Hindawi Publishing Corporation
Journal of Nanomaterials
Volume 2011, Article ID 178921, 8 pages
doi:10.1155/2011/178921

Research Article

Effect of Superhydrophobic Surface of Titanium on *Staphylococcus aureus* Adhesion

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Received 24 January 2011; Accepted 16 April 2011

Academic Editor: Xing J. Liang

Despite the systemic antibiotics prophylaxis, orthopedic implants still remain highly susceptible to bacterial adhesion and resulting in device-associated infection. Surface modification is an effective way to decrease bacterial adhesion. In this study, we prepared surfaces with different wettability on titanium surface based on TiO_2 nanotube to examine the effect of bacterial adhesion. Firstly, titanium plates were calcined to form hydrophilic TiO_2 nanotube films of anatase phase. Subsequently, the nanotube films and inoxidized titaniums were treated with 1H, 1H, 2H, 2H-perfluorooctyl-triethoxysilane (PTES), forming superhydrophobic and hydrophobic surfaces. Observed by SEM and contact angle measurements, the different surfaces have different characteristics. *Staphylococcus aureus* (SA) adhesion on different surfaces was evaluated. Our experiment results show that the superhydrophobic surface has contact angles of water greater than 150° and also shows high resistance to bacterial contamination. It is indicated that superhydrophobic surface may be a factor to reduce device-associated infection and could be used in clinical practice.

1. Introduction

The number of trauma and aged patients requiring internal fixation devices or joint replacements is increasing steadily. In the USA, about 2 million fracture-fixation devices are inserted annually. Infection is generally the most common serious complication of fixation devices. Although infection of initially inserted internal fixation devices is about 5% [1], such infection is associated with a high potential for chronic osteomyelitis or limb loss, even death. Since cure of infections associated with internal fixation devices commonly requires removal of the infected devices, managements could be rather difficult and quite expensive. The serious medical complications, problematic managements, and economical sequelae of infections associated with internal fixation devices have prompted a keen interest in exploring innovative preventive approaches [2–5].

Mechanism of device-related internal fixation caused by bacteria is very complex, but bacteria adherence on device surfaces is an initial crucial step and a prelude to clinical infection [6]. Many studies show that wettability of surface is one of the reasons of bacteria adherence. Hydrophilic surface (contact angle <90°) attracted most of bacteria such as *Staphylococcus aureus*, *Escherichia coli* [7], whereas hydrophobic surface (contact angle >90°) attracted *Psx. Taiwanensis* and *Staph. epidermidis* and reduced the adherence of bacteria including *D. geothermalis* and *M. Silvanus* [8], *Staphylococcus aureus* [9], and *Streptococcus mutans* [10]. Whether attraction or reduction depends on the type of bacteria and the surface characteristics, in particular contact angle.

Staphylococcus aureus (SA) is the main pathogenic bacteria in the internal fixation-related infection in clinic [6]. How to reduce SA adherence and how to prevent infection most concerned things by orthopaedic surgeons. To surface characteristics, why hydrophobic surface could reduce some bacteria and attract some others? We assume that the degree of hydrophobicity is not enough and being increased to superhydrophobicity (contact angle >150°) could be attributed to the reduction of bacteria adherence

substantially at the interfaces. Common approaches to enhance the degree of hydrophobicity at biomaterial surfaces were raising the surface roughness and modifying low surface energy [11–15]. But both tended not to exhibit water contact angle values more than 150°.

In this work, we used a more facile method to fabricate superhydrophobic surface on the titanium, and we compared with other techniques including etching [16], sol-gel [17], chemical vapor phase deposition [18], and mechanical stretching method [19]. Firstly, anodic oxidation was conducted to generate the nanotube on the titanium surface in the HF acid system, with the surface wettability being moderate hydrophilicity. And then PTES was modified on the surface of the nanotube by layers self-assembly technique, and the superhydrophobic surface with high contact angle was formed. Then we investigated the ability of different surfaces to prevent bacterial adherence *in vitro*.

