Investigation of antibacterial effect of copper introduced titanium surface by electrochemical treatment against facultative anaerobic bacteria

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This study investigated the efficacy of copper (Cu) as an antibacterial element incorporated on titanium (Ti) surface by electrochemical treatment. Cu was incorporated onto Ti surface by micro-arc oxidation (MAO). A small amount of Cu was incorporated into the oxide layer and was found to be in oxidized states. Cu-incorporated samples exhibited no-harmful effect on the proliferation of osteoblast-like cells. Moreover, the difference in antibacterial property between fresh and incubated samples was evaluated using gram-positive and gram-negative facultative anaerobic bacteria. The specific antibacterial property of Cu incorporated into the Ti surface were confirmed. The antibacterial property prolonged upon immersion in physiological saline for 28 days. In other words, MAO-treated Ti containing Cu in this study is expected to achieve long-term antibacterial property in practical usage.

Keywords: Copper, Titanium, Antibacterial, Biofilm, Micro-arc oxidation

INTRODUCTION

Titanium (Ti) and its alloys are widely used as major implant materials owing to their excellent mechanical properties and biocompatibility. Recently, biomaterial-associated infections (BAI) caused due to the formation of a biofilm on biomaterial surface have been recognized as a major cause of failure in implant surgeries¹⁻⁴. The formation of biofilms is usually a result of bacterial adhesion, growth, and colony formation. Biofilms weaken the effect of antibiotic agents due to the variability of bacterial species and the barrier effect of extracellular polysaccharides⁵⁻⁹. Biofilm formation is the final phase of infection and it is almost impossible to eliminate the biofilm grown on the implanted device from the living body. The only solution is the removal of the contaminated device from the patient. This further surgery is necessary to avoid subsequent undesirable biological reactions such as infectious diseases. The prosthetic joint infection is divided into the early infection phase *i.e.* within 3 weeks after surgery, and the late-onset infection, which occurs later. Coventry reported that it takes around three to eight weeks after surgery until late-onset infection may become evident, and this period is considered as the incubation stage¹⁰. Thus, preventing bacterial invasion is necessary for assured implantation surgery, and imparting antibacterial properties to the implant materials is strongly desired. An ideal biomaterial surface with antibacterial properties will prevent bacterial adhesion at the initial

stages of infection and also inhibit subsequent bacterial growth at later stages.

Recent studies on antibacterial surfaces have employed various surface treatments with silver (Ag) species¹¹⁻¹⁹. Ag is known as a strong antibacterial element and its inhibitory effect on bacteria has been studied extensively. Many researchers have reported that Ag can strongly affect various kinds of fungal and bacterial strains including multidrug resistant strains of bacteria¹⁸⁻²¹. However, excess Ag exerts toxic effects on the surrounding cells and tissues²²⁻²⁷. Therefore, control of Ag release from implants is required for application of implant surface treatment. Copper (Cu) is another important trace element in living organisms and is known to be one of an antibacterial element²⁸⁻³². The application of Cu for surface modification of implant devices may be utilized for achieving antibacterial properties similar to Ag.

Micro-arc oxidation (MAO) is an electrochemical surface treatment in a specific electrolyte under high voltage. The valve metal surface is covered by the unique porous structured oxide layer after the treatment. The resultant oxide layer formed by MAO treatment also consists of additional elements that are incorporated from the electrolyte solution. Therefore, many studies involving MAO as surface modification techniques for the purpose of improvement on the hard-tissue compatibility of Ti has been reported³³⁻³⁹. Recently, several studies have been reported that MAO treated Ti with Ag shows good antibacterial property against both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*)^{17,34}. Moreover, Cochis *et al.* reported that the MAO treated Ti

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with Ag showed effective antibacterial property against *Acinetobacter baumannii*¹⁸⁾. It has been proven that Ag-incorporated surfaces exhibit antibacterial effects against various bacteria.

