red was excited by a 561 nm laser and the emission spectrum from 620 nm to 650 nm was collected” [35]. Three random regions corresponding to each of the ten areas on the chip were taken pictures under the CLSM using a 3D imaging mode. Each CLSM image showed a view field of 848.53 × 848.53 μm² by employing a ×10 objective lens. A software named ImageJ (version 1.43u, National Institutes of Health, USA) was employed to count the bacterial occupied area of each CLSM image. The ratio of the bacterial occupied area out of the whole examined area in each CLSM image was normalized by dividing the average ratio corresponding to TiO₂ Flat (control), and then the results were expressed in percentages which represented the anti-bacterial ability of different areas on the chip.

2.4.3. SEM observation

The experimental procedure was conducted according to that in our previous study [35]. Briefly, the detailed morphologies of bacteria on the TiO₂ periodic micro/nano-pillar arrays and the TiO₂ flat area were observed using the FESEM (JSM-6700F, JEOL, Japan). “After the CLSM examinations, samples were fixed in a solution (pH = 7.4) mixed with glutaraldehyde (C₅H₈O₂, 2.5 wt%) and sodium cacodylate buffer (Na(CH₃)₂AsO₂·3H₂O, 0.1 M) at 4 °C for 2 h [65,66], dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, and 100%; each step for 5 min), dried using a critical point dryer (Model CPD-2, Pelco), coated with a thin layer of gold, and then observed by the SEM” [35].

2.5. Statistical analysis

All statistical data were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), which was clearly indicated in relevant figure captions. Significant differences (p ≤ 0.05) among the groups were detected by a One-way analysis of variance (ANOVA) followed by a Student’s t-test. Notably, there was an independent Student’s t-test between TiO₂PA_0.6μm and each of the other nine areas of TiO₂PA_20μm, TiO₂PA_10μm, TiO₂PA_5μm, TiO₂PA_2μm, TiO₂PA_1.4μm, TiO₂PA_1.2μm, TiO₂PA_1.0μm, TiO₂PA_0.8μm, and TiO₂PA_0.6μm (Fig. 2c). The flat area was named as Si Flat (Fig. 2c). Moreover, the location arrangement of the ten areas on a chip was random (Fig. 2a), not according to the pillar array motif size order to avoid potential edge/central effects which might interfere the results obtained from the following bacterial experiments [35]. So far, a Si scaffold had been successfully obtained.

After the RF magnetic sputtering and the thermal oxidation, the chip color changed as shown in Fig. 3a and b, which was an indirect evidence of a thin film formation on the Si scaffold. Fig. 3c shows the morphology of flat Si substrate under a magnification of ×50,000. While after the Ti thin film deposition and the thermal oxidation, it could be seen under the same magnification (×50,000) that a continuous and dense thin film with small and homogenous size of grains was formed on the flat Si substrate (Fig. 3d). In addition, when comparing morphologies before (Fig. 3e, Fig. S2a, and Fig. S2c) and after (Fig. 3f, Fig. S2b, and Fig. S2d) the Ti thin film deposition and the thermal oxidation, we could find that a thin film with the identical morphology shown in Fig. 3d also formed on the side-wall of the pillar (Fig. 3f), the top of the pillar (Fig. S2b), and the surface of the substrate (Fig. S2d). All these SEM observations provided direct evidences to demonstrate that a conformal thin film was successfully formed on the whole Si pillar array. Since the thin film thickness was only about 52 nm as shown in a cross-section view (Fig. 3g), the topography of the Si scaffold (Fig. 2c) was maintained after the thin film formation.

There was no obvious difference between the broad range XPS spectra of the thin film coated pillar array and the thin film coated flat surface (Fig. 3h). The broad range XPS spectra (Fig. 3h) indicate that the thin film contains the components of Ti and O together with C which is a common surface contamination element. Moreover, the high-resolution XPS spectra corresponding to Ti 2p (Fig. S3) can help to better understand the Ti chemical state. Similarly, there was no obvious difference between the high-resolution XPS spectra of the thin film coated pillar array and the thin film coated flat surface either (Fig. S3). There are two typical electron binding energies of Ti which are 453.6–454.2 eV (oxidation state 0, Ti metal) and 458.8–459.2 eV (oxidation state +IV, TiO₂). The high-resolution XPS spectra corresponding to Ti 2p (Fig. S3) show that the binding energy for Ti 2p peak is at ~458.8 eV, which indicates that all Ti components are Ti⁴⁺ and TiO₂ do not exist in the thin film. These results confirmed that the sputtered Ti thin film had been completely transformed to TiO₂ by the thermal oxidation.