2. Materials and Methods

2.1. Materials—Substrates. Medical purity titanium was purchased from Zheng tian Co., Ltd in Beijing, China. 1H, 1H, 2H, 2H-perfluorooctyl-triethoxysilane (PTES) used for surface hydrophobic modification was purchased from Degussa Co., Ltd.

2.2. Surface Treatment. At the first step, the purity titanium samples were cut into dimensions of 1 cm × 2 cm × 0.2 cm by flame incision machine (Zheng tian Co., Ltd, China). Different sizes of sand papers were used to polish the samples until their surfaces become smooth. Then the polished samples were washed by ultrasonic (90 kHz, output power 90 W), propanone, ethanol, and deionized water, successively.

Secondly, the superhydrophobic nanotube structure TiO_2 film was fabricated through electrochemical oxidation and self-assembled technique. Purity titanium (99.5%) sheets were electrochemically anodized with Pt counterelectrode in 0.5 wt% HF electrolyte under certain voltage for 1 h, rinsed with deionized water, and dried with dry nitrogen stream. The prepared amorphous TiO_2 nanotube films were calcined at 450°C for 2 h to form anatase phase and then treated with a methanolic solution of hydrolyzed 1 wt% 1H, 1H, 2H, 2H-perfluorooctyl-triethoxysilane (PTES, Degussa Co., Ltd.) for 1 h and subsequently heated at 140°C for 1 h to remove residual solvent and promote chemisorption of the SAM. Sample 1 was treated by surface anodic oxidation only (NT), sample 2 was treated by PTES after the anodic oxidation (NTS), and sample 3 was treated by PTES on the surface of titanium (TiS).

2.3. Surface Characterization. The surface morphology of the samples was examined with Hitachi S-4800 Field Emission scanning electron microscope. The samples were pressed slightly and coated with gold. The water contact angles of the samples were measured by First Ten Angstroms dynamic contact angle analyzer (FTA 200) at ambient temperature with a droplet volume of 0.013 mL.

2.4. Biological Analysis Materials. S. aureus 8325 strain was purchased from Academy of Military Medical Sciences; Luria-Bertani (LB) medium and agar which were used for growing and maintaining bacteria cultures were purchased from Sigma-Aldrich; Osmium tetroxide (OsO_4) was purchased from Simec (Switzerland); Glutaraldehyde was purchased from AGAR (Stansted, UK), Tannin Acid was purchased from Fluka (Buchs, Liechtenstein), and The BacLight RedoxSensor CTC Vitality Kit was purchased from Invitrogen detection technologies (USA).

2.5. Bacterial Culturing and Adhesion. Got 1 mL *S. aureus* 8325-4 into a tube, followed by 5 mL Luria-Bertani (LB) medium (no antibiotics) and placed it on the swing bed overnight (37°C, 250 r.p.m., 12 h) and then stored in 4°C for use. Multiple proportion dilution and plate counting methods were conducted to adjust the density of 1 μL liquid into 1×10^9 cfu/mL. LB mediums were then added into the liquid until the final density of 1×10^6 cfu/mL. Metal samples were placed into the six-well culture dish to be sterilized by γ-ray (25 kGy), and liquid at the density of 1×10^6 cfu/mL was added into every well with 10 mL. Then the dish was put into the incubation case with the relative humidity above 90% at 37°C. Scanning electron microscopy (SEM) and fluorescence microscopy (FM) were conducted after 2 h to 4 h.

2.6. Visualization of S. Aureus on Different Surfaces. After being rinsed with 0.01 M PBS to remove the float bacterium on the surface, the sample was moved into the six-well culture dish and 2.5% glutaraldehyde was added and then kept in the freezer at 4°C overnight. After being washed with 0.01 M PBS (three times for 30 min each), 1% osmic acid was added to fix for 2 h. After being washed with 0.01 M PBS (three times for 30 min each), samples were kept in the 2% Tannin solution. 30 min later, the solution was refreshed, and, after another 30 min, samples were washed with 0.01 M PBS (three times for 30 min each). Then samples were dehydrated by 50% alcohol, 70% alcohol, 90% alcohol and absolute alcohol and had a transition using isoamyl acetate. Visualization with a Hitachi S-4800 Field Emission SEM operated in secondary electron (SE) detection modes.