On the other hand, studies based on the usage of Cu to attain antibacterial property by MAO treatment is limited. Yao *et al.* and Wu *et al.* reported that MAO treated Ti with Cu exhibits antibacterial effects against *S. aureus* and *E. coli*^{40,41)}. Moreover, Zhu *et al.* reported that Cu-incorporated Ti surface has no-harmful effects on calcification of osteoblasts⁴²⁾. However, the duration of antibacterial effect exerted by Cu incorporation is still unknown. The investigation of long-term antibacterial effect is important to combat late-onset infection, which develops more than 3 weeks after the implant surgery. Therefore, the present study aims to investigate the efficacy of Cu as an antibacterial element incorporated on Ti surface by MAO treatment. The difference in antibacterial property between fresh and four-week incubated samples was evaluated using gram-positive and gram-negative facultative anaerobic bacteria. In addition, the relationship between the presence of Cu on the treated surface, Cu-ion release behavior, and biological effects on the bacteria and osteogenic cells were investigated. Surface characterization was also performed to examine the presence of Cu, chemical states of Cu, and release of Cu-ions from the Ti surface. The long-term antibacterial effects associated with the biosafety of Cu related to osteogenic cells are discussed based on the surface analyses of the MAO-treated Ti surface.

MATERIALS AND METHODS

Sample preparation

Two types of Ti disks with diameters 8 and 25 mm were obtained by cutting rods of commercially pure grade 2 Ti. The disk surfaces were mechanically grinded using #150, #320, #600, and #800 grid SiC abrasive papers followed by ultrasonication using acetone and ethanol. The disks were then kept in an auto-dry desiccator, until further use. The Ti disk was fixed onto the polytetrafluoroethylene holder with an O-ring. The area in contact with the electrolyte was 39 mm² (7.0 mm in diameter) or 398 mm² (22.5 mm in diameter). Details of the working electrode are described in the previous study⁴³⁾. A type 304 stainless steel plate was used as a counter electrode. The base composition of the electrolyte for MAO treatment was 100 mM calcium glycerophosphate and 150 mM calcium acetate. In this study, 0, 0.5, 1.0 and 2.5 mM CuCl₂ were added to the base electrolyte. After pouring the electrolyte into the electrochemical cell, both electrodes were connected to a DC power supply (PL-650-0.1, Matsusada Precision, Shiga, Japan), and a positive voltage with a constant current density of 251 Am⁻², was applied for 10 min. After MAO treatment, the sample surfaces were thoroughly washed in ultrapure water to remove the electrolyte solution remaining inside the porous oxide structure. In this study, a major part of the Ti disk was MAO-treated

except an annular untreated area of 0.5 mm width from the edge. All the surface characterization described below were performed within the MAO-treated area.

Incubation in a simulated body fluid

The samples treated in the electrolyte with various concentrations of Cu were incubated in simulated body fluid for 28 days. Physiological saline (5 mL, 0.9% NaCl) was used, which is the simplest composition in simulated body fluid. The samples were fixed onto a polyethylene container to allow the release of Cu ions from the porous oxide layer. The experiment was performed at 310 K in a temperature-controlling chamber under roundly-shaking condition (100 rpm). The physiological saline was periodically exchanged every 7 days using the stored solution stock. After incubation, the samples were picked up from the solution to evaluate antibacterial property. In addition, the remaining solutions were used to investigate the amount of Cu ions released from the Cu incorporated surface.

Inductively-coupled plasma atomic emission spectroscopy (ICP-AES, ICPS-7000 ver. 2, Shimadzu, Kyoto, Japan) was performed to investigate the amount of Cu ions released from the Cu-incorporated Ti sample surface.

Surface characterization and Cu ion release evaluation

Scanning electron microscopy with energy dispersive X-ray spectrometry (SEM/EDS; S-3400NX, Hitachi High-Technologies, Tokyo, Japan) was used to investigate the surface morphology of each sample. X-ray photoelectron spectroscopy (XPS; JPS-9010MC, JEOL, Tokyo, Japan) was performed using Mg K α X-ray source (1,253.6 eV, 10 kV, 10 mA). Instrument base pressure was 8 $\times$ 10⁻¹⁰ Torr. All binding energies mentioned in this article are relative to Fermi level. The spectrometer was calibrated against Au 4f_{7/2} of pure gold, Ag 3d_{5/2} of pure Ag and Cu 2p_{3/2} of pure Cu. Spectra were obtained using an analysis area of 1 mm ϕ and 20 eV pass energy. The detection angle was fixed at 90°. The binding energies were calibrated using that of C 1s photoelectron energy region peak derived from the contamination carbon (285.0 eV). To estimate the integrated intensities of the peaks, the background was subtracted from the measured spectrum, according to Shirley's method⁴⁴⁾. The chemical state of Cu inside the MAO-treated Ti surface were determined by modified auger parameter (α') on Wagner plot with those of typical Cu compounds. The α' of each sample was calculated using equation (1),

$$\alpha' = E_K(\text{Cu } L_3VV) + E_B(\text{Cu } 2p_{3/2}) \quad (1)$$

where E_K shows kinetic energy of Cu L₃VV, $E_K(\text{Cu } L_3VV)$, and E_B shows binding energy of Cu 2p_{3/2}, $E_B(\text{Cu } 2p_{3/2})$. The quantitative analysis for determination of the sample compositions were performed according to the methods described in the previous study⁴⁵⁾. Empirical data^{46,47)} and theoretically calculated data⁴⁸⁾ were used for quantification.

