Antimicrobial effects of oxygen plasma modified medical grade Ti–6Al–4V alloy

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ABSTRACT

Biomedical titanium alloy (Ti–6Al–4V) has good mechanical properties and cytocompatibility but postoperative implant-related bacterial infection is a big concern. Therefore, it is very important to suppress adhesion of bacteria on the implants or even kill the bacteria by proper surface modification. In this study, oxygen plasma immersion ion implantation (O-PIII) is employed to produce antibacterial effects on medical grade titanium alloy. The correlation between the surface chemistry and topography on antibacterial behavior is systematically investigated. Colony forming unit (CFU) counting is carried out to evaluate the adhesion of bacteria on the surface and enhanced green fluorescent protein (EGFP) mouse osteoblastic cells are used to study the cytocompatibility after the plasma treatment. Our results suggest that the nanostructured TiO2 layer produced by O-PIII can significantly suppress bacterial adhesion while the original cytocompatibility can be retained. The nanoscale TiO2 layer is promising in the prevention of implant-related bacterial infection on orthopedic implants.

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1. Introduction

Metallic biomaterials continue to play an essential role in the repair or replacement of bony tissues that have become diseased or damaged [1], particularly in load-bearing applications. Titanium alloys such as pure Ti, Ti–6Al–4V, NiTi, Ti–Nb, and Ti–Zr are widely used in the biomedical fields because of their high mechanical strength, good corrosion resistance, superior fatigue properties, as well as good biocompatibility [1–9]. In spite of the superior compatibility of Ti–6Al–4V alloy, post-operative implant-related bacterial infection has been reported. Although the risk of bacterial infection can be minimized by performing the surgery in a clean environment and adopting the proper sterilization procedure, the prevalence of implant-related bacterial infection in joint replacement surgery and bone fracture fixation is still about 2% and 5%, respectively [10]. In some clinical studies, implant-related bacterial infection is the second most common cause for repeated surgery in total knee replacements [11–13]. The scenario can be further aggravated by the aging population and increased use of orthopedic implants.

Bacterial adhesion on the implant surface is an important step in the pathogenesis of bacterial infection [14]. The mechanism involves non-specific and specific adherence [15–19]. Non-specific adherence is thought to involve non-specific processes mediated by physico-chemical interactions such as hydrophobic interactions and electrostatic interactions, whereas specific adherence is mediated by extracellular polysaccharides and lectin-like substances [16]. Deadly bacteria usually secrete polymeric-like materials after adhering to the implant forming protective layers known as biofilms which can further reduce the effectiveness of antibiotic therapy as well as the host defence mechanism. In serious cases, implant removal is the last and most effective option. Clinical data reveal that Staphylococcus aureus (S. aureus) and Coagulase-negative staphylococci (CoNS) account for 40–55% of implant-related infection and S. aureus is responsible for the majority of biofilms accounting for 20–25% of micro-organisms observed in the infection cases [20–26]. Bacterial infection is known to cause problems such as superficial skin lesions and deep wound infection and can sometimes lead to severe life-threatening complications [24]. Successful treatment of bacterial-related infection is challenging and usually requires prolonged antimicrobial therapy, multiple surgical debridement and intervention, and even total removal of implants. Besides S. aureus, Pseudomonas aeruginosa is the second most common micro-organism isolated from implant-related infection [27]. Mixed infection by P. aeruginosa and S. aureus has also been reported [28]. To prevent the formation of
biofilms that resist most of the current antibiotics treatments, inhibition of initial bacteria adhesion on the implant surface is the crucial step and an implant surface with anti-bacterial activity is an advantage over other treatments such as antibiotic therapy.

Many techniques have been employed to improve the antibacterial characteristics of implants. Antibiotic coatings have been used to reduce implant-related bacterial infection by the release of antibiotics and nitric oxide (NO) from sol–gel films [29,30] or by solid phase peptide coating that prevents biofilm formation at the site of the implant [31]. Killing of bacteria using silver and copper plasma immersion ion implantation has been investigated and the mechanism is believed to be the action of the released ions [32–35]. These positively charged metallic ions attach to the negatively charged bacteria cell wall causing cell lysis and death [35,36]. Another technique involving photo-catalysis of titanium dioxide has been widely used in many antibacterial applications such as sterilization of water and surface coatings [37–40]. Under ultraviolet (UV) light irradiation, the anti-bacterial function of TiO2 can be triggered by free radicals of TiO2 released to attach to the bacteria cell wall or cell membrane resulting in bacteria death. However, release of such ions inevitably poisons the surrounding cells at the same time and tissue necrosis may occur.