Notably, both the thin film coated pillar array and the thin film coated flat surface did not exhibit any obvious difference in their XRD diffraction patterns (Fig. 3i). Typical TF-XRD patterns (Fig. 3i) confirm that the crystalline phase of the TiO₂ thin film covering the Si scaffold is rutile. The diffraction patterns (Fig. 3i) do not show any peak of Ti, which double confirms that the thermal oxidation have completely transformed the deposited Ti thin film to TiO₂. In addition, it is reasonable that the Si signal from the substrate shows in the diffraction patterns (Fig. 3i), because the TF-XRD can obtain signals from the bulk material ~1 μm away from the material top surface while the TiO₂ thin film thickness was only about 52 nm as mentioned above.

Finally, well-defined TiO₂ periodic micro/nano-pillar arrays were obtained. Similar to the naming rule of Si scaffold, the ten areas were named as TiO₂PA_20μm, TiO₂PA_10μm, TiO₂PA_5μm, TiO₂PA_2μm, TiO₂PA_1.4μm, TiO₂PA_1.2μm, TiO₂PA_1.0μm, TiO₂PA_0.8μm, TiO₂PA_0.6μm, and TiO₂ Flat. The results of WCA measurement revealed a significant influence of pillar array motif size on the wettability of patterned TiO₂ surface. As shown in Fig. 3j, the WCA measurements were obtained. The WCA of TiO₂ Flat was 56.4° (Fig. 3j) which was slightly higher than that of TiO₂PA_20μm. Therefore, all the ten areas were hydrophilic since their WCs were all smaller than 90°, which indicated that the surface energies (γ) of all the ten areas were relatively high.

3.2. Bacterial adhesion

3.2.1. S. aureus cultured for 30 min

The experimental results of S. aureus adhesion exhibited an evident
correlation with the motif size of TiO$_2$ periodic micro/nano-pillar array. CLSM images (Fig. 4a) depict an obvious difference in S. aureus occupied areas corresponding to different patterned areas with distinct pillar array motif sizes, as illustrated by a sharp boundary between every two patterned areas. Qualitatively, the CLSM images clearly indicate that the smaller pillar array motif size such as 0.6μm results in less bacterial adhesion. However, the TiO$_2$ periodic micro/nano-pillar array with larger motif size and the flat surface seem favorable for S. aureus adhesion. Quantitative statistical analysis (Fig. 4b) of the CLSM images confirms that the normalized bacterial occupied area percentage first increases with decreasing the pillar array motif size and then decreases with further decreasing the pillar array motif size down to the sub-micron level. Comparing to the TiO$_2$ flat surface, the bacterial adhesion decreases dramatically if the pillar array motif size reduces down to 0.6μm (only 38% of the bacterial occupied area on TiO$_2$-Flat, as shown in Fig. 4b), while TiO$_2$PA$_{1.4μm}$ has the most obvious bacterial anti-adhesive effect since it exhibits only 27% of the bacterial occupied area on TiO$_2$-Flat (Fig. 4d).

3.3. Bacterial growth and proliferation

3.3.1. S. aureus cultured for 12 h and 24 h

The experimental results (Fig. 5a and b) of S. aureus cultured for 12 h reveal an approximately monotonic decreasing tendency that the normalized bacterial occupied area percentage reduces with decreasing the pillar array motif size. Consistently, the TiO$_2$PA$_{0.6μm}$ also has the least bacterial occupied area which is only 12% of that on TiO$_2$-Flat (Fig. 5b). Nevertheless, the experimental results (Fig. 6a and b) of S. aureus cultured for 24 h exhibit a non-monotonic trend of bacterial adhesion with decreasing the pillar array motif size. The trend indicates that the normalized bacterial occupied area percentage first slightly increases with decreasing the pillar array motif size, reaches the maximum (118% of the bacterial occupied area on TiO$_2$-Flat, as shown in Fig. 6b) when the pillar array motif size equals 2μm, and then decreases with further decreasing the pillar array motif size down to the sub-micron level. Notably, the TiO$_2$PA$_{0.6μm}$ also has the most obvious effect on inhibiting the bacterial proliferation and colonization since it exhibits only 40% of the bacterial occupied area on TiO$_2$-Flat (Fig. 6b).

