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The biomimetic apatite-cefalotin coatings on modified titanium

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Dental implant failure often occurs due to oral bacterial infection. The aim of this study was to demonstrate that antibiotic efficacy could be enhanced with modified titanium. First, the titanium was modified by anodization and heat-treatment. Then, a biomimetic coating process was completed in two steps. Surface characterization was performed with scanning electron microscopy, energy dispersive spectroscopy, and X-ray diffraction. Release of antibiotic was evaluated by UV/VIS spectrometry, and the antibacterial effect was evaluated on *Streptococcus mutans*. After the second coating step, we observed a thick homogeneous apatite layer that contained the antibiotic, cefalotin. The titanium formed a rutile phase after the heat treatment, and a carbonated apatite phase appeared after biomimetic coating. We found that the modified titanium increased the loading of cefalotin onto the hydroxyapatite coated surface. The results suggested that modified titanium coated with a cefalotin using biomimetic coating method might be useful for preventing local post-surgical implant infections.

Keywords: Anodization, Antibacterial effect, Biomimetic coating, Cefalotin, Heat-treatment

INTRODUCTION

Several attempts have been made to improve the bonding between bone and a dental implant by coating the titanium of the implant with bioactive materials, like hydroxyapatite, which can form a chemical bond with bone tissue[1-4]. Despite the development of this coating method, implants have continued to fail. An exposed implant can interact with negative bacteria, which can lead to infection around the implant. In addition, some patients that receive implants have had periodontitis in the past. A history of periodontitis and the presence of bacteria are risk factors for peri-implant infections[5,6]. And this may ultimately result in implant failure. Therefore, many studies have addressed this problem with limited success[7-12]. Therefore, to solve this problem, local drug delivery system through antibiotic coatings was developed[13-17]. Recently, precipitation coating method was introduced. In this biomimetic precipitation method, the titanium implant is immersed into saturated solutions of calcium and phosphate that also contain antibiotics. When the calcium-phosphate crystals were precipitated, the antibiotics were coprecipitated[13-16]. Antibiotics that contain a carboxylic group, like cefalotin, have strong binding interactions with calcium. *In vitro* tests have shown that biomimetic coatings have high antibiotic incorporation efficiency and a slow release rate from the coated surface[14]. The cefalotin antibiotic is a first generation cephalosporin antibiotic. It is used to prevent infection during surgery and to treat many kinds of infections of the blood, bone or joints, respiratory tract, skin, and urinary tract. The bactericidal activity of cefalotin results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs). The PBPs are transpeptidases which are vital in peptidoglycan biosynthesis. Therefore, their inhibition prevents this vital cell wall compenent from being properly synthesized[18].

In this study, the titanium surface was modified by anodizing and heat-treatment before applying a biomimetic coating. We hypothesized that modifying the titanium surface could increase the quantities of antibiotic loaded into the coating. We reasoned that anodization would increase the porosity and the surface area compared to polished titanium[19-21]. In addition, the heat-treatment could increase the crystallinity of the titania layer[22]. Thus, these morphological and phase modification could enhance the precipitation of calcium-phosphate.

The aim of this study was to demonstrate that modifying the titanium surface would result in an increased loading of the antibiotic cefalotin onto a biomimetic hydroxyapatite coating. In addition, antibiotic efficacy was evaluated to determine whether this method would improve antibacterial effect.

MATERIALS AND METHODS

Preparation of specimen

The present study was performed with commercially pure titanium (cp-Ti; 10×10×0.25 mm). The titanium surfaces were polished mechanically with SiC paper with grits of 100, 600, and 1,200 and cleaned ultrasonically in acetone, ethanol, and distilled water for 15 min. Then, the titanium was anodized at 300 V for 2 min with a DC power supply (Genesys 600-2.6, Densi-Lambda, TDK, Tokyo, Japan). As the electrolyte, 0.4 M calcium acetate (CA) and 0.04 M beta-glycerol phosphate disodium salt *n*-hydrate (β-GP) mixed solution was used. The anodizing apparatus is shown schematically in Fig. 1. After

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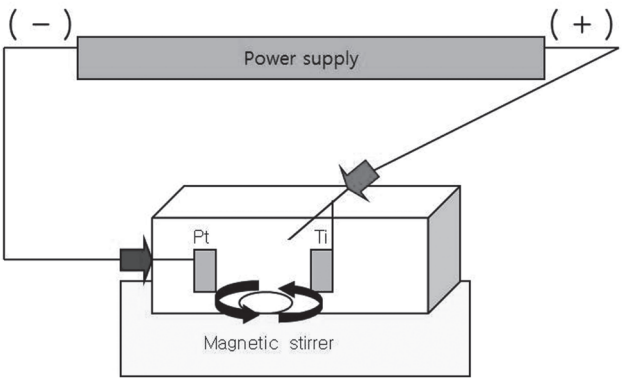

Fig. 1 Schematic diagram of the apparatus for anodization.