The most convenient way to suppress the early phases of bacterial adhesion is the modification of the surface chemistry and topography of the substrate [41]. Bacteria proliferation can be inactivated when the surface roughness is small [41,42]. It has been reported that surfaces with a titanium nitride layer and ethylene oxide group exhibit significant reduction of bacteria and these coatings can decrease the risk of inflammation of peri-implanted soft tissues [43,44]. Therefore, modification of surface properties such as topography and chemistry using chemical vapor deposition (CVD), physical vapor deposition (PVD), plasma immersion ion implantation (PIII), and laser-assisted treatments are possible alternatives in addition to antibiotic coatings, NO, or silver and copper ion release. On the other hand, in order to maintain a high degree of biocompatibility and anti-bacterial property at the same time, surface modification is an attractive means. Among these techniques, plasma immersion ion implantation (PIII) is a viable technique for the surface modification of orthopedic implants having a complex geometry. It can be used to tailor the surface topography, surface chemistry, layer thickness, and surface energy of the materials. This study aims at investigating the anti-bacterial properties of medical grade titanium alloy after oxygen PIII in an effort to reduce the possibility of implant-related bacterial infection on orthopedic implants.

2. Experimental procedures

2.1. Materials preparation and characterizations

Circular titanium (Ti–6Al–4V) bars containing 4% vanadium, 6% aluminum, and less than 0.2% iron and oxygen (ASTM F136, Nitinol Device Company, Fremont, USA) were cut into disks 5 mm in diameter and 1 mm thick. They were ground and mechanically polished to a shiny surface, and then ultrasonically cleaned with acetone and ethanol before implantation was conducted in plasma immersion ion planter in the Plasma Laboratory in City University of Hong Kong [45,46]. The implantation parameters are displayed in Table 1. All the treated samples were ultrasonically cleaned again after PIII.

Table 1: Oxygen plasma immersion ion implantation parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control sample</th>
<th>O-PIII sample</th>
<th>O-PIII sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas type</td>
<td>–</td>
<td>O2</td>
<td>O2</td>
</tr>
<tr>
<td>RF</td>
<td>–</td>
<td>1000 W</td>
<td>1000 W</td>
</tr>
<tr>
<td>High voltage</td>
<td>–</td>
<td>–40 kV</td>
<td>–40 kV</td>
</tr>
<tr>
<td>Pulse width</td>
<td>–</td>
<td>100 μs</td>
<td>100 μs</td>
</tr>
<tr>
<td>Frequency</td>
<td>–</td>
<td>50 Hz</td>
<td>100 Hz</td>
</tr>
<tr>
<td>Duration (min)</td>
<td>–</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Base pressure</td>
<td>–</td>
<td>7.0 × 10^-4 Torr</td>
<td>7.0 × 10^-4 Torr</td>
</tr>
<tr>
<td>Working pressure</td>
<td>–</td>
<td>6.4 × 10^-4 Torr</td>
<td>6.4 × 10^-4 Torr</td>
</tr>
</tbody>
</table>

0.8 eV per step were first acquired to identify the elemental species. High-resolution scans were subsequently obtained with a pass energy of 11.75 eV and 0.1 eV per step. A Gaussian–Lorentzian peak-fitting model was used to deconvolute the spectra. The surface topography of the untreated and O-PIII samples were characterized by atomic force microscopy (AFM, Auto Probe CP, Park Scientific Instruments). The measurement was operated in the contact mode. The scanning area was 5 μm × 5 μm under ambient conditions. Nano-indentation tests (MTS Nano Indenter XP, USA) were conducted on nine areas to determine the average hardness of the samples. A three-sided pyramidal Berkovich diamond indenter was employed.

