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Chemical and Biological Roles of Zinc in a Porous Titanium Dioxide Layer Formed by Micro-Arc Oxidation

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Abstract: This study investigated the time transient effect of zinc (Zn) in the porous titanium dioxide formed by micro-arc oxidation (MAO) treatment routinely performed for Zn-containing electrolytes. The aim of our analysis was to understand the changes in both the chemical and biological properties of Zn in physiological saline. The morphology of the Zn-incorporated MAO surface did not change, and a small amount of Zn ions were released at early stages of incubation in saline. We observed a decrease in Zn concentration in the oxide layer because its release and chemical state (Zn^{2+} compound to ZnO) changed over time during incubation in saline. In addition, the antibacterial property of the Zn-incorporated MAO surface developed at late periods after the incubation process over a course of 28 days. Furthermore, osteogenic cells were able to proliferate and were calcified on the specimens with Zn. The changes related to Zn in saline had non-toxic effects on the osteogenic cells. In conclusion, the time transient effect of Zn in a porous titanium dioxide layer was beneficial to realize dual functions, namely the antibacterial property and osteogenic cell compatibility. Our study suggests the importance of the chemical state changes of Zn to control its chemical and biological properties.

Keywords: zinc; titanium oxide; micro-arc oxidation; antibacterial activity; osteogenic cell compatibility

1. Introduction

Recent studies have reported that biomaterial-associated infections caused by the formation of biofilms on biomaterial surfaces were a major cause of failure in implant surgeries [1–6]. The biofilms are generally formed as a result of bacterial adhesion, growth, colony formation, extracellular polysaccharides, quorum sensing signals, and formation of nutrition channels. Biofilms can weaken the effect of antibiotic agents due to the presence of a wide variety of bacterial species and the barrier effect of the extracellular polysaccharide [7–12]. After the formation of biofilms, it becomes almost impossible to remove the matured biofilms from implanted devices in the human body. The only way of preventing sepsis is by retrieval of the device on which the biofilm was formed from the patient. To avoid this, it is necessary to inhibit biofilm formation during implantation of devices in

advance. Prosthetic joint infection is divided into early infection (within three weeks after surgery) and late-onset infection (around three to eight weeks after surgery); this period is considered the incubation stage [13]. Moreover, infections associated with hip implants have been reported in the case of dental treatments [14,15]. Thus, the long-term antibacterial activity of the implant material is strongly desired. An ideal biomaterial surface with antibacterial activity can not only prevent the initial stages of infection such as bacterial adhesion, but also inhibit subsequent bacterial growth at later stages.

Recent studies on antibacterial surfaces have used various surface treatments with silver (Ag) species [16–23]. Ag is a well-known antibacterial agent and its effects on various bacteria have been studied extensively. Many researchers have reported that Ag can strongly influence various kinds of fungal and bacterial strains, including multidrug-resistant bacteria [23–27]. In addition to the efficacy of Ag for bacteria, copper, zinc (Zn), gallium, selenium and silicon have been recently used as antibacterial elements owing to their good antibacterial activity [23,28–35]. Among them, Zn is one of the most important trace elements in living organisms and an effective antibacterial element [30,31,36–42]. Therefore, we expected that the application of Zn for the surface modification of implant devices might offer antibacterial activity as effective as that of Ag.

Titanium (Ti) and its alloys are widely used as major implant materials owing to their excellent mechanical properties and biocompatibility [43]. Recent studies related to the bio-functionalization of a Ti surface has been widely reported and well-summarized elsewhere [44]. Among them, Micro-arc oxidation (MAO) is an electrochemical surface treatment technique performed in a specific electrolyte under high voltage. After MAO, the surface of the substrate metal is covered by a connective-porous oxide layer. The resultant oxide layer formed by MAO treatment contained additional elements that were contained in the electrolyte solution. Therefore, MAO treatment improved the osteogenic cell compatibility of titanium (Ti) when the electrolyte contained calcium and phosphate ions [45–51]. The biocompatibility of MAO coatings has been demonstrated by numerous in vitro and in vivo tests [52–57].

Several studies have focused on the incorporation of Zn onto a Ti surface by MAO treatment [58–64]. Hu et al. [59], Zhang et al. [61], Zhang et al. [62] and Du et al. [63] reported that Zn-incorporated TiO₂ coatings showed good antibacterial activity against both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus*. Moreover, Zhao et al. [60] reported that a Zn coating on a Ti surface showed bacteriostatic activity against *Streptococcus mutans*. These experiments provide adequate evidence to demonstrate that MAO-treated Ti in the electrolyte with Zn inhibited both bacterial adhesion as well as growth. In addition, Hu et al. [59], Zhao et al. [60] and Zhang et al. [64] reported that suitable amounts of Zn could promote adhesion, proliferation, and differentiation of osteogenic cells (e.g., rat bone marrow stem cells, MG63 cells and MC3T3-E1 cells). Thus, Zn plays a key role in the development of both antibacterial activity and osteogenic cell compatibility, and the incorporation of Zn by MAO treatment is a promising approach for surface treatment to achieve antibacterial activity in orthopedic and dental implants. Nevertheless, little attention has been given to the chemical and biological changes of Zn in the MAO coatings on their bio-function. Therefore, for the prevention of late-onset infections and the application of Zn to medical and dental implants, it is important to evaluate the long-term behavior of Zn in the body.