Table 1 Experimental groups used in this study

Group	Description
1	Polishing
2	polishing+Anodizing
3	polishing+Anodizing+Heat treatment

anodization, the titanium was heated to 800°C for 4 h in the air atmosphere (BF51800 sreies, Lindberg/Blue M, TPS, White Deer, PA, U.S.A) to change the crystalline phase. Therefore, three experimental groups were divided according to the different procedures, as shown in Table 1.

Biomimetic coating process

The coating process was performed in two steps. In the first step, the titanium was coated with a layer of calcium phosphate by immersing five times in concentrated simulated body fluid (SBF A; 733 mM NaCl, 21 mM $NaHCO_3$, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 7.5 mM $MgCl_2 \cdot 6H_2O$, 12.5 mM $CaCl_2 \cdot 2H_2O$) at 37°C for 24 h[14]. The SBF A was adjusted to a pH of 7.4 with 1 M NaOH and 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer. The salts were dissolved by bubbling gas CO_2 in the solution. When the pH dropped to 6.0, the CO_2 bubbling was terminated. After 24 h, the pH had reached approximately 8.0; then, the titanium was rinsed in distilled water and dried overnight at room temperature. In the second step, the titanium was coated with a layer of calcium phosphate that contained an antibiotic. Titanium from the first step was immersed in another calcium phosphate supersaturated solution (SBF B; 146 mM NaCl, 1 mM $NaHCO_3$, 2 mM $NaH_2PO_4 \cdot 2H_2O$, 0.05 mM $MgCl_2 \cdot 6H_2O$, 4 mM $CaCl_2 \cdot 2H_2O$) containing 800 mg/L of cefalotin[14]. After 48 h at 37°C, the titanium was removed from the solution, and then dried at 50°C for 30 min.

Surface characterization

The surface morphology and elements were examined by field-emission scanning electron microscopy (FE-SEM, JSM-6700F, Jeol, Tokyo, Japan) and electron dispersive spectroscopy (EDS), respectively. The surface layer phases were analyzed with a High Resolution X-ray Diffractomer (HRXRD, Bruker D8 DISCOVER, Karlsruhe, Germany).

In vitro release of antibiotic

Each specimen was immersed in 5 mL distilled water in a glass vial. The vials were stored at 37°C for 1, 2, 4, 7, 14, 30 and 60 days. At the indicated time intervals, the distilled water was collected to measure antibiotic concentration, and then refreshed. The antibiotic concentration was measured with an UV/VIS spectrometer (Spectro UV-VIS double beam PC, USD-3200, Labomed Inc, Culver city, CA, U.S.A.) at 236 nm (n=10).

Evaluation of antibacterial activity

Antibacterial activity was evaluated with a film adhesion method. This method modified ISO 22196: Plastics-measurement of antibacterial activity on plastic surfaces and JIS Z 2901; Antibcaterial products– Test for antibacterial activity and efficacy. Firstly, *Streptococcus mutans* (*S. mutans*; IFO 13955) which were abundant bacteria found almost universally in the mouths and the earliest colonizer were cultured aerobically in a conical tube that contained 100 mL Brain Heart Infusion (BHI) medium at 37°C. The initial concentration of bacteria was adjusted to 10^6–10^5 colony-forming units (CFU)/mL by dilution with phosphate-buffered saline (Invitrogen, GIBCO, gland Island, NY, U.S.A.) (PBS, pH 7.2). To test for antibiotic efficacy, 50 μL of bacterial solution was pipetted onto each specimen. And specimen was covered with polyethylene film. This procedure was conducted under dark condition to avoid photocatalytic activity. After 4 h, the specimen was rinsed with 1 mL PBS and bacterial cells were detached by sonication in PBS. And 100 μL of harvested cells were plated onto Bacto-Agar plates. Plates were incubated for 48 h at 37°C to determine the number of viable *S. mutans* (expressed in CFUs). (n=15). The uncoated titanium was used as a control.