2.7. Bacterial Adherence and Fluorescence Microscopy Counting. Metal samples were placed into the six-well culture dish to be sterilized by γ-ray (25 k Gy), and liquid at the density of 1×10^6 cfu/mL was added into the wells with 10 mL each. The dish was sealed and put into standard bacteria culture incubator (at a 37°C, humidified, 5% CO_2, and 20% O_2 environment). *S. aureus* 8325 was cultured on the surfaces for prescribed time period (2 and 4 h), washed twice with PBS (0.01 M) to remove any loose or unattached bacteria, and stained with fluorescent redox dye, CTC for 30 minutes at 37°C and protected from light. Bacteria cell counts were then completed using a fluorescence microscope in a field of view of 200 mm × 200 mm. Colony-forming

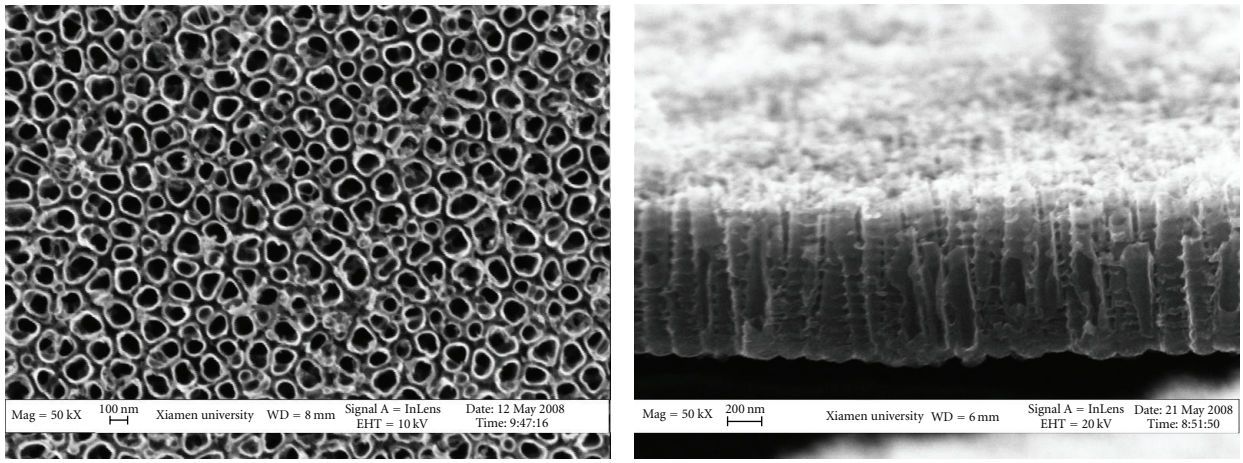

Figure 1: SEM images of the nanotube layer formed on titanium in 0.5 wt% HF electrolyte solution at 20 V: (a) top view; (b) cross-section.

