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ORIGINAL PAPER

# Inhibition of biofilm formation on iodine-supported titanium implants

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**Abstract**
*Purpose* We have developed iodine-supported titanium implants that suppress microbial activities and conducted in vivo and in vitro studies to determine their antimicrobial properties.
*Methods* The implants were Ti-6Al-4 V titanium implants either untreated (Ti), treated with oxide film on the Ti surface by anodization (Ti-O), or treated with an iodine coating on oxidation film (Ti-I). The strain of bacteria used in this study was Gram-positive *Staphylococcus aureus* strain ATCC 25923. We analyzed the antibacterial attachment effects in vivo by using rats. The attachment bacteria on the implant surface were evaluated using a spread-plate method assay. A biofilm study was performed in vitro. The biofilm formed after bacterial attachment was qualitatively studied with fluorescence microscopy (FM) and scanning electron microscopy (SEM). Also, the formed biofilm was quantitatively studied with a spread-plate method assay.
*Results* In vivo analysis of antimicrobial attachment effects showed that the mean viable bacterial number was significantly lower on Ti-I than Ti or Ti-O surfaces. In the in vitro biofilm study, FM and SEM images showed thick and mature biofilm formation on Ti and Ti-O and thin, small biofilm formation on Ti-I. A quantitative biofilm analysis found a significant difference in the number of viable bacteria between Ti-I and Ti or Ti-O.
*Conclusions* This study showed that iodine-supported implants have a good antibacterial attachment effect and inhibit biofilm formation and growth. Iodine-supported implants may have great potential as innovative antibacterial implants that can prevent implant related infection in orthopaedic surgery.

**Keywords** Iodine-supported implant · Implant related infection · Biofilm · Antibacterial attachment effect

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## Introduction

In orthopaedic surgery, infection is one of the most common and most challenging complications following implant placement. Studies have shown that the postoperative infection rate after total hip arthroplasty is 2.2%, while infection rate after spinal surgery is 2.0% despite strict antiseptic procedures [1, 2]. Other studies show that almost 50% of patients experience pin tract infection after pin insertion for external fixation [3, 4]. Once bacteria adhere to metal surfaces, they produce an extracellular polymeric matrix instead of substance and form a biofilm. The biofilm on the metal surface is resistant to almost all antibiotics except rifampicin and protected from immune surveillance. Such infections can be difficult to cure without removing the medical devices, along with extensive bone or soft tissue debridement.

On the premise that modification of the implant surface could help prevent bacterial adhesion, a variety of biomaterials with antimicrobial effects have been developed as implant surface coatings including: either quaternary ammonium compounds, human serum albumin or ion implantation (Ag, Cu and F),

chitosan-nanoparticle loaded implants, phosphatidylcholine-based material, and antibacterial loaded (vancomycin, etc.) implants [5–13]. In particular, the usefulness of silver as an antibacterial agent has been widely investigated because of its strong broad-spectrum antibacterial properties [11]. Although silver-coated implants definitely have good antibacterial effects, the possibility of low biocompatibility and toxic effects on human cells cannot be ignored [14–16]. In an effort to develop a biomaterial with both good antibacterial effects and low toxic effects, we developed iodine-supported titanium implants that suppress microbial activities. Shirai et al. demonstrated the antibacterial attachment effect and cytocompatibility of iodine-supported implants in an in vitro study [17]. However, no reports to date have shown the antibacterial attachment effect of iodine-supported implants in an in vivo study, nor has anyone yet addressed the inhibition of biofilm formation and growth, which is a clinically important factor.

In this study, the authors investigated the following questions: (1) Do iodine-supported implants demonstrate antibacterial attachment effects in an in vivo study? (2) Do iodine-supported implants inhibit biofilm formation and growth in an in vitro study?