Statistical analysis

Results of the antibacterial activity test were analyzed with the one-way ANOVA test to compare significant differences. The significance level was set at 95%.

RESULTS AND DISCUSSION

Surface characterization

FE-SEM images showed the modified titanium specimens before the coating process (Fig. 2a–c). The treatment for groups 2 and 3 increased the surface area compared to group 1 (polished titanium). The mean surface roughness (R_a) of modified titanium was about 0.4 μm. In contrast, the R_a of polished titanium was 0.2 μm (data not shown).

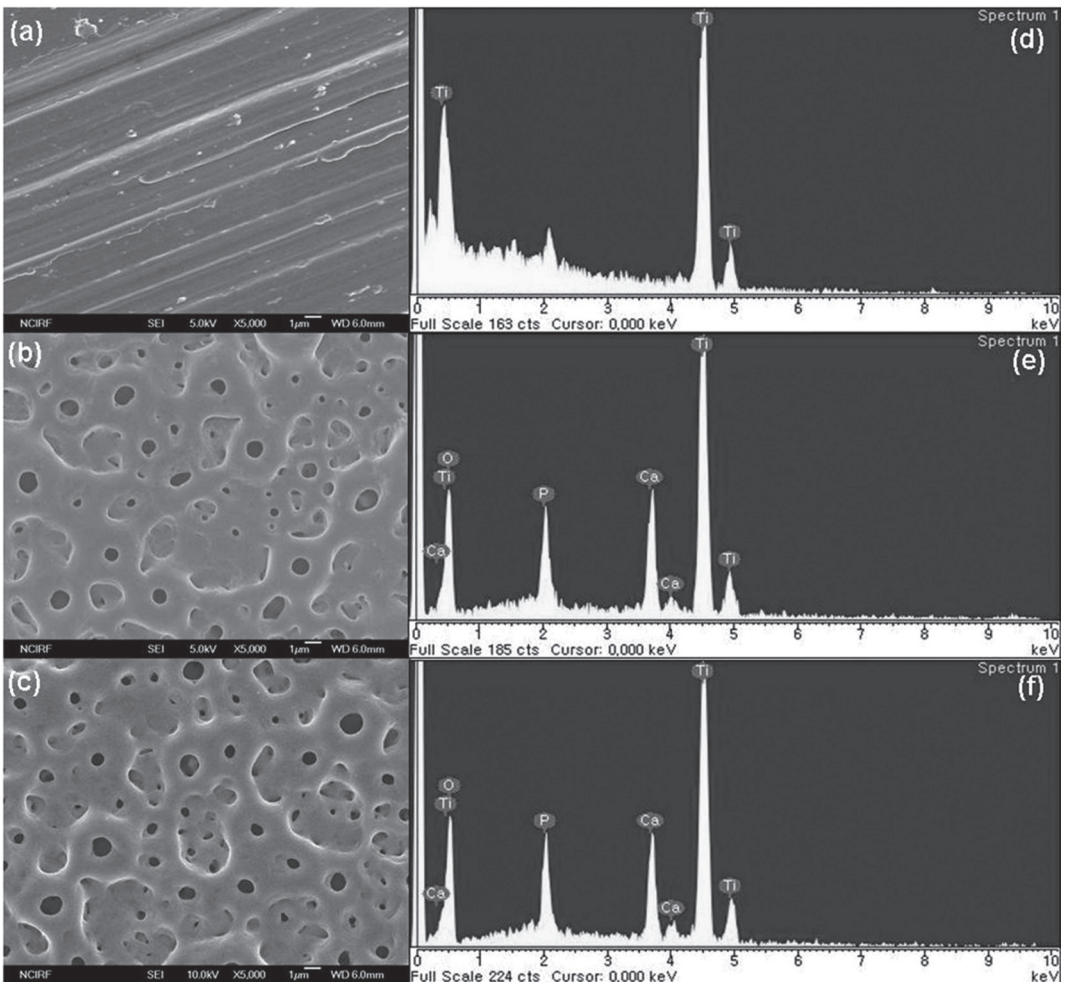

Fig. 2 Characteristics of modified titanium. (Left) Field-emission scanning electron microscopy images show the surface morphologies of modified titanium implant models (×5,000): (a) Group 1: polished, (b) Group 2: polished and anodized (c) Group 3: polished, anodized, and heat-treated. (Right) Electron dispersive spectroscopy analyses identified elements on the surfaces of modified titanium: (d) Group 1, (e) Group 2, (f) Group 3.