Cell culture

As described in our previous work⁴⁹, MC3T3-E1 cells (RIKEN BioResource Center, Tsukuba, Japan) were maintained in a cell culture medium, which was an alpha modification of Eagle's minimum essential medium (α -MEM, GIBCO, Grand island, CA, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, and 0.25 mg mL⁻¹ amphotericin B (GIBCO). In this study, cells were seeded on the sample at an approximate initial density of 10,000 cells cm⁻². Then cells were incubated at 37°C in a fully humidified atmosphere containing 5% CO₂ in air. All of the samples were sterilized in advance in 70% ethanol for 20 min and thoroughly rinsed with deionized water before the test. The MAO-treated Ti without Cu (0 mM Cu) was used as a control.

Cellular proliferation

Numbers of the cells attached to the sample surface after 1, 2, and 3 days after seeding were counted using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). The attached cells on the tissue-culturing treated polystyrene (TCPS) were harvested by treating with Trypsin/EDTA and then resuspended into the cell culture medium. Trypan blue (Trypan Blue Stain 0.4%, GIBCO) was used to count the attached cells on TCPS. These cell suspensions were then diluted progressively and counted on a hemocytometer. On the other hand, these cell suspensions were also transferred into a 96 well microplate, in which the cell counting assay was performed. A reaction reagent was added to each sample, according to the manufacturer's instructions, and the reaction was continued for 4 h, at 37°C. The absorbance of the samples was measured at 450 nm using a microplate reader (ChroMate® Microplate Reader, Awareness Technology, Palm City, FL, USA) and a reference wavelength of 630 nm was used. Finally, the standard line calibrating the cell number and the light absorbance was obtained from the results.

Cellular calcification

The calcification behavior of MC3T3-E1 cells in each sample was evaluated by staining the calcified deposits by a color change reaction with alizarin red S reagent employing the following method. After removal of the culture medium, the samples along with the cells were rinsed thrice with phosphate-buffered saline. The cells were then fixed on the samples by immersing in 4% formalin for 1 h and rinsing thrice with ultra-pure water. Each sample was stained with 1% alizarin red S solution (adjusted to pH 4.2 with ammonium hydroxide) at 25°C for 30 min. After removing the alizarin red S solution, the samples were repeatedly rinsed with ultrapure water. An optical microscope (OLYMPUS SZX12, Olympus, Tokyo, Japan) was used to observe whole surface area of the samples.

Antibacterial property evaluation

The antibacterial property test was performed in accordance with ISO 22196:2007 method. To examine

the antibacterial effect of MAO-treated Ti with various concentration of Cu, *S. aureus* (NBRC122135) and *E. coli* (NBRC3972) were used in this study. The experiment was approved by Pathogenic Organisms Safety Management Committee of Tokyo Medical and Dental University (22012-025c). *S. aureus* and *E. coli* are used in antibacterial property tests as representative gram-positive and gram-negative bacteria, respectively, as described in ISO 22196. *S. aureus* are gram-positive cocci frequently found in the nose, respiratory tract, and on the skin. However, *E. coli* is a gram-negative, rod-shaped bacterium, commonly found in the gut. Each strain was cultured according to its specific requirements. *S. aureus* and *E. coli* were cultured on tryptic soy broth (TryptoSoya Broth, Nissui, Tokyo, Japan) and Luria-Bertani (LB) broth (LB-Medium, MP Biomedicals, Santa Ana, CA, USA), respectively, for 24 h at 37°C, under ambient conditions. The optical densities of the bacterial suspensions were measured at 600 nm using an ultraviolet-visible (UV-vis) spectrometer (V-550, JASCO, Tokyo, Japan) to obtain the concentrations of 3–5×10⁶ colony-forming units (CFU) mL⁻¹. Prior to the antibacterial property tests, all samples were sterilized with 70% ethanol, washed with distilled water, and dried. Bacterial suspensions were dropped on all samples, which were subsequently covered with a sterilized plastic film and incubated at 37°C for 24 h (*n*=3). After collecting bacteria from the incubated samples, the obtained suspensions were 100-times diluted by physiological saline, pipetted onto nutrient agar plates, and incubated overnight at 37°C. The number of viable bacteria was determined by counting the number of colonies formed on the petri plates.