2.2. Mammalian cell cultures

To investigate the cytocompatibility of the plasma-treated and untreated samples, osteoblasts isolated from calvarial bones of 2-day-old enhanced green fluorescent protein (EGFP) mice were employed in our culture in a Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Biowest, France), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), and 2 mM l-glutamine at 37°C in an atmosphere of 5% CO2 and 95% air. The specimens (1 mm thick and 5 mm in diameter) were put on the bottom of a 96-well tissue culture plate (Falcon). A cell suspension consisting of 5000 cells was seeded onto the surface of the untreated Ti alloy samples, the oxygen-implanted Ti alloy for normal culturing conditions. Cells were grown in 1 mL of medium and changed every three days. Cell attachment was examined after the first day of culture, and cell proliferation examined after 3 and 7 days of culture. Three samples were taken at each time point to obtain better statistics. Cell proliferation was observed by a fluorescent microscope (Axioplan 2, Carl Zeiss, Germany). The attached living EGFP-expressing osteoblasts were visualized using a 450–490 nm incident filter and the fluorescence images emitted at 510 nm captured using a Sony DKS-STS digital camera.

2.3. Bacterial cultures — colony forming unit (CFU) counting with the use of clinically relevant bacteria

To investigate bacterial adhesion on the samples, clinically isolated Staphylococcus aureus bacteria (RN6390 from Professor Ambrose Cheung at the Dartmouth Medical School, Hanover, USA) were used in the culture. Brain heart infusion broth (BHI) was prepared to culture S. aureus cells and BHI agar was also prepared for the growth of the single colony before growing an overnight culture. The BHI broth powder and agar powder were weighted at 7.4 g/200 ml and 9.4 g/200 ml, respectively. 200 ml of distilled water were added to each bottle and mixed. The solutions were sterilized by autoclaving at 121°C for 15 min. About 20–25 mL of the agar solution was poured into the culture dish to allow solidification and formation of an agar plate. The streaking plate isolated
single colony was incubated for 24 h at 37 °C. Then, one single colony was taken and put into the BHI broth. The overnight culture was grown at 37 °C in a temperature control shaker (Thermo Forma, Thermo Fisher Scientific Inc., Waltham, MA, USA) operated at 250 rpm for 16–20 h, unless otherwise specified.

The number of bacteria in the overnight culture was counted. As the overnight culture contained $10^{10}$/ml bacteria ($S. aureus$), they were diluted to $10^{8}$/ml to test bacterial adhesion on the treated Ti alloy surface and untreated Ti alloy surface (5 mm diameter). 20 µL of BHI with $10^{8}$/ml bacteria, which was about $2 \times 10^6$ bacteria, were added to the Ti alloy surface. After incubation for 1 h, the unattached bacteria were removed by rinsing 3 times in 1 ml of PBS. The adherent bacteria were then removed from the surface by sonication. 1 ml of 0.01% Tween 80 in 0.01 M PBS was used to detach the bacteria from the surface and mixed with the sample for 1 min sonication. Afterward, the suspension containing the bacteria was collected. The sample surface was rinsed 3 times again with 1 ml of PBS. Some of the suspension was diluted 50 times. Moreover, 50 µL of the suspension and the one diluted 50 times were added to the BHI agar for overnight culture and the colonies were counted afterward.

LIVE/DEAD BacLight Viability Kit staining (Invitrogen Inc.) was used to stain the bacteria. $2 \times 10^5$ bacteria were put on the surface and incubated for 1 h. The suspended and non-adhered bacteria were removed by rinsing in distilled water. The stain was added on the surface and incubated for 20 min before taking the fluorescent images.