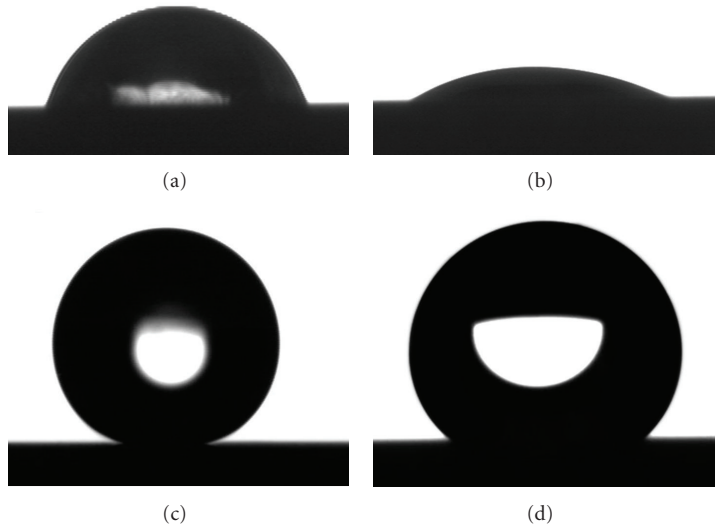

Figure 2: Photographs of water droplet shape on (a) NT film; (b) NT film and the rapid spread and wet the NT film; (c) NTS film, PETS-treated on the surface of nanotube array; (d) TiS film, PETS-treated on the surface of titanium.

units were determined using Image Pro software. Ten fields were averaged for each substrate. Statistical analysis was done using a one-way ANOVA with Tukey's test. Experiments were completed in triplicate and were repeated three times [2].

2.8. Surplus Bacterium Solution Count. Ultraviolet spectrophotometer was conducted to determine the OD value of the bacteria solution that was soaked in the samples at the wave length of 600 nm.

3. Results and Discussion

It is generally believed that what we are able to do is to minimize its deleterious clinical consequences, once an implant infection has developed [20, 21]. However, there is no doubt that prevention represents a main goal. For implant materials, the interface biomaterial surface-surrounding tissue represents the real ground where the battle takes place and where accidental contamination can first develop into colonization [6], and so many routes of surface coating and modification developed. Recently researchers [8–10] report that wettability (hydrophilicity and hydrophobicity) may be a factor of deceasing bacteria adherence on surfaces. But few people discussed the superhydrophobic surfaces of titanium or the effect of bacteria. In this work we have fabricated superhydrophobic nanostructured TiO_2 surfaces with electrochemical and self-assembly method [22], validated the character of superhydrophobicity, and compared with hydrophilic and hydrophobic surfaces on bacteria adherence. In SEM morphology, anodic oxidation was conducted in our solution containing fluorine to generate the regulated