## Materials and methods

### Implant preparation

The implants used in the in vivo antibacterial study were Kirschner wires (K-wire) with a length of 20 mm and a diameter of 1.25 mm. The implants used in the in vitro biofilm study were metallic washers with a diameter of 6 mm and a thickness of 0.5 mm. The implants were solid, smooth, Ti-6Al-4 V titanium implants (Ti), either untreated, treated with oxide film on the Ti surface by anodization (Ti-O), or treated with an iodine coating on oxidation film (Ti-I). The iodine supports were produced at the Chiba Institute of Technology (Narashino, Chiba, Japan) using a technique described by Hashimoto et al. [18]. The anodic oxide film shows a thickness of 5–10 μm with more than 50,000 pores/mm$^2$, and the capacity to support 10–12 μg/cm$^2$ iodine. As for the surface area, microscopic observations showed that the surface area of Ti-O and Ti-I clearly increased more than the surface area of Ti; there was no change between Ti-O and Ti-I (Fig. 1a–c). All implants were processed by Promedical Instruments Company (Kanazawa, Ishikawa, Japan).

### In vivo analysis of antimicrobial attachment effect

The strain of bacteria used in this study was Gram-positive *Staphylococcus aureus* strain ATCC 25923. The bacteria were incubated in 5 ml of fresh brain-heart infusion (BHI, Bacto™, Becton Dickinson) in a shaking incubator for 24 h at 37 °C. The culture was subjected to 10-fold serial dilution with phosphate buffered saline (PBS) to finally get to about 5 × 10$^4$ colony forming unit (CFU) /ml.

Eighteen male, ten-week-old Sprague-Dawley rats (Japan Charles River, Japan) weighing 300–350 g were used in this study. This animal procedure was performed with the approval of the animal ethics committee at our institution (approval date: 3 September 2013; approval number: 132,928). This in vivo study followed the method outlined in a previous report with slight modifications [19]. The operation was performed under general anaesthesia injected intraperitoneally with pentobarbital (0.3 mg/kg body weight). The operation field (bilateral knee joint) was shaved and disinfected with povidone-iodine. A medial parapatella approach was made over the bilateral knee joint. We accessed the knee joint, and a hole was hand-drilled with an 18-gauge needle through the centre of the knee. Next, 10 μl of PBS containing *S. aureus* (almost 5 × 10$^2$ CFU) was inoculated into the distal femoral canal. After bacterial inoculation, the implant was press-fit into the canal. In this study, we randomly inserted either Ti, Ti-O, or Ti-I in both knee joints. Finally, skin, fascia, and joint capsule were closed using 4–0 nylon.

The *S. aureus* infected rats used in the experiments were euthanised at 24, 48 and 72 hours post-operatively by intraperitoneal injection with pentobarbital (9 mg/kg body weight). All inserted K-wires were removed under sterile conditions and placed in 1.5 ml microtubes containing 1 ml PBS. The tubes were subjected to rapid vortex mixing for one minute to detach adhered bacteria on the implant surface. The solution was serially diluted 10-fold with PBS and the final bacterial suspensions were plated on BHI agar plates for 24 hours at 37 °C. The number of viable cells was counted for the three types of implants.

### Biofilm formation assay

The biofilm study in vitro was performed as described in the past report, with a slight modification from Braem et al. [20]. The implant was exposed to Gram-positive *Staphylococcus aureus* strain ATCC 25923 which is known to have a tendency to form biofilm [21]. The bacteria were incubated in 5 ml of fresh Tryptic Soy Broth [TSB, Bacto™, Becton Dickinson] with aeration at 37 °C for 24 hours. This culture was diluted 100-fold in TSB with 1% glucose (wt./vol) and re-incubated at 37 °C for 75 minutes. The re-incubating medium corresponded to the early exponential growth phase and was adjusted to obtain an OD600 = 0.2–0.3, giving a bacterial suspension of 1–5 × 10$^7$ CFU/ml. The metal washers were sterilized in an autoclave and placed into 24-well plates, and 1 ml of the bacterial suspension was added to each well. The material was statically incubated for 24 hours at 37 °C to allow bacterial adhesion to the metal surface. After initial bacterial adhesion, the medium was replaced with fresh TSB medium