3.3.2. E. coli cultured for 30 min

The experimental results of E. coli adhesion also exhibited an evident correlation with the motif size of TiO$_2$ periodic micro/nano-pillar array, though E. coli possesses a different shape (rod) other than sphere. Qualitatively, CLSM images (Fig. 4c) intuitively show an obvious difference in E. coli densities on different patterned areas with distinct pillar array motif sizes. Similar to the S. aureus case, quantitative statistical analysis (Fig. 4d) of the CLSM images also shows a non-monotonic trend of bacterial adhesion with decreasing the pillar array motif size. The trend indicates that the normalized bacterial occupied area percentage first increases with decreasing the pillar array motif size, reaches the maximum (128% of the bacterial occupied area on TiO$_2$-Flat, as shown in Fig. 4d) when the pillar array motif size equals 5 μm, and then decreases with further decreasing the pillar array motif size down to the sub-micron level. In particular, the TiO$_2$PA$_{0.6μm}$ has the most obvious bacterial anti-adhesive effect since it exhibits only 27% of the bacterial occupied area on TiO$_2$-Flat (Fig. 4d).

3.3.3. E. coli cultured for 12 h and 24 h

The results of bacterial growth and proliferation assay reveal an evident pillar array motif size dependent effect on E. coli occupied area evidenced by the CLSM images (Fig. 5c cultured for 12 h, and Fig. 6c cultured for 24 h, respectively). Quantitative analysis shown in Fig. 5d (cultured for 12 h) and Fig. 6d (cultured for 24 h) indicates similar trends which first slightly increase and then decrease with decreasing the pillar array motif size. The TiO$_2$ periodic micro/nano-pillar array shows an obvious effect on inhibiting E. coli proliferation and
colonization compared to the TiO₂ flat surface when the pillar array motif size reaches the sub-micron level. Again, the TiO₂PA_0.6μm exhibits the most excellent effect on decreasing *E. coli* population no matter the culture time is 12 h (only 22% of the bacterial occupied area on TiO₂_Flat, as shown in Fig. 5d) or 24 h (only 30% of the bacterial occupied area on TiO₂_Flat, as shown in Fig. 6d).

3.4. Bacterial viability

In this study, the CLSM images corresponding to TiO₂PA_2μm cultured for 24 h was used as an example for demonstration, since the same conclusion could be drawn from the results of the other nine areas. As depicted in Fig. S4a, there is no red bacteria shown in this merged CLSM image, which suggests that the death rate of *S. aureus* is close to 0 in 24 h. Similarly, the TiO₂PA_2μm is also not lethal to *E. coli* in 24 h (Fig. S4b).

4. Discussion

4.1. Characteristic contour for certain pattern vs. bacterial strain

It is noteworthy that the contours of bar charts for *S. aureus* (red curves in Figs. 4b, 5b and 6b) and those for *E. coli* (red curves in Figs. 4d, 5d and 6d) reveal distinct characteristic tendencies in the same motif size range of the same pattern type. This phenomenon may be attributed to the distinct shapes, sizes, and physiological behaviours of the two bacterial strains. Therefore, the characteristic contour corresponding to certain pattern type and bacterial strain deserves to be archived in a database. The database can be used for the surface topography design of medical implants in the future to reduce the infection risk by involving a specific surface topography (certain pattern type + specific motif size) with an antibacterial ability against certain bacterial strains in a real clinical situation.