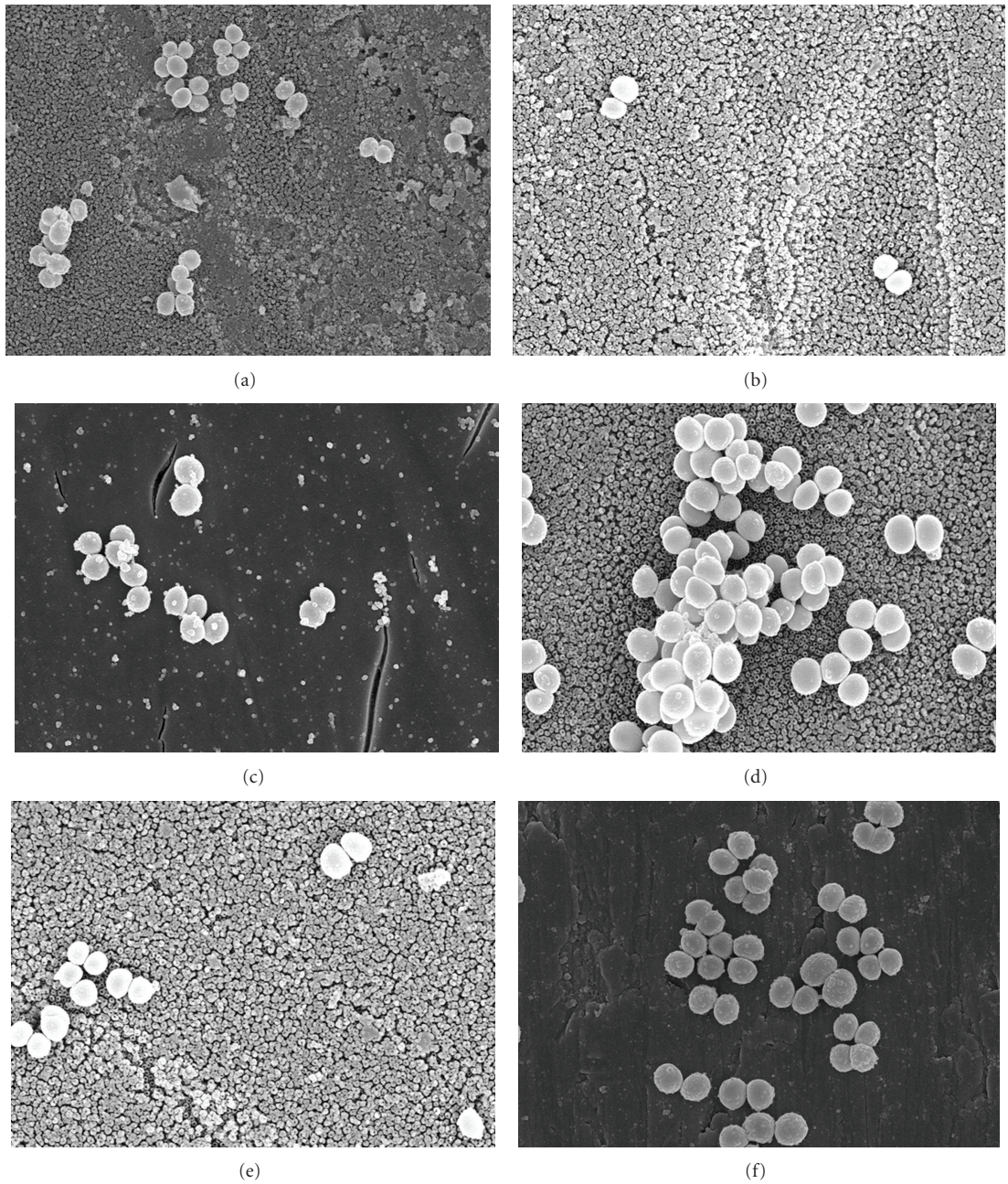

Figure 3: SEM images of bacteria colonies after 2 h on (a) NT, (b) NTS, and (c) TiS, and after 4 h on (d) NT, (e) NTS, and (f) TiS (×10 K).

nanotubes array structure on the surface of titanium by the etching of fluoride ion. Figure 1 shows an SEM image of TiO_2 nanotubes with uniform and regular array. This nanotube is about 400 nm long, with an 8 to 10 nm vessel wall and an inner diameter ranging from 80 to 100 nm. There are no difference between nanotube film treated with or without PETS on SEM image.

Electrochemical anodizing is verified to be a convenient and effective method for fabricating nanostructured TiO_2 films with powerful mechanical strength directly on pure titanium (Ti) substrates, compared with sol-gel technique, sputtering, and chemical vapor deposition [23]. The nanotube created in HF acid system using this way was uniform, thus a rough surface formed, just as Crimes [24] described. A detailed study on crystallization and structural transformation of these nanotube arrays upon thermal annealing is reported elsewhere [25], and the wettability of the rough surface was moderate hydrophilic (the contact angle was 54°) after being calcined forming anatase phase. Figure 2(a) was captured when the water dropped on the surface instantly, and then water rapidly spreads and wets the film due to side penetration of the liquid by capillary