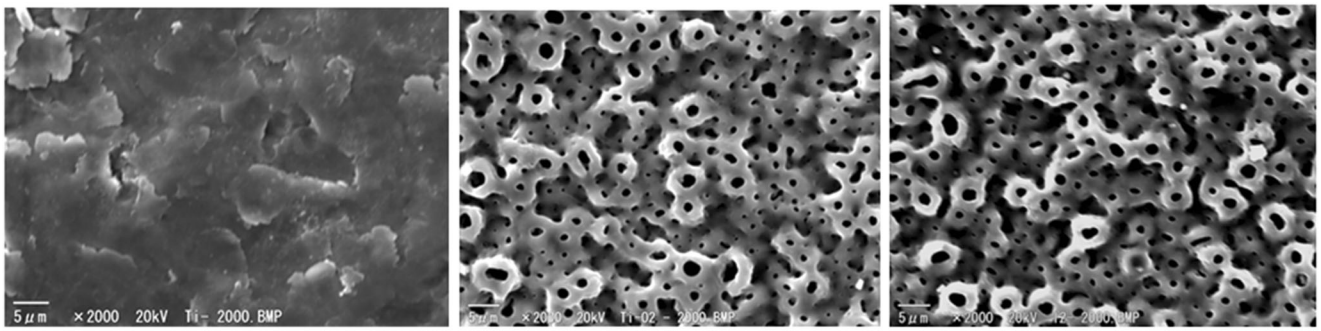


**Fig. 1** Electron microscopic evaluation of metal implant surfaces: titanium implant (**a**), Ti-O implant (**b**), Ti-I implant (**c**). Original magnification × 2000 (scale bar = 5 μm)

every 24 hours to form a mature biofilm and remove planktonic cells under sterile conditions. Finally, further incubation was performed under the same conditions for 24 hours and 72 hours after bacterial attachment.

The biofilm formed at 24 hours after bacterial attachment was studied with fluorescence microscopy, scanning electron microscopy (SEM), and the spread-plate method assay. The biofilm at 72 hours after bacteria attachment was studied with the spread-plate method to investigate biofilm growth for each type of implant. In this study, we used 57 wells (nine wells used in SEM analysis, 12 wells used in fluorescence microscopy analysis, and 36 wells in quantitative analysis by the spread plate method).

### Observation of biofilm by fluorescence microscopy

All washers were gently rinsed in ultrapure water to remove planktonic bacteria, and the biofilms that had formed on the metal surfaces were stained using FilmTracer™FM® 1–43 green biofilm cell stain (Invitrogen Life Science), basically according to the manufacturer's protocol [22]. In this study, 100 μl of the staining solution was applied to the material surface; the metal washers were then incubated for 45 minutes in the dark with a slight modification according to Nganga et al. [11]. The stained metal washers were rinsed with sterile ultrapure water to remove excess stain. They were investigated by using BIOREVO BZ-9000 fluorescence microscopy (Keyence, Tokyo, Japan). The lens magnification was 20 × (four replicates). The color photographs were converted to gray-scale images with Adobe Photoshop Elements 12.0 (Adobe Systems, San Jose, CA, USA), and biofilm coverage rate (BCR) was measured with Image J to calculate the percentage of surface covered by biofilm, with a slight modification according to past reports [23, 24]. The BCR value of eight areas were averaged for each implant.

### Observation of biofilm by SEM

SEM (JSM 5400; JEOL Ltd) was used to analyze the morphology and distribution of the biofilm formed on the surface. After PBS washing to remove planktonic cells, the biofilm on each washer was fixed by immersion in 2.5% glutaraldehyde at room temperature for 24 hours, and then rinsed with deionized water. For dehydration, the metal washers were passed through an ascending series of ethanol solutions (50 - 75 - 95 - 100%) for ten minutes at each interval with a final pass through t-butyl alcohol. Then the washers were dried using a freeze-dryer. Finally, the biofilm was sputter-coated with platinum palladium using an ion-sputtering system. The fixed washers were attached to metal folders and viewed with an SEM at an accelerating voltage of 20 kV and × 2000 magnification (three replicates).