After anodization, micro-structures had formed on the surface. This did not change after heat-treatment. The pore sizes both before and after heat treatment were less than 2 μm[23]. In the elemental analysis by EDS (Fig. 2d–f), Ca and P were detected on anodized, heat-treated titanium, but not polished titanium. It could explain that Ca and P ions contained in the electrolyte solution would penetrate the inside of the oxide layer[19]. After the biomimetic coating process, morphological and elemental analyses of the titanium were also performed (Fig. 3 and 4). After the first step (Fig. 3a–c), the titanium was covered with loose calcium phosphate particles. The coated surface appeared globular. These globules would be nucleation sites for the growing calcium phosphate crystals[24]. And more Ca and P could detect on the titanium surface (Fig. 3d–f) compared to titanium before the coating process (Fig. 2d–f). After the second step (Fig. 4), the modified titanium surface (Group 2 and 3) were homogeneously covered with well-formed calcium phosphate crystals; this contrasted with the polished titanium surface because modified titanium had higher number of nucleation sites due to larger surface area compared to polished titanium as shown Fig.3. In contrast, the coating on polished titanium formed a heterogeneous layer[24]. This biomimetic coating reaction could occur due to an increased solubility of calcium phosphate salts with the addition of the acidic gas, CO_2[25]. In addition, the SBF B solution had lower Mg and HCO_3 ion content than the SBF A solution. These ions can inhibit crystallization. Therefore, the second coating formed a thick, homogenous calcium phosphate layer compared to the first coating[24-29]. As shown in the EDS results, Mg was not detected in the second coating, unlike the first coating. Ca and P were increased after second coating. And C peak was showed also. The Table 2 shows the composition of specimen from EDS.

Figure 5 shows the XRD results of modified titanium prior to the biomimetic coating. The anodization process