Statistical analysis

The experimental data of the biological tests are presented as means±SD. The software, KaleidaGraph (Synergy Software, Reading, PA, USA) was used for statistical analysis. One-way analysis of variance was used to make multiple comparisons employing the Student-Newman-Keuls method to assess the data, and *p*<0.05 indicated statistical significance.

RESULTS

Surface characterization and Cu ion release evaluation

Figure 1 shows SEM images of the MAO-treated Ti surfaces in the electrolyte with various Cu ion concentrations. The porous oxide layer formation was confirmed by all MAO-treatment conditions. The morphology of the oxide layer was almost same among the MAO treated samples with different Cu ion concentrations in the electrolyte. Figure 2 shows the XPS wide-scan spectra of the MAO-treated samples. Several peaks originated from C 1s, O 1s, P 2p, Ca 2p and Ti 2p orbital binding energies were detected from all samples. The binding energies of the peaks of P 2p and Ca 2p_{3/2} were 133.7 eV and 347.6 eV, respectively. It suggests that phosphorus and calcium in the oxide layer exist as a phosphate species and as divalent ions, respectively. The

Fig. 1 SEM images of each sample surface. Scale bar is 10 μ m.

Fig. 2 XPS survey spectra of each sample.

binding energy of Ti $2p_{3/2}$ peak was 458.9 eV, indicating that Ti existed as TiO_2 . Moreover, Cu 2p photoelectron peaks and Cu L_3VV Auger electron peaks were detected in the samples treated with the electrolyte containing Cu ions. Figure 3 shows the Wagner plot of Cu in the oxide layer prepared by MAO treatment, based on E_B of Cu $2p_{3/2}$ peaks and E_K of Cu L_3VV peaks. The reference plots reported in other literature^{50–53)} using standard Cu chemical compounds are also shown in the Figure. The chemical states of the elements in different compounds are usually distinguished by reference to both E_B and α' values (parallel solid lines) in Wagner plot. The binding energy of Cu in the oxide layer was almost the same as that of CuO. Moreover, the modified auger parameter values of Cu in the oxide layer were almost the same as that of Cu_2O . Therefore, the incorporated Cu element into the oxide layer is assumed to be in the complex of oxidized states.

Figure 4 shows the relative composition (atomic percentage) of Cu in the oxide layer, calculated by XPS quantitative analysis. It was confirmed that small amounts of Cu can be introduced into the surface oxide layer of Ti and its amount apparently increases with increase in the Cu-ion concentration of the electrolyte for MAO treatment.

Cu in all tested solutions remained undetected, even though the detection limit of the machine was less than 0.1 ppm. In other words, extremely small amounts of Cu, which were below the detection limit of ICP-AES, may be released from the porous oxide layers containing Cu.

Fig. 3 The Wagner plot of Cu in the oxide layer based on Cu $2p_{3/2}$ peaks and Cu L_3VV Auger peaks.

Cellular proliferation

Figure 5 shows MC3T3-E1 cell growth on the samples for 1, 2, and 3 days after seeding. The living cell numbers

Fig. 4 Atomic percentage of Cu in the oxide layer calculated by quantitative analyzes of XPS spectra.

Fig. 5 Number of MC3T3-E1 cells on Ti MAO-treated in the electrolyte containing various concentration of Cu with culture time. Data are shown as the mean±SD. n.s.: No significant difference ($p < 0.05$).

on the all samples tested in this study increased with increase in culture time. The growth rate of the cells showed no statistical differences among the samples with and without the Cu incorporation.

Calcification

Figure 6 shows the sample surface after the alizarin red S staining of calcified deposits in MC3T3-E1 cells

Fig. 6 Color scale stained by alizarin red S of calcified deposits by MC3T3-E1 cells on MAO-treated Ti with or without Cu cultured for 14 and 28 days. Scale bar represents 2 mm.