4.2. Antibacterial mechanism

On the basis of statistical analysis results, the TiO₂ periodic micro/nano-pillar array with sub-micron motif size could effectively inhibit or retard the adhesion, growth, and proliferation of *S. aureus* and *E. coli*. A proposed main antibacterial mechanism of periodic micro/nano-pillar array is illustrated in Fig. 7 and Fig. 8. Briefly, *S. aureus* can adhere (Fig. 7a), elongate & proliferate (Fig. 7b), and colonize (Fig. 7c) on a flat surface as usual, while the bacterial behaviours (Fig. 7d, e, and f) are significantly interfered by the periodic micro/nano-pillar array as evidenced by the compressed shapes of *S. aureus* shown in Fig. 7e and f. Rather than occupying a large area of surface by proliferating to form a close-packed colony (Fig. 7c), the *S. aureus* is imprisoned by a narrow space (Fig. 7f) constructed by the periodic micro/nano-pillar array. Similarly, both the hypothesis models and the SEM actual observation (Fig. 8a, a', b, b', c, and c') indicate that the elongation and binary fission of *E. coli* are normal when an *E. coli* is on a flat surface. However, when an *E. coli* locates at the bottom of a periodic micro/nano-pillar array, its elongation and binary fission seem to be constrained, as shown in Fig. 8d, d', e, e', f, and f'.
It is believed that one main reason for the antibacterial property of periodic micro/nano-pillar array is a spatial confinement size-effect which traps bacteria between pillars, limits the attachment area for bacteria, and impedes the bacterial cell-cell interactions [26,35]. Nevertheless, the antibacterial property cannot be attributed to the photocatalytic effect of TiO\textsubscript{2} since the bacteria were cultured with the samples in the dark.

In addition, the wettability of nine TiO\textsubscript{2} patterned surfaces and one TiO\textsubscript{2} flat surface was systematically investigated since different wettability might result in different affinity for bacteria. Specifically, the

![Image of CLSM images and statistical analyses for S. aureus and E. coli after culturing for 30 min on TiO\textsubscript{2} periodic micro/nano-pillar arrays.](image)

**Fig. 4.** Characteristics of *S. aureus* and *E. coli* after culturing for 30 min on the TiO\textsubscript{2} periodic micro/nano-pillar arrays. (a) Typical CLSM images of *S. aureus* on one flat area and nine patterned areas, respectively; (b) Quantitative statistical analysis of relative *S. aureus* occupied area percentage which is normalized by that on TiO\textsubscript{2} Flat. Error bar = SEM; (c) Typical CLSM images of *E. coli* on one flat area and nine patterned areas, respectively; (d) Quantitative statistical analysis of relative *E. coli* occupied area percentage which is normalized by that on TiO\textsubscript{2} Flat. Error bar = SEM. One asterisk (*) indicates significant difference at $p < 0.05$, two asterisks (**) indicate significant difference at $p < 0.01$ in t-tests.

![Image of CLSM images and statistical analyses for S. aureus and E. coli after culturing for 12 h on TiO\textsubscript{2} periodic micro/nano-pillar arrays.](image)

**Fig. 5.** Characteristics of *S. aureus* and *E. coli* after culturing for 12 h on the TiO\textsubscript{2} periodic micro/nano-pillar arrays. (a) Typical CLSM images of *S. aureus* on one flat area and nine patterned areas, respectively; (b) Quantitative statistical analysis of relative *S. aureus* occupied area percentage which is normalized by that on TiO\textsubscript{2} Flat. Error bar = SEM; (c) Typical CLSM images of *E. coli* on one flat area and nine patterned areas, respectively; (d) Quantitative statistical analysis of relative *E. coli* occupied area percentage which is normalized by that on TiO\textsubscript{2} Flat. Error bar = SEM. One asterisk (*) indicates significant difference at $p < 0.05$, two asterisks (**) indicate significant difference at $p < 0.01$ in t-tests.
pillar array motif size plays a vital role in the wettability of patterned TiO$_2$ surfaces, since the WCAs monotonically reduce with decreasing the pillar array motif size, as depicted in Fig. 3j. All the ten areas are hydrophilic because their WCAs are smaller than 90° (even smaller than 60°). Among them, the TiO$_2$ Flat has the largest WCA as 56.4°, while the TiO$_2$PA 0.6μm possesses the smallest WCA as 16.1°. It is generally believed that super-hydrophobic surfaces have an effective ability in decreasing bacterial adhesion [67,68]. However, in this work, the experimental results (Figs. 4–6) revealed that the TiO$_2$PA 0.6μm with the smallest WCA (super-hydrophilic) possesses the greatest ability to resist bacterial adhesion. This controversy will not be a surprise. Xu et al. [69] reported that a hydrophilic polyurethane pillar array with a sub-micron motif size has a higher resistance to bacterial adhesion compared with a flat surface or micron-sized patterns. The reductions in bacterial adhesion are dependent on the pattern motif size which determines the contact area availability for bacteria [69]. Notably, the contact area availability is a principal decisive factor affecting bacterial adhesion on hydrophilic patterned surfaces [69], which could also be used to perfectly explain the experimental results presented in this study.