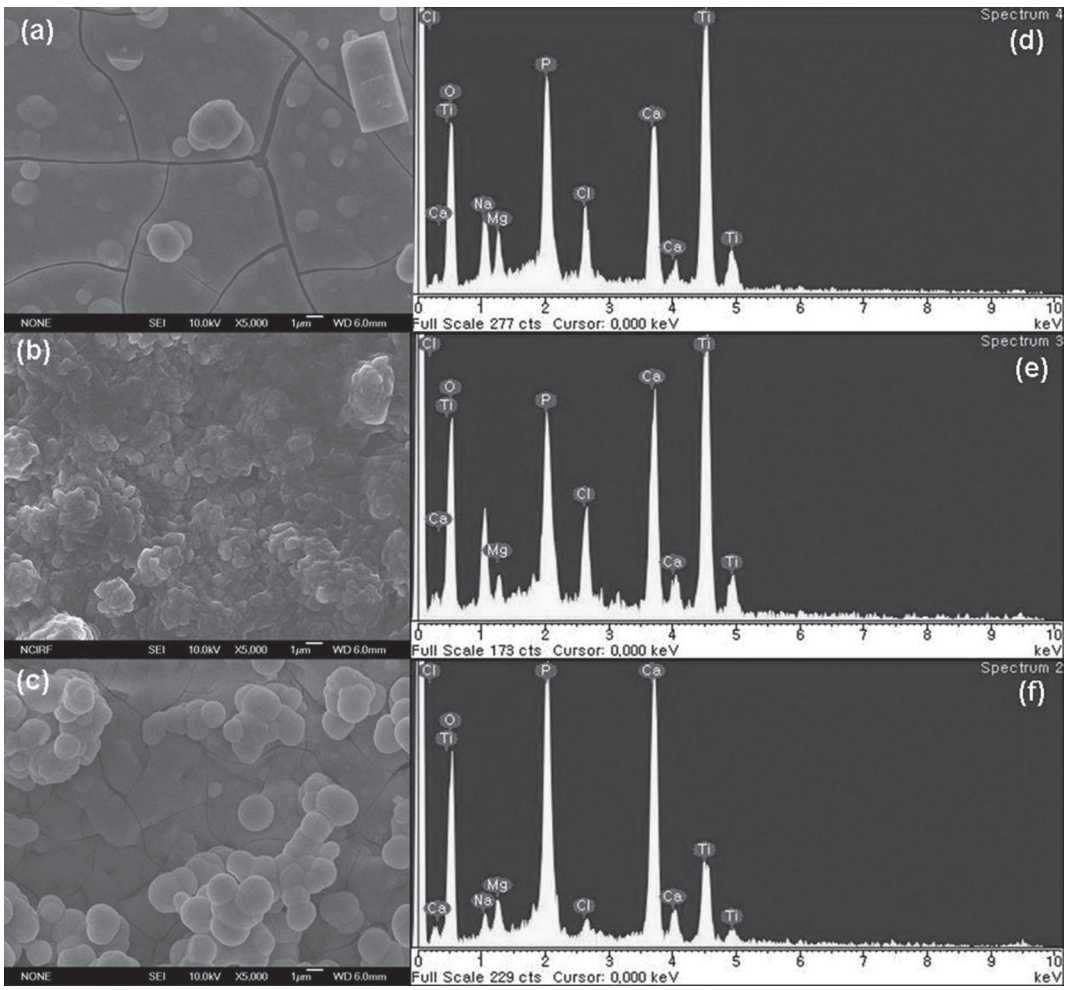

Fig. 3 Characteristics of modified titanium after first biomimetic coating. (Left) Field-emission scanning electron microscopy images show the surface morphologies of the titanium after the first biomimetic coating (×5,000): (a) Group 1: polished, (b) Group 2: polished and anodized, (c) Group 3: polished, anodized, and heat-treated. Electron dispersive spectroscopy analyses identified elements on the surfaces of modified titanium after the first biomimetic coating: (d) Group 1, (e) Group 2, (f) Group 3.

induced the titanium to form an anatase phase. Titanium and oxygen ion underwent a redox reaction that resulted in the formation of a titanium oxide film. The heat-treated titanium exhibited both anatase and rutile phases together. In addition, $Ca_3(PO_4)_2$ was also observed. These results indicated that heat treatment improved the crystallinity of titanium oxide and calcium phosphate (Fig. 5)[30]. In contrast, polished titanium showed only an alpha titanium phase (Fig. 5). The phase of surface could have influence on accumulation of calcium phosphate. As shown Fig. 6, after the biomimetic coating process, negatively charged HPO_4^{2-} ions in the solution were chemically absorbed into the $CaTiO_3$ surface (Fig. 6). TiO_2 on Ti surface can be transformed in to $CaTiO_3$ which is in contact aqueous solution containing sufficient Ca, at a suitable pH and temperature. This chemical bond is capable of strengthening the adhesion between the apatite and $CaTiO_3$ surfaces[31]. In addition, titanium with an anatase and/or rutile crystal structure also showed excellent apatite-forming ability[22,25,32]. In other words, the apatite-forming ability was related to the crystallinity of the titanium surface. The anodized titanium surface had a lower crystal order compared to heat-treated titanium[33]. Therefore, Fig. 4 showed that carbonated apatite formed more readily on the heat-treated titanium surface than on the polished surfaces. The carbonate was able to substitute for phosphate, which results in the transformation of HA in to carbonated apatite. This carbonated apatite is similar to bone[24].

Release of antibiotic

Figure 7 shows the release of antibiotic from the coated titanium surface as a function of time. Most of the loaded cefalotin was released during the first day. A burst release of antibiotic will influence initial bacterial