Fig. 7 Results of antibacterial evaluation using *E. coli* or *S. aureus*. Data are shown as the mean±SD. *: Significant difference ($p < 0.05$).

after culturing for 14 and 28 days. The central part (7 mm in diameter) of the sample is the MAO-treated area covered with the oxide layer. The margins (0.5 mm) of the sample remains as untreated surface. The calcified cells were observed in all the samples. The whole MAO-treated area was evenly stained without any segregated area. There was no apparent difference between the MAO-treated samples with and without Cu.

Antibacterial property evaluation

Figure 7 shows the antibacterial property evaluation using both *E. coli* and *S. aureus*. The vertical axis of the

Fig. 8 The effect of sample aging by physiological saline in which samples were incubated for 28 days on the antibacterial property.

A and B show the number of *E. coli* (A) and (B) *S. aureus* on each sample. Data are shown as the mean $\pm$ SD. *: Significant difference ($p < 0.05$). n. s.: No significant difference.

Figure represents the normalized bacterial number: The bacterial number on the tested sample is divided by that on the MAO-treated control sample without Cu in the electrolyte. The normalized bacterial number below 1 (broken line in the Figure) depicts that tested sample has antibacterial property and inhibits bacterial growth. The numbers of both *E. coli* and *S. aureus* on the samples containing Cu were smaller than those on the control samples without Cu. The number of *E. coli* significantly decreased with increase in the Cu amount. Moreover, the growth of *S. aureus* was drastically inhibited on the Cu-containing samples, even though they were prepared with the smallest Cu-ion concentration.

Figure 8 shows the effect of sample aging by physiological saline in which samples were incubated for 28 days on the antibacterial property. The white colored bars in the Figure show the results of aged samples that were incubated in physiological saline for 28 days at 310 K, simulating the living body environment. The number of *E. coli* shown in Fig. 8A were not significantly different between the fresh and the aged samples. On the other hand, the number of *S. aureus* shown in Fig. 8B on the aged samples containing Cu was found to be significantly smaller than those on the fresh samples prepared with the same Cu-concentration.

DISCUSSION

SEM observation confirmed that MAO treatment could be successfully performed in Cu-ion containing electrolytes, without changing the surface morphology of the Ti sample (Fig. 1). In addition, the presence of Cu in the MAO-treated samples was confirmed by the XPS wide scan spectra (Fig. 2). The atomic fraction of Cu

inside the oxide layer, calculated by the XPS quantitative analysis using the narrow spectra (Cu 2p), was found to increase with the increase in Cu-ion concentration in the electrolyte for MAO treatment (Fig. 4). However, when the Cu ion concentration in the electrolyte exceeded 5 mM, the voltage applied during the treatment did not rise sufficiently and no porous oxide layer was formed on the Ti sample. It is believed that the anodic current was preferentially supplied by the electrolysis of water, which occurred on the surface of the sample because the oxide attained electro-conductivity. Thus, it was confirmed that Cu could be incorporated into the Ti surface oxide layer, to some extent, with the MAO treatment condition applied in this study. Moreover, the fraction of the incorporated Cu could be limited by adjusting the Cu-ion concentration of the electrolyte. The behavior of Cu ion release in physiological saline was evaluated to simulate *in vivo* implantation period. The amount of Cu ions released from the sample surface could not be fully evaluated because the concentration of the Cu ions in the all tested solutions was lower than the detection limit of 10 ppb (it corresponds to be 0.0126 $\mu\text{g cm}^{-2}$ for this experimental condition). These results indicate that Cu ions were hardly released from the Cu-incorporated porous oxide layer, unlike the Ag ions from the Ag-incorporated samples, which was previously reported by our group¹⁷. The chemical state of the incorporated Cu atoms in the porous oxide layer formed by MAO treatment was found to be oxidized with complex state, as depicted by XPS Wagner plot analyses (Fig. 3). Both the oxides of Cu (Cu_2O and CuO) are known to be poorly soluble in neutral aqueous solution. Thus, the dissolution test results of undetectable Cu ions may be attributed to the poor solubility of Cu inside the oxide

layer.

The number of living cells on the samples containing Cu inside the porous oxide layer was found to be the same as that on the sample without Cu (Fig. 5). It was clarified that MC3T3-E1 cells can proliferate on the Cu-incorporated sample similar to the MAO-treated ones without Cu. Moreover, the calcification behavior of the cells in MAO-treated samples was confirmed. There was no difference between the samples with and without Cu (Fig. 6). In other words, the Cu-containing samples showed no harmful effects on the calcification of osteogenic cells even after long-term culture period (14 and 28 days). The amount of the incorporated Cu species into the porous oxide layer and the amount of Cu ions released were negligibly small for osteoblast-like cells. Therefore, all samples prepared in this study may be considered as biosafe for the surrounding osteogenic cells and tissues, if implanted in a living body.