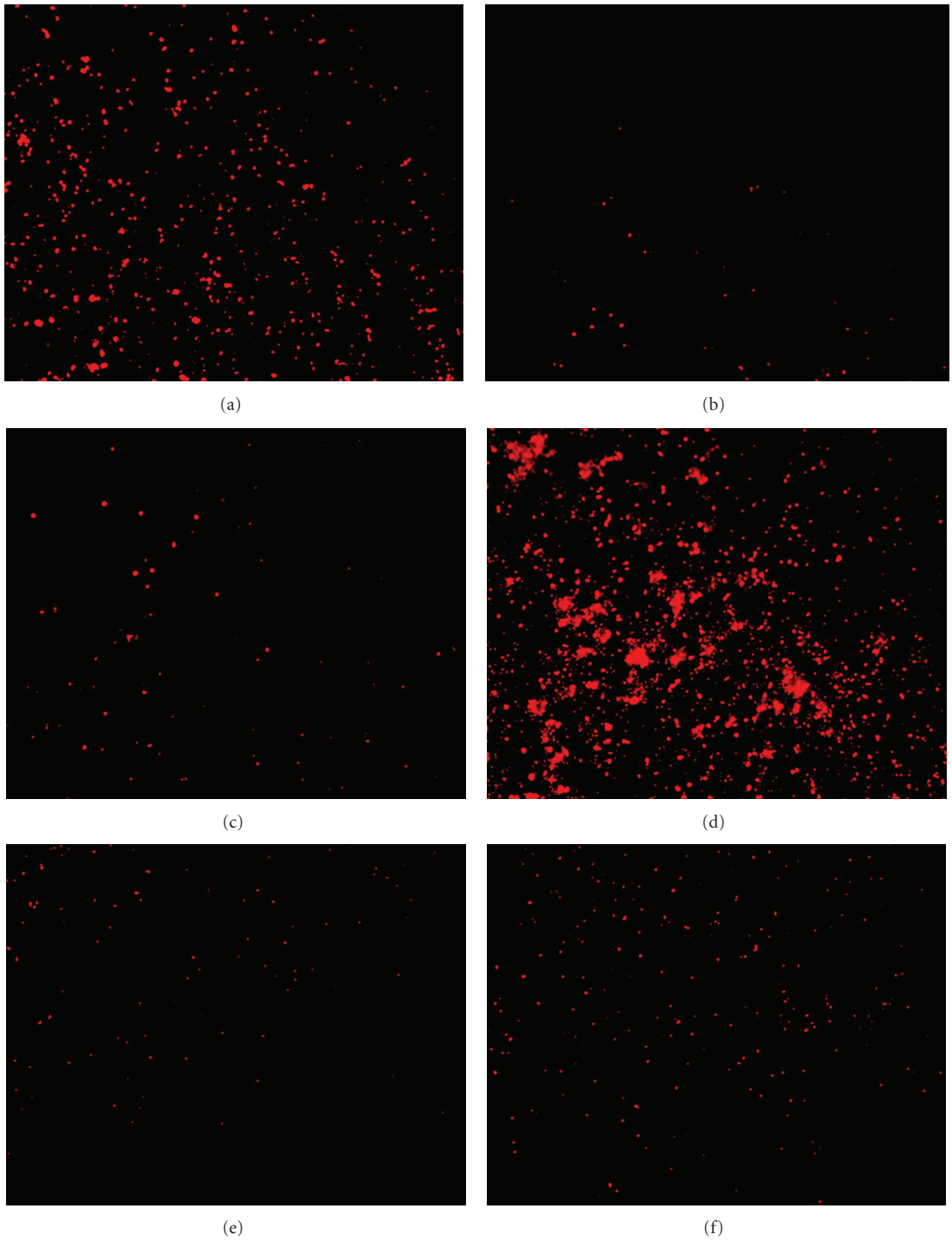

Figure 4: Fluorescence microscope images of bacteria stained with CTC after 2 h (a–c) and 4 h (d–f) of culture on NT, NTS, and TiS.

forces [26], showed in Figure 2(b). Figure 2(c) shows that the contact angle of NTS is increased to 156° after it was modified by PETS, which is superhydrophobicity, while the contact angle of TiS is only 133° (Figure 2(d)).