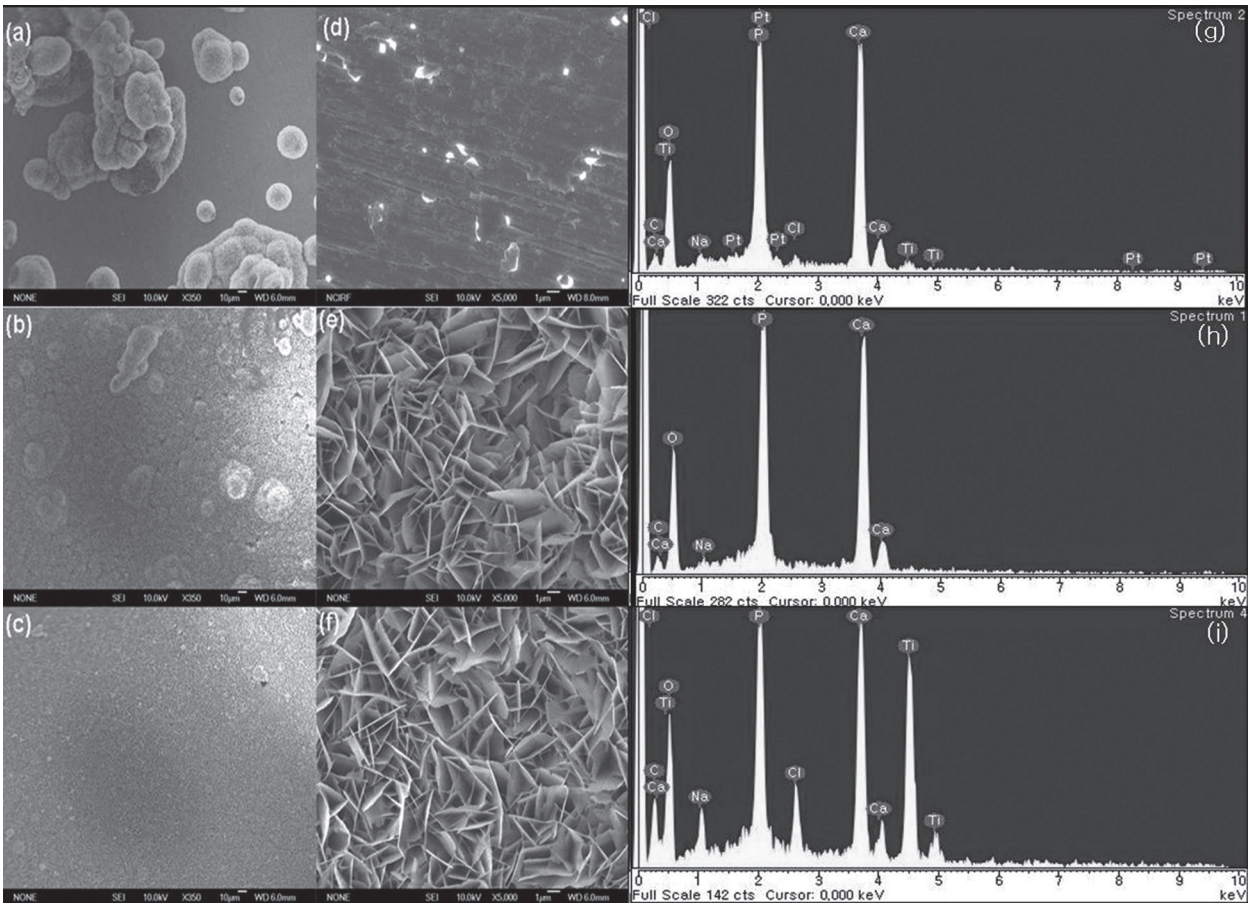

Fig. 4 Characteristics of modified titanium after second biomimetic coatings. (Left) Field-emission scanning electron microscopy images show the surface morphologies of the titanium after the second biomimetic coating (×350, ×5,000): (a, d) Group 1: polished, (b, e) Group 2: polished and anodized, (c, f) Group 3: polished, anodized, and heat-treated. Electron dispersive spectroscopy analyses identified elements on the surfaces of titanium after the second biomimetic coating: (g) Group 1, (h) Group 2, (i) Group 3.

Table 2 Chemical composition of biomimetic coated titanium

Group	1	2	3
Element composition (atm. %)	First biomimetic coating	First biomimetic coating	First biomimetic coating
	Second biomimetic coating	Second biomimetic coating	Second biomimetic coating
Ti	16.83	16.22	10.92
	11.66	5.7	4.62
Ca	4.64	7.2	9.03
	12.94	20.01	23.45
P	5.64	5.92	8.08
	12.31	21.99	23.98
O	61.00	63.05	68.47
	30.29	37.99	16.74
Mg	1.94	1.52	1.58
	–	–	–
Cl	2.51	3.09	0.57
	2.31	2.21	2.83
Na	3.95	3.0	1.35
	2.94	2.41	3.19
C	–	–	–
	10	10.69	13.2

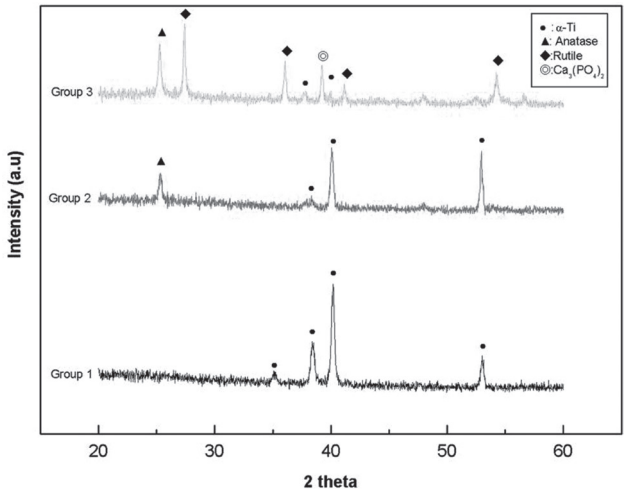

Fig. 5 High resolution X-ray diffraction patterns of modified titanium before the biomimetic coating. Group 1: polished, Group 2: polished and anodized, Group 3: polished, anodized, and heat-treated.