3. Results and discussion

The high-resolution O 1s XPS spectrum in Fig. 1(a) can be deconvoluted into three peaks at 530.8, 532.2 and 534.2 eV corresponding to the metal oxide bonds, carbonyl bond (C=O), and...
carbon–oxygen single bond (C–O), respectively [47]. It is obvious that the signal of the peak at 530.8 eV increases significantly when the pulsing frequency in the O-PIII experiments is increased to 100 Hz. This observation may be attributed to the formation of more metal oxide bonding on the top layer. As shown in Fig. 1(b), the measured binding energy of Ti2p3/2 on the three types of samples is 458.9 eV and the difference in the binding energy between Ti2p1/2 and Ti2p3/2, Δ, is 5.5 eV. It reveals that TiO2 mainly exists on the surface layer of the samples, although traces of other chemical states such as Ti2+ (TiO) can also be observed. The results are consistent with previous findings [3] implying that the top layer is composed of predominantly TiO2 and a trace of TiO. The depth profile in Fig. 2(a) reveals that the top layer of the untreated sample contains only titanium oxide with a thickness of several nanometers due to natural oxidation. Compared to the untreated sample, the titanium oxide layer on the 50 Hz and 100 Hz O-PIII samples is about 150 nm thick as shown in Fig. 2(b) and (c).

Fig. 3 exhibits the surface topographies of the O-PIII and untreated samples. The untreated sample has a smooth surface (Fig. 3(a)), whereas the 50 Hz O-PIII sample exhibits irregular nanostructured TiO2 with a height of about 5 nm (Fig. 3(b)). When the implantation frequency is increased to 100 Hz, the height of the TiO2 structure increases to about 10 nm as shown in Fig. 3(c).

Cell attachment on the untreated control and O-PIII samples (50 and 100 Hz) is observed after culturing for one day (Fig. 4) and proliferation is monitored after 3 and 7 days (Figs. 5 and 6). The TiO2 nano structure does not alter cell attachment and proliferation compared to the untreated control.

Fig. 7 shows the number of adhered bacteria on the treated and untreated sample surfaces after 1 h of incubation. The TiO2 nano structures fabricated at frequencies of 50 Hz and 100 Hz reduce bacterial adhesion. Fig. 8(a) and (b) reveals the morphology of adhered bacteria on the untreated and oxygen plasma treated samples observed by fluorescent microscopy, respectively. Live
Fig. 4. Microscopic view of green fluorescent protein mouse osteoblasts on the titanium alloy samples after 1 day: (a) untreated titanium alloy, (b) 50 Hz O-PIII sample, and (c) 100 Hz O-PIII sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Microscopic view of green fluorescent protein mouse osteoblasts on the untreated titanium alloy in 3 days: (a) untreated titanium alloy, (b) 50 Hz O-PIII sample, and (c) 100 Hz O-PIII sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
bacteria are stained in green color, whereas dead bacteria manifest in red (Fig. 8). The results suggest that the number of bacteria on the TiO₂ nano-structure surface are significantly smaller than that on the untreated titanium alloy. These results are consistent with those obtained by CFU counting. Fig. 9 reveals the surface hardness of the untreated, 50 Hz and 100 Hz oxygen plasma treated samples. The hardness scales of the samples with oxygen plasma treatment ranged from 5.0 to 9.0 GPa, whereas the hardness of the untreated sample was from 4.6 to 6.8 GPa only. The treated surfaces were significantly harder than the untreated control.