### Quantitative analysis of biofilm formation assay by the spread plate method

We used the spread plate method to conduct a quantitative analysis of biofilm formation. The washers were gently rinsed by dipping in PBS to remove non-adherent cells on the surface. Next, they were placed into 1 ml PBS in 1.5 ml microtubes. The solution was subjected to rapid vortex mixing for 15 seconds and then sonicated for five minutes (Bransonic Branson 5210, Kanagawa, Japan) at a frequency of 40 Hz to disrupt the formed biofilm. Finally, rapid vortex mixing of the solution was performed again for one minute. This method of disrupting the biofilm was performed with a slight modification according to Braem et al. [20]. Quantitative analysis was done using the standard plate count method. The solution containing bacteria derived from the biofilm was subjected to ten-fold serial dilution using PBS, and the bacterial suspensions were plated on TSB agar plates for 24 hours at 37 °C. The number of colonies was counted on all plates. This study was repeated six times.

#### *Statistical analysis*

Statistical analysis was performed using SPSS software (PASW Statistics Base version 19; SPSS, Chicago, Illinois). Antimicrobial attachment effect, BCR, and quantitative

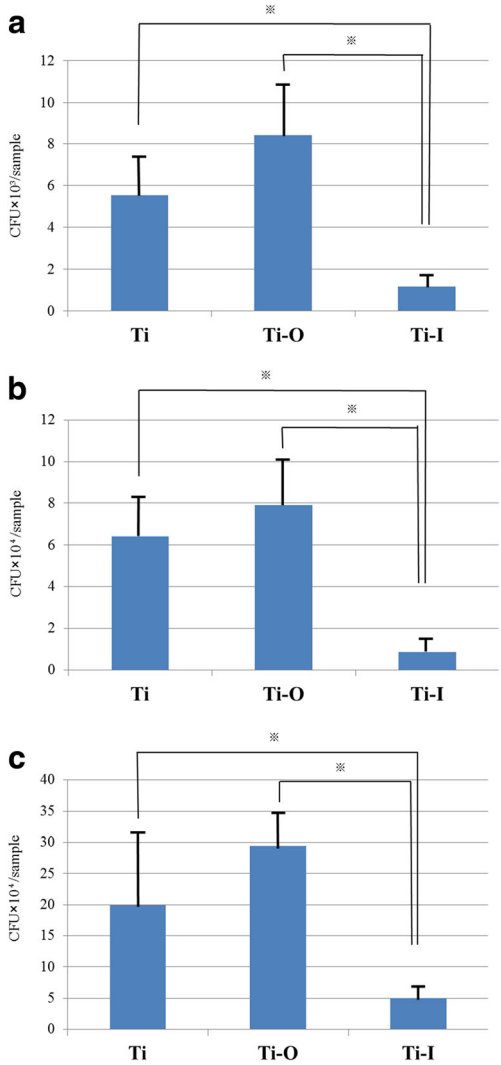


**Fig. 2** Antimicrobial attachment test results ($n$ = 4). **a** 24 hours after inoculation. **b** 48 hours after inoculation. **c** 72 hours after inoculation. Data are expressed as mean ± standard deviation. ※ p<0.05

biofilm formation analysis by the spread plate method were compared between groups by Mann-Whitney U test. A $p$-value < 0.05 was considered statistically significant.

## Results

1) Do iodine-supported implants demonstrate antibacterial attachment effects in an in vivo study?

All rats were alive and all implants were explanted under sterile conditions. Mean viable bacterial numbers in Ti, Ti-O, and Ti-I were $5.6 \pm 2.1 \times 10^3$ CFU, $8.4 \pm 2.4 \times 10^3$ CFU, and $1.2 \pm 0.7 \times 10^3$ CFU at 24 h (Fig. 2a); $6.4 \pm 2.1 \times 10^4$ CFU, $7.9 \pm 2.3 \times 10^4$ CFU, and $8.6 \pm 2.6 \times 10^3$ CFU at 48 h (Fig. 2b); and $2.0 \pm 0.6 \times 10^5$ CFU, $2.9 \pm 0.6 \times 10^5$ CFU, and $5.0 \pm 2.1 \times 10^4$ CFU at 72 h (Fig. 2c). The mean viable bacterial number was significantly lower on Ti-$I_2$ than on Ti or Ti-$O_2$ implants; there was no significant difference between the mean viable bacterial numbers on the Ti and Ti-O implants.

2) Do iodine-supported implants inhibit biofilm formation and growth in an in vitro study?