Generally, the interactions between bacteria and material surfaces can be affected by various properties of the material surface, including wettability, surface energy, charge, chemical components, elastic modulus, topography, and so on [67]. Bacterial behaviours on the material surface could be synergistically influenced by these properties, hardly manipulated by any single property unless it is dominant. This is why mild hydrophobic surfaces cannot always decrease bacterial adhesion [67]. Thus, the spatial confinement size-effect and the limited bacterial adhesion on a flat surface...
Contact area availability are the main antibacterial mechanisms for the hydrophilic TiO₂ periodic micro/nano-pillar array with sub-micron motif size.

5. Conclusions

Here, we designed a novel model substrate which was a combination of periodic micro/nano-pillar array and TiO₂ for basically understanding the topographical bacteriostatic effects of periodic micro/nano-pillar array and the photocatalytic bactericidal activity of TiO₂. Such innovation may potentially exert the synergistic effects by integrating the persistent topographical antibacterial activity and the non-invasive X-ray induced photocatalytic antibacterial property of TiO₂ to combat against antibiotic-resistant implant-associated infections. This work systematically studied how TiO₂ periodic micro/nano-pillar array affected the bacterial behaviours, including bacterial adhesion, growth, proliferation, and viability. The TiO₂ periodic micro/nano-pillar array with sub-micron motif size can significantly inhibit the adhesion, growth, and proliferation of S. aureus and E. coli. Specifically, TiO₂PA_0.6μm can reduce 62% of S. aureus adhesion at 30min, 73% of E. coli adhesion at 30min, 60% of S. aureus colonization at 24h, and 70% of E. coli colonization at 24h compared with the TiO₂ flat surface. Such antibacterial ability is mainly attributed to a spatial confinement size-effect and limited contact area availability generated by the special topography of TiO₂ periodic micro/nano-pillar array, but cannot be ascribed to the photocatalytic effect of TiO₂ since the bacteria were cultured with the samples in the dark. Rather than using a model material as Si, this study employed a common biocompatible and bioactive biomaterial (TiO₂) to further confirm that the topographical antibacterial effects are independent of substrate chemistry. Moreover, the TiO₂ periodic micro/nano-pillar array is not lethal to S. aureus and E. coli in 24h, no matter what the pillar array motif size is (from 20μm to 0.6μm). Then, the X-ray induced photocatalytic antibacterial property of TiO₂ periodic micro/nano-pillar array in vitro and in vivo will be systematically studied in a future work.

Nevertheless, the multi-step fabrication process of the model substrate is somewhat laborious. Besides, the bulk material of the model substrate is still Si instead of Ti of which strength is high enough for clinical applications. Additionally, the photolithography plus magnetron sputtering may not adapt well to a more complicated 3D substrate other than flat surface. Although the model substrate is still far from clinical applications owing to the above-mentioned shortcomings, this study could shed light on the direction of surface topography design for future medical implants to combat against antibiotic-resistant implant-associated infections without using antibiotics, which may eliminate health concerns caused by the widespread, long-term, and excessive usage of antibiotics.

Author contributions

X.G. and Y.L. conceived the experimental system and analyzed the figures and data; X.G. carried out the material synthesis and characterization; and Y.D. performed part of material synthesis and characterization. All authors contributed to the manuscript preparation and revision.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper. Additional data related to this paper may be requested from the authors.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2019.10.006.

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