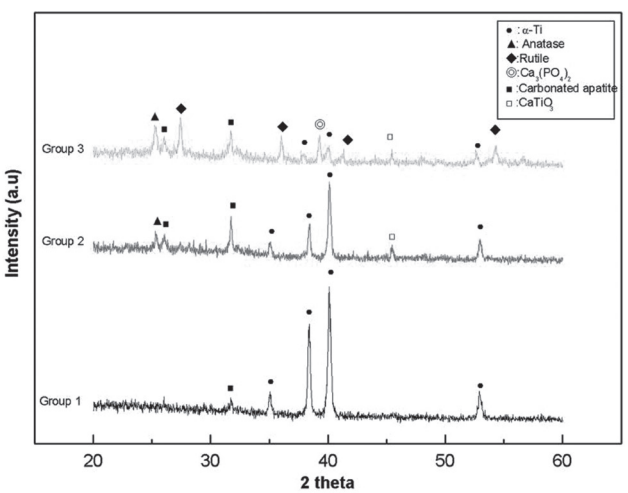

Fig. 6 High resolution X-ray diffraction patterns of modified titanium after the second biomimetic coating. Group 1: polished, Group 2: polished and anodized, Group 3: polished, anodized, and heat-treated.

colonization. The heat treated titanium (Group 3) enhanced the ability of antibiotic to incorporate onto the surface, which increased the loading of cefalotin. Because the rutile structure of heat-treated titanium played an important role in inducing calcium phosphate deposition. In addition, the rutile structure allowed lattice matching between the titanium and apatite[22,25,32]. Therefore, as the coated layer grew, the deposition of antibiotic on the heat-treated titanium might have been accelerated. This co-precipitation mechanism was also corroborated by Stigter and coworkers. They demonstrated that

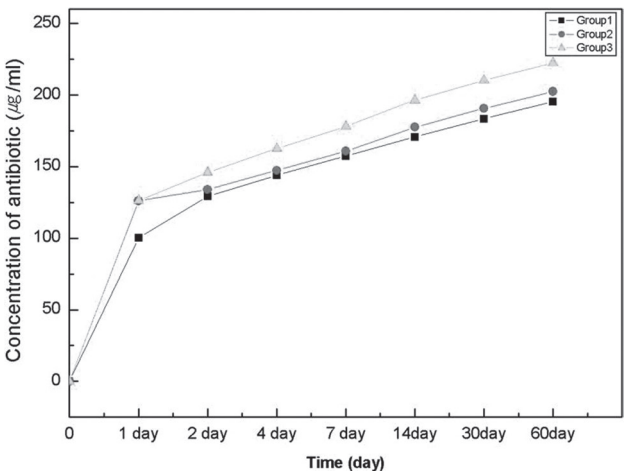

Fig. 7 Cumulative release of antibiotic from coated titanium over time. Group 1: polished; Group 2: polished and anodized; Group 3: polished, anodized, and heat-treated.

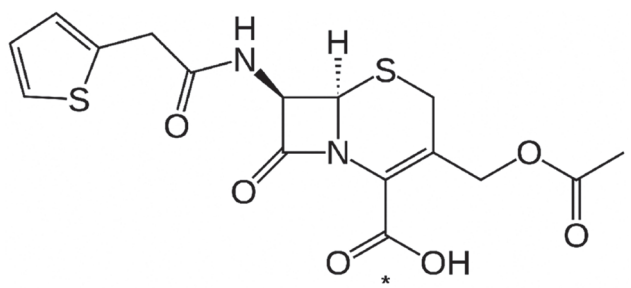

Fig. 8 Structure of the antibiotic, cefalotin. *Indicates the carboxyl group that binds to calcium.

tobramycin had an affinity for calcium phosphate surfaces and showed a correlation between the concentration of antibiotic in solution and the loading of tobramycin onto a calcium phosphate coated surface[13]. In this study, carboxyl group in the chemical structure of cefalotin (Fig. 8) would confer strong binding to calcium ions; this facilitated the coprecipitation with apatite. In addition, the modified titanium (Group 2 and 3) had calcium ions on the surface (Fig. 2). Therefore, modified titanium could have enhanced the incorporation efficiency of cefalotin compared to polished titanium. The release mechanism of cefalotin is related to apatite dissolution though we didn't evaluate it yet. So, to observe the release rate, weight loss should be measured in the future.