In fluorescence microscopy images, biofilms were observed on all surfaces and were stained green by using FilmTracer™FM® 1–43 green biofilm cell stain (Fig. 3). BCR in Ti, Ti-O, and Ti-I were 11.4 ± 2.4%, 39.6 ± 3.4%, and 3.1 ± 1.0%. This result demonstrated that a wide area was covered by the stained biofilm on the surface of Ti and Ti-O compared with the surface of Ti-I (Fig. 4). This tendency was observed across the entire surface of all three metals.

The biofilm formation observed in SEM images as the mass of the microcolonies confirmed this difference in biofilm morphology or distribution. The biofilm formation was also observed on all surfaces in fluorescence microscopy images. In particular, these images showed that the bacteria on Ti and Ti-O was accumulated and more tightly colonized than for Ti-I. The bacterial slime formed on Ti or Ti-O clearly included many microcolonies compared with Ti-I although bacterial colonization could be seen on all implants (Fig. 5).

Viable bacteria within the biofilm tended to increase over time on all metal surfaces using the spread plate method. At

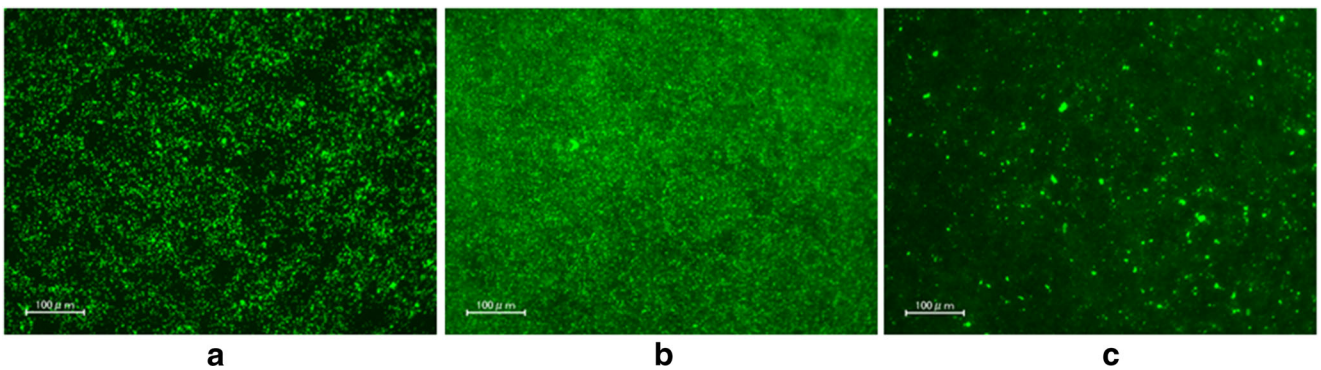


**Fig. 3** Biofilm formation in fluorescence microscopy images. **a** Titanium implant. **b** Ti-O implant. **c** Ti-I implant. Original magnification × 20 (scale bar = 100 μm)

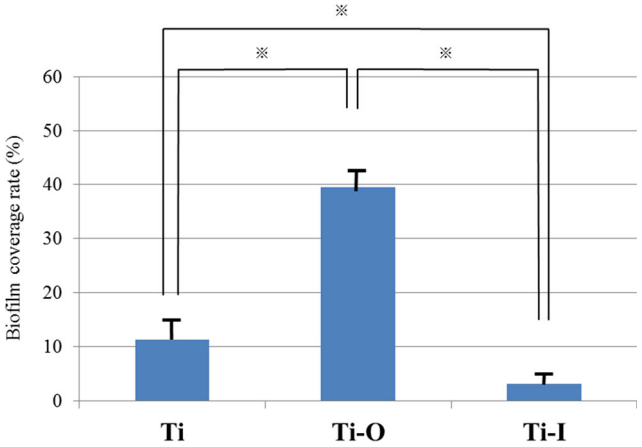


**Fig. 4** Biofilm coverage ratio. **a** Titanium implant. **b** Ti-O implant. **c** Ti-I implant. Data are expressed as mean ± standard deviation. ※ p<0.05

24 hours or 72 hours after bacterial attachment, there was a significant difference in the number of viable bacteria between Ti-I and Ti or Ti-O (Fig. 6a, b). On the other hand, there was no significant difference between Ti and Ti-O in the amount of viable bacteria within biofilms.