The moderate hydrophilic nanotube films can be fabricated through self-assembly technique, and the hydrophobicity of the films is also improved greatly. It is well known that a flat surface with low surface energy tends to exhibit high water contact angle values on the order of 100–120°. However, this is insufficient to produce a water repellent or superhydrophobic surface, which requires a water contact angle larger than 150° [27]. Generally speaking, hydrophobic surfaces can be generated by being roughed and being modified by low-energy materials. Numerous nanotube surfaces increase the surface area and the rough degree and also modify the low surface energy material PTES, in

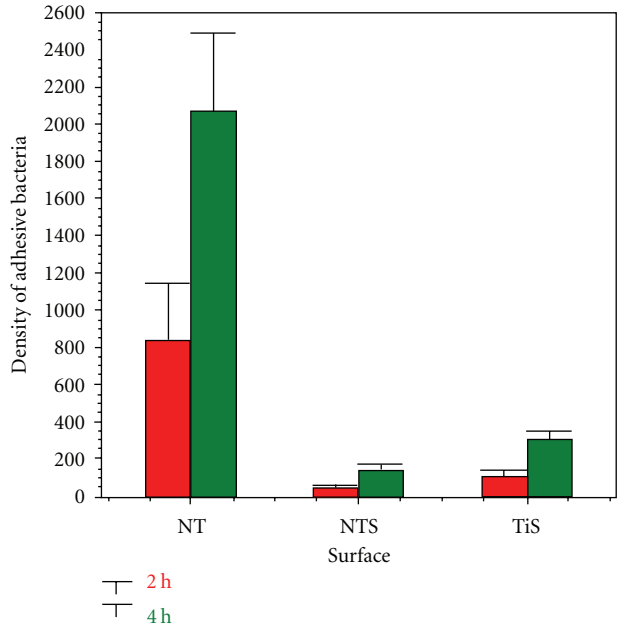

Figure 5: Decreased *S. aureus* adhesion on nanotubes self-assembled with PETS (NTS) compared to TiS and NT; NTS ($P < .05$) compared with growth on TiS and NT after 2 h of culture; NTS ($P < .05$) compared with growth on TiS and NT after 4 h of culture.

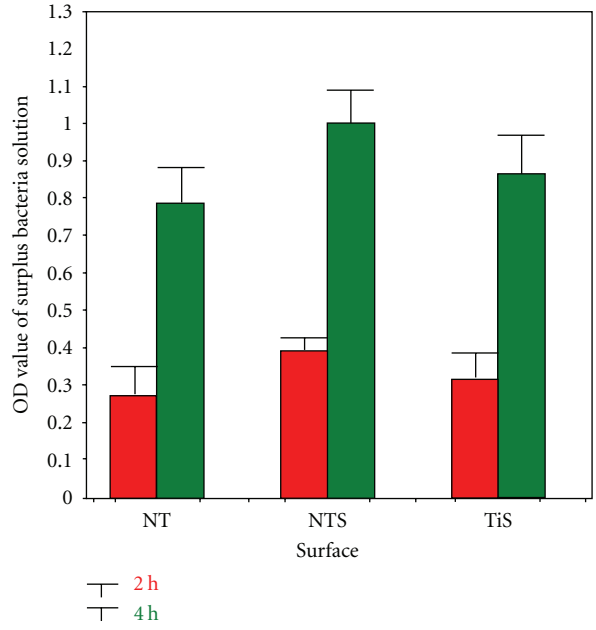

Figure 6: Graph showing OD_{600} of the surplus bacteria solution soaking the tested samples after 2 h and 4 h of culturing. The concentration of *S. aureus* solution was evaluated with the ultraviolet spectrophotometer. The value of OD_{600} is higher than the other two ($P < .05$) at either 2 h or 4 h.

which the lotus leaf effect of superhydrophobic surface was generated.