The experimental results using anaerobic bacterial species clearly demonstrate the efficacy of the incorporated Cu on inhibiting their proliferation. Figure 7 shows the antibacterial effect of MAO-treated Ti samples using Cu-containing electrolyte against both *E. coli* and *S. aureus*. Moreover, the inhibition effect of incorporated Cu on *S. aureus* was stronger than that on *E. coli*. Generally, the minimum inhibitory concentration (MIC) of antibacterial agents is different for each bacterial species. The efficacy of Cu against *S. aureus* is higher than *E. coli*⁵⁴, therefore, the viability of *S. aureus* was lower than *E. coli* under similar exposure condition to Cu incorporated into the oxide layer. It is notable that the antibacterial effect of the incorporated Cu, against *S. aureus*, was strengthened after incubation in physiological saline for 28 days. All samples showed smaller numbers of *S. aureus* after incubation treatment, as compared to the fresh samples (Fig. 8). Therefore, it was confirmed that the MAO-treated samples exhibited sustainable antibacterial property for a long period. Moreover, the specific effectiveness against *S. aureus* might be enhanced during the implantation period and this unique phenomenon is expected to be a solution for late-onset infection disease. On the other hand, Cu-ion release was not observed in the dissolution test because the Cu concentrations in tested solutions were below detection limit of ICP-AES measurement. Therefore, it may be concluded that almost no Cu ion can dissolve into physiological environment from the MAO-treated Ti surface, when it is implanted to a living body. The antibacterial property of the sample prepared in this study can be explained by contact-killing effect on Cu species incorporated in the oxide layer rather than the nonspecific Cu-ion release. The contact killing effect of Cu is introduced by specific Cu elution behavior triggered by the bacterial contact, followed by the metabolic activities and it finally causes critical damage to the cell membrane^{55,56}. In other words, the Cu ion release might have been locally accelerated where the bacteria were attached. From the XPS analyses, the chemical state of the incorporated Cu was found to be of complex oxides (Fig. 3). The enhancement of the antibacterial property

against *S. aureus*, after the incubation period, may be attributed to the transition of Cu oxides during the immersion in physiological saline. The osteoblast-like cell evaluation shows that the MAO-treated samples containing Cu exert no toxic effect on proliferation and calcification (Figs. 5 and 6). It was found that the porous oxide layers consist of not only Ti oxide but also incorporated Ca, P, and Cu (Fig. 2). Relatively large amounts of Ca and P in the MAO-treated Ti were also reported in other studies^{57,58}. Ca and P are the main components in living bone tissues as calcium-phosphate compounds. The presence of Ca and P in the porous oxide layer formed by MAO treatment enhances the activity of osteogenic cells. In our previous study, MAO-treated samples showed osteogenic cell compatibility which can accelerate the calcification process of MC3T3-E1 cells, without any cytotoxicity¹⁷. The Cu-containing samples in this study may have similar biofunction as they contain Ca and P without preventing cell proliferation and calcification. In addition, the specific antibacterial property of the Cu which incorporated into Ti surface, was confirmed. The antibacterial activity prolonged upon immersion in physiological saline for 28 days. Thus, it may be concluded that MAO-treatment of Ti using Cu is suitable to attain long-term antibacterial property and osteogenic cell compatibility. This may be a promising technique for the development of novel dental materials and devices.

CONCLUSION

The Cu-incorporated porous oxide layer could be successfully prepared on Ti surface by MAO treatment in the electrolyte containing CuCl₂. A small amount of Cu which presents complex oxide states was incorporated into the porous oxide layer. The amount of Cu ions released from the oxide layer into physiological saline was negligibly small and it was less than the detection limit of ICP-AES. Cu incorporated samples exhibited no harmful effect on the proliferation and calcification of osteoblast-like cells. On the other hand, the inhibition effect on the growth of anaerobic bacteria on the Cu-incorporated samples was confirmed. The specific antibacterial property that prolonged and strengthened during immersion in physiological saline against *S. aureus* was also revealed by this study. Therefore, it could be concluded that the utilization of Cu with MAO treatment is expected to be promising technique for development of novel dental materials.

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