## Discussion

Inhibition of biofilm formation on iodine-supported implants is very effective in preventing early post-operative implant related infection. Povidone-iodine, the coating material used in this study, is a broad-spectrum antimicrobial agent frequently used in orthopaedic surgery. The antibacterial spectrum of iodine includes not only general bacteria including *staphylococcus*, but also viruses, tubercle bacilli, and fungi [25]. Furthermore, it has low potency for developing resistance and adverse reactions because iodine is a trace metal and an essential component of the thyroid hormone [26]. We would expect iodine-supported implants to have good antibacterial effects and low toxic effects on human cells in comparison to silver.

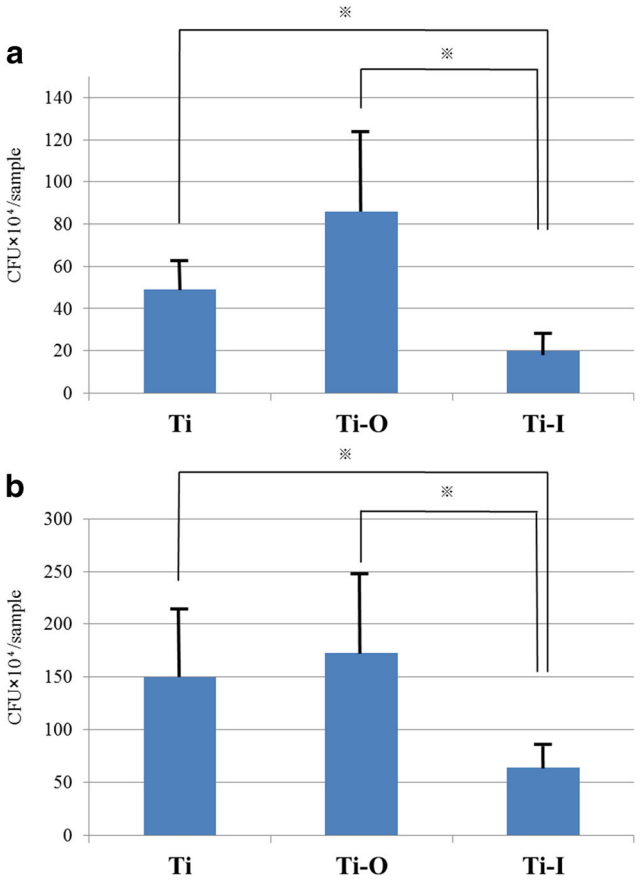


**Fig. 6** Quantitative biofilm formation analysis by the spread plate method ($n$ = 6). **a** 24 hours incubation after bacterial adhesion. **b** 72 hours incubation after bacterial adhesion. Data are expressed as mean ± standard deviation. ※ p<0.05

Our study had several limitations. First, there was no long-term investigation of the antimicrobial attachment effect or the inhibition of biofilm formation on iodine-supported implants. However, the postoperative surgical site infection was established by attaching the metal surface firstly in the acute phase, and therefore showed that the iodine-supported implants were very effective in preventing initial microbial attachment. Further study would be needed to assess the long-