In this experiment, three kinds of surfaces were created, moderate hydrophilic surfaces (NT), superhydrophobic surfaces (NTS), and hydrophobic surfaces (TiS), which were modified by PETS on flat titanium as a control. In visualization of *S. aureus* on different surfaces (Figures 3(a)–3(f)) are shown the SEM images of bacterial colony formation after 2 h and 4 h of culture on NT, NTS, and TiS, respectively. At 2 h, there were more bacteria on the hydrophilic surfaces (NT, Figure 3(a)) than those on the hydrophobic surfaces (TiS, Figure 3(c)), and in comparison to the superhydrophobic surfaces (NTS, Figure 3(b)), more bacteria were observed on both the surfaces above. Furthermore, bacteria on hydrophobic surfaces, including NTS and TiS, were scattered, while bacteria on hydrophilic surfaces tended to gather. By 4 h, bacteria on the three surfaces all increased and those on the hydrophilic surface (NT, Figure 3(d)) were still more and in clumps compared with the other two (NTS and TiS, Figures 3(e) and 3(f)). The images of SEM showed that the amount of bacteria on superhydrophobic surfaces was less and bacteria were mostly scattered, which indicated that interaction among bacteria was less than that between bacteria and material surfaces. It means that bacteria were not easily adhesive on them. Further studies of CTC dye bacteria indicate that there is an approximately 50%–90% decrease in bacteria colonies on TiS compared to NT and TiS after 4 h of culture. CTC (5-cyano-2, 3-ditolyl tetrazolium chloride) has been used to evaluate the respiratory activity of many bacterial populations derived from environmental sources. Healthy cells respiring via the electron transport chain will absorb and reduce CTC into an insoluble, red fluorescent formazan product. Only live and healthy bacteria could be stained and observed on the surfaces. On the images of fluorescence microscope, the amount of bacteria on hydrophilic surfaces was much more than that on the other two and tended to gather. Once bacteria were in clump, biological membrane could be formed, which kept them away from the effect of body immunity and antibacterial agent, and thus it caused the infection. There was no aggregation of bacteria on the superhydrophobic surface, which suggested that the interaction was tiny and there was little chance to form the biological membrane that was easy to be cleared by body immunity and antibacterial agent. In this way, incidence of infection was decreased [28].

We also tested the OD_{600} value of surplus soaking solution. Figure 4 shows the fluorescence microscopy images of bacteria colonies formed on NT, NTS, and TiS surfaces after 2 h (Figures 4(a)–4(c)) and 4 h (Figures 4(d)–4(f)) of culture. These images suggest that hydrophobic and superhydrophobic surfaces (NTS and TiS) have fewer and smaller bacteria colonies compared to hydrophilic surface (NT). In particular, much less bacteria were again observed by FM on the PETS-coated surfaces compared with the other two. Figure 5 shows the bacteria colony count obtained by Image Pro software. Statistical analysis using one-way ANOVA with Tukey's test showed that the differences between NT and TiS significant ($P < .05$), as were the differences observed between NTS and TiS ($P < .05$). We collected the surplusbacteria solution of tested samples at 2 h and 4 h. And the OD value at the wave length of 600 nm was determined

using the ultraviolet spectrophotometer. Figure 6 shows the changes of OD_{600} of the surplus bacteria solution soaking the samples. Statistic analysis using Tukey's test of the one-way ANOVA showed that the surplus bacteria solution of the superhydrophobic sample was significantly higher than that of the hydrophilic and hydrophobic ones.

Bacteria are in the lag phase 4 h before, according to the growth curve of bacteria. It means that it was not the amount of bacteria that increased but the body that became bigger in this period. So the total number of bacteria in each dish of 2 h or 4 h was the same. As the superhydrophobic surfaces were not profitable for the bacteria adherence, there were more bacteria in the liquid than on the sample surfaces. However, the results in the liquid soaking hydrophilic samples were on the contrary.

4. Conclusion

Electrochemistry anodic oxidation on the titanium surface could generate regulated Tio_2 nanotube array to roughen the surface, while modifing the low surface energy material PTES could easily generate the superhydrophobic surface. Our experiments showed that the amount of bacteria adherence on these surfaces was different. With the contact angle increased, the amount of bacteria decreased. Superhydrophobic surface may decrease the incidence of infection by inhibiting the adherence of *Staphylococcus aureus in vitro*. However, *Staphylococcus aureus* was not totally absent on the superhydrophobic surface; they were less and more scattered compared with those on the hydrophilic and hydrophobic surfaces. So superhydrophobicity was one of the factors affecting bacterial adherence. Further research will focus on the reduction of some other bacteria, such as streptococcus, Gram-negative, on superhydrophobic surface of titanium and the effect *in vivo*.

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