Evaluation of antibacterial activity

Next, antibacterial activity test of coated titanium was performed (Fig. 9). The viability of *S. mutans* was tested after a 4-h exposure to each specimen. The CFUs of *S. mutans* decreased after exposure to titanium specimens,

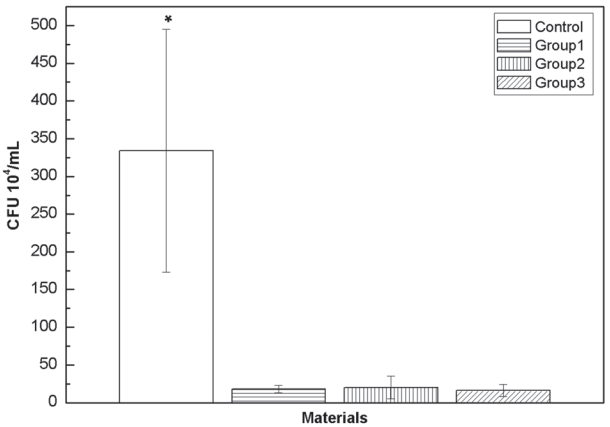

Fig. 9 Comparison of the number of *S. mutans* CFUs in the antibacterial activity test after exposure to coated titanium. Group 1: polished; Group 2: polished and anodized; Group 3: polished, anodized, and heat-treated.
* : Statically significant difference to experimental group (p<0.05).

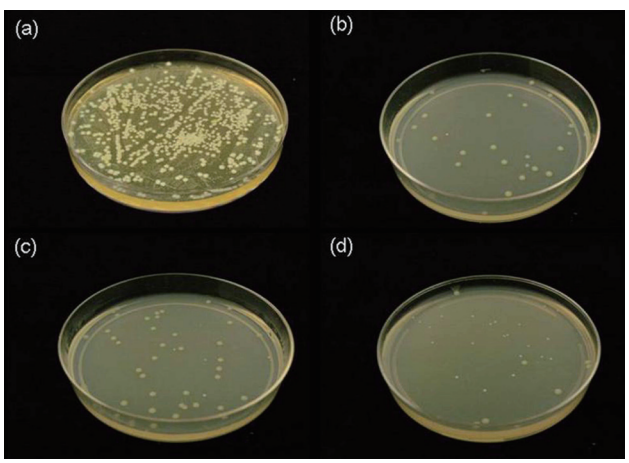

Fig. 10 Representative agar plates with *S. mutans* colonies displays the antibacterial effects of the coated titanium. (a) control (untreated) (b) Group 1: polished, (c) Group 2: polished and anodized, (d) Group 3: polished, anodized, and heat-treated.

in the following order: uncoated titanium (control) > polished, anodized titanium (group 2) > polished titanium (group 1) > polished, anodized, and heat-treated titanium (group 3). All the experimental groups showed reduced CFUs compared to the control group (p<0.05) (Fig. 9). However, agar plates for the experimental group 1, 2 and 3 showed a similar number of *S. mutans* CFUs (p>0.05) (Fig. 10); this might be related to the burst release of antibiotic. Thus, all experimental groups had a sufficient antibacterial effect against *S. mutans* in that time that corresponded to immediately after surgery. However, it is necessary to ensure a sustained antibiotic release from the coated surface, because peri-implantitis is slow developing[5]. Also, a sustained antibiotic release may improve the safety of the dose and avoid side effects that can cause problems[16].

CONCLUSIONS

In this study, cefalotin was co-precipitated with carbonated apatite applied with a biomimetic coating method. A modified titanium surface increased the quantity of antibiotic that could be loaded onto the coated surface. Therefore, these results suggested that a cefalotin coating applied with a biomimetic coating method on modified titanium might be a promising material for preventing local post-surgical implant infections. However, further study is necessary to ensure the cytocompatibility and safety of this material.

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