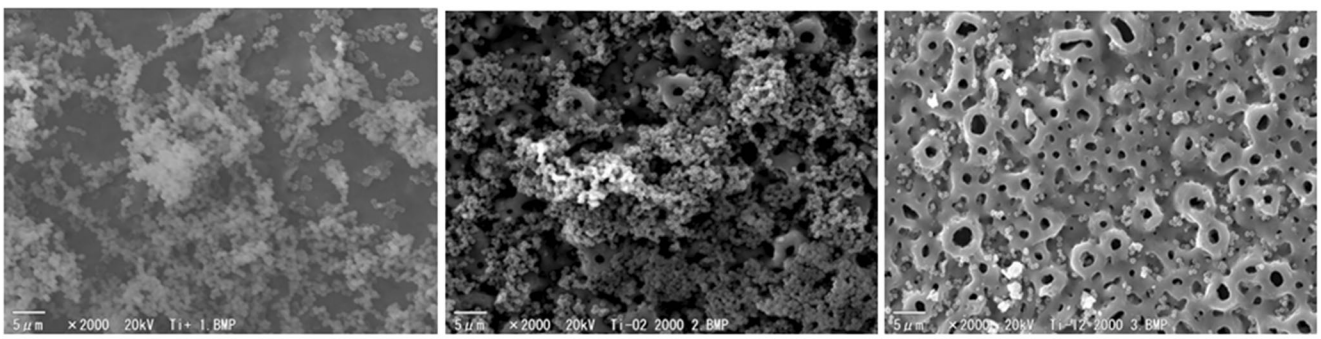


**Fig. 5** Biofilm formation in scanning electron microscopy images. **a** Titanium implant. **b** Ti-O implant. **c** Ti-I implant. Original magnification × 2000 (scale bar = 5 μm)

term effects. Secondly, in this study, the implants were exposed to only methicillin-sensitive *Staphylococcus aureus* (MSSA). The antimicrobial attachment effect of methicillin-resistant *Staphylococcus aureus* (MRSA) or *Pseudomonas aeruginosa*, which are well known to form biofilms on metal surfaces, also should be evaluated in the future. Finally, we did not investigate the chronological influence of iodine release in basic research. Further study would be also needed.

The results of our study in vivo indicate that iodine-supported implants have strong antimicrobial attachment effects. Shirai et al. reported that iodine-supported implants have favourable antimicrobial attachment properties in vitro, and our results in vivo were consistent with this report [17]. However, the mean viable bacterial number on Ti-O surfaces tended to increase compared with Ti implants, although there was no statistical difference in the mean viable bacterial number between Ti and Ti-O. Several previous reports showed that surface area is related to bacterial attachment [27, 28]. Ti implants were anodized to support the iodine on the Ti implant surface, and apparently the anodization resulted in an increased surface area (Fig. 1a–c). Necula et al. reported that Ti implants anodized to support Ag and increase the surface area of implants resulted in the complete killing of methicillin-resistant *Staphylococcus aureus*, whereas many viable bacteria were recorded on the Ti or Ti-O implants in our in vitro study [29]. Because Ti-I was impregnated with povidone-iodine more widely due to the increased surface area, the contact area between the povidone-iodine and bacteria was increased, so rather than increasing the bacterial attachment, Ti-I could obtain good antimicrobial attachment effects compared with Ti and Ti-O.

In fluorescence microscopy images and SEM images, our results showed the bacteria on Ti and Ti-O was accumulated and more tightly colonized than for Ti-I (Figs. 3, 4, and 5). Furthermore, our quantitative biofilm analysis by the spread plate method found a significant difference in the number of viable bacteria between Ti-I and Ti or Ti-O at 24 h or 72 h after bacterial attachment (Fig. 6a–b). Consequently, we can conclude that Ti-I has a stronger anti-biofilm effect than Ti or Ti-O. Past reports showed that biofilm formation is related to the initial bacterial attachment on a metal surface [30, 31]. Therefore, our in vivo results showing the antimicrobial attachment effect on Ti-I were associated with inhibiting biofilm formation on the metal surface. There was significant difference between BCR on the Ti and Ti-O implants. Because the Ti-O surface area tended to increase compared with Ti implants, a wide area was covered by the stained biofilm on the surface of Ti-O compared with the surface of Ti [27, 28]. Microscopic analysis showed a clearer difference than the spread plate method. This is because with the spread plate method, sonicating the biofilm on the metal surface may excessively destroy viable bacteria. Even considering this possibility, the in vivo and *vitro* results showed that the iodine-supported implant inhibits biofilm formation by preventing initial bacterial attachment on the metal surface. Clinically, we believe that iodine-supported implants may have the potential to prevent, or at least dramatically reduce, postoperative implant related infection.

## Conclusions

This study showed that iodine-supported implants have a good antibacterial attachment effect in vivo and inhibit biofilm formation and growth. Our results indicate that iodine-supported implants may have great potential as innovative antibacterial implants that can prevent implant related infection in orthopaedic surgery.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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**Ethical approval** This study was performed with the approval of the animal ethics committee at our institution (Approval date: 3 September 2013; Approval number: 132,928).

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