Photo-catalytic titanium dioxide with nano-particles size [48], mixed with a polymer film [49], as well as deposited on a metal substrate has been extensively studied for antibacterial applications when irradiated by UV light [50]. With regard to the antibacterial mechanism of titanium dioxide, Matsunaga et al. suggest that light irradiation of TiO₂ powders induces the formation of an electron hole pair in the particles. The excited TiO₂ powders generate electrons in the conduction band that are transferred to oxygen as the acceptor, and the hole in the valence band of TiO₂ receives an electron from coenzyme A (CoA) as the donor, finally resulting in the formation of dimeric CoA [37,38]. However, this explanation is controversial. Maness et al. propose that ROS such as OH⁻, O₂⁻, and H₂O₂ generated on the irradiated TiO₂ surface operate in concert to attack polyunsaturated phospholipids in *Escherichia coli*. Since the cell membrane is composed of various lipids with various degrees of unsaturation and relies on these lipids to carry out essential functions, the lipid peroxidation reaction induces a breakdown in the cell membrane structure, thus killing the bacteria [51]. Cai et al. suggest that photo-excited TiO₂ damages the cells not only the cell membrane but also the inside of the cytoplasm by the photo-generated holes and the hydroxyl and perhydroxyl radicals from water by the irradiated TiO₂ [39]. In reality, in order to prevent implant-related bacterial infection on orthopedic implants, TiO₂ in the form of powder particles cannot be directly applied clinically because the TiO₂ must bond rigidly to the surface of the biomedical implants. In order to maintain the antibacterial property, this protective layer should have a superior bonding strength with the implant surface to avoid peeling off during handling and surgical implantation. Furthermore, the effectiveness and longevity of the antibacterial property are important and essential to prevent post-operative implant-related bacterial infection, since it has been reported that infection can occur even two years after surgery.
Previous studies suggest that TiO$_2$ exhibits antibacterial function only after UV light irradiation [37–40,50,51]. This approach may not be appropriate to biomedical implants since UV light is harmful to people. Therefore, an implant surface with self-contained antibacterial functions is better clinically. In our study, the medical grade titanium surface with TiO$_2$ nano structure fabricated by oxygen PIII can significantly reduce bacterial adhesion. XPS reveals that this film is composed of a stable and 75 nm thick layer of TiO$_2$ and TiO with a graded interface between 75 nm and 150 nm in depth. AFM discloses that the outermost layer is composed of one-dimensional TiO$_2$ nano structure with heights of 5–10 nm. Our results suggest that the one-dimensional titanium oxide nano structure possess antibacterial function even without UV irradiation. It is believed that the titanium oxide layer produced by O-PIII is quite different from previously reported TiO$_2$ particles. According to theories proposed previously [37,39,51], one possible factor is that this structure intrinsically disfavors bacterial adhesion. Another possibility is that the free hydroxyl and perhydroxyl radicals from water can easily attach onto the surface of these TiO$_2$ nano structure to prevent adhesion of bacteria. The antibacterial mechanism of 1D TiO$_2$ nano structure is being investigated in more details in our laboratory and will be reported in the future.

The cytocompatibility of implantable materials is another critical issue. Although photocatalytic TiO$_2$ particles stimulated by UV light illumination can kill bacteria such as E. coli, Maness et al. have also pointed out that the proposed killing mechanism may kill other types of cells as well [51]. It implies that these excited TiO$_2$ particles may kill surrounding cells such as osteoblasts, thereby potentially inducing tissue necrosis. Therefore, an ideal implantable antibacterial surface should not be only able to resist bacterial adhesion and its subsequent biofilm formation, but also possess good biocompatibility. The results of our cell culture tests suggest that osteoblast cells attach and proliferate well on the surface of the nanostructured TiO$_2$ produced by oxygen plasma treatment and the biocompatibility is comparable to that of medical grade (untreated) Ti alloy.

Coating with antibiotics such as vancomycin and gentamicin on the implant surface is an alternative [29,52]. Price et al. have coated gentamicin on metallic implants with polylactic-co-glycolic (PLGA) as the biodegradable carrier to reduce the risk of bacterial infection [52]. Their results reveal that this antibiotic coating can successfully kill 99.9% of the bacteria. It has also been reported that antibiotics may even directly bond to the metallic surface using chemical treatment, i.e. solid phase coupling in which this surface-bound antibiotics effectively prevents bacterial colonization [53]. The reported effectiveness of the vancomycin-coated titanium surface only lasts for 45 days and the antibacterial function disappears completely afterward due to degradation of vancomycin. The bacterial killing effect of these antibiotics coatings depends on the release mechanism, which is typically only short term, but implant-related bacterial infection may occur up to 2 years or more after surgery [54]. In this respect, the antibacterial mechanism of the TiO$_2$ nano structure does not stem from a release mechanism and in principle, this antibacterial surface can suppress bacterial adhesion for a longer time. The nano structure is not expected to degrade after implantation. As shown in Fig. 9, the nano-indentation tests demonstrate that the TiO$_2$ nano structure substrate possesses higher hardness than the untreated titanium alloy and good wear resistance is expected.

4. Conclusion

This study reveals that a nanostructured surface layer composed of predominant TiO$_2$ and a trace of TiO is formed on the titanium alloy after oxygen plasma immersion ion implantation. This one
dimensional titanium oxide nano structure possesses good anti-bacterial property and good cytocompatibility. Because the anti-bacterial properties do not stem from UV irradiation or a release mechanism, the effect will be more long lasting for orthopedic implantation.

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