The purpose of this study was to investigate the long-term behavior of Zn-incorporated surface oxide produced by MAO for the prevention of late-onset infections. We investigated the time transient effect of Zn on chemical property, antibacterial activity, and osteogenic cell compatibility. The detailed chemical state of Zn in physiological saline was characterized by X-ray photoelectron spectroscopy (XPS). The change in antibacterial activity of the specimens before and after incubation for four weeks in saline was evaluated by the ISO method using *E. coli* as typical gram-negative facultative anaerobic bacteria. In addition, calcification by the cells on the specimens were evaluated at 14 and 28 days after seeding. In this study, we considered different time points and investigated changes in the chemical and biological properties of the Zn-incorporated MAO surface in a simulated body fluid environment.

2. Materials and Methods

2.1. Specimen Preparation

Commercially pure Ti (CP Ti; Rare Metallic, Tokyo, Japan) with grade 2 was used as a substrate material in this study. Two kinds of CP Ti disks with diameters 8 mm and 25 mm were obtained by cutting from a rod of CP Ti. The surfaces of these disks were mechanically polished using #150, #320, #600, and #800 grid SiC abrasive papers, followed by ultra-sonication in acetone and ethanol for 10 min. The disks were then stored in an auto-dry desiccator till further use. Each Ti disk was then fixed onto a 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 were as described earlier [65]. An AISI304 type 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. Varying concentrations of ZnCl₂ (0, 0.5, 1.0, and 2.5 mM) 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 Inc., Shiga, Japan) and a positive voltage with a constant current density of 251 Am⁻² was applied for 10 min. The resultant voltage during the MAO treatment was 400 V at all conditions. After the MAO treatment, the surfaces were thoroughly washed in ultrapure water in order to remove any electrolyte solution remaining in the porous oxide layer. A major part of the Ti disk was MAO-treated; an annular untreated area was 0.5 mm from the margin. All surface characterization described below was performed in a MAO-treated area.

2.2. Incubation in Saline

The specimens treated in the electrolyte that had various concentrations of Zn were immersed in physiological saline (0.9% NaCl) for 28 days. Saline is the simplest simulated body fluid, which is suitable for long-period measurement. Moreover, chloride ion (Cl⁻) is unexceptionally contained in all simulated body fluids. The specimens were fixed onto a polyethylene container to allow the release of Zn ions from the surface into physiological saline. Incubation was performed at 37 °C in a humidified chamber under constant shaking (100 rpm). Every 7th day, the pooled solution was changed with fresh physiological saline. After immersion, the immersed specimens were used further for surface characterization, evaluation of antibacterial activity, and quantification of Zn ions.

2.3. Surface Characterization and Evaluation of Zinc Ion Release

Scanning electron microscopy with energy dispersive X-ray spectrometry (SEM/EDS; S-3400NX, Hitachi High-Technologies Corp., Tokyo, Japan) was used to observe the surface morphology and composition before and after incubation. X-ray diffraction (XRD, BRUKER D8 DISCOVER, Bruker AXS KK, Yokohama, Japan) was performed to characterize the crystal structure of the specimens before and after incubation. The diffractometer was used with Cu-K α radiation (40 keV, 40 mA). XPS was performed using a spectrometer (JPS-9010MC, JEOL, Tokyo, Japan) with Mg K α X-ray source (energy: 1253.6 eV; acceleration voltage: 10 kV; current: 10 mA). The pressure of the measurement chamber was 1×10^{-7} Pa. All binding energies mentioned in this paper are relative to the Fermi level. The spectrometer was calibrated against Au 4f_{7/2} of pure gold, Ag 3d_{5/2} of pure silver, and Cu 2p_{3/2} of pure copper. Spectra were obtained using an analysis area of 1 mm ϕ with a pass energy of 20 eV. The detection angle to the specimen surface was 90°. The binding energies were calibrated with C 1s photoelectron energy region peak derived from contaminating carbon (285.0 eV). To calculate the integrated intensity of peaks, the background was subtracted from the measured spectrum according to Shirley's method [66]. The chemical state changes of Zn were determined by the modified Auger parameter (α') on the Wagner plot with those of typical Zn compounds. The α' of each specimen was calculated using the following Equation (1).

$$\alpha' = E_K(\text{Zn } L_3M_{45}M_{45}) + E_B(\text{Zn } 2p_{3/2}) \quad (1)$$

Equation (1) contains both kinetic energy of Zn $L_{3M_{45}M_{45}}$, $E_K(\text{Zn } L_{3M_{45}M_{45}})$ and binding energy of Zn $2p_{3/2}$, $E_B(\text{Zn } 2p_{3/2})$. The composition of the specimens were calculated according to a method described previously [67]. The photoionization cross-section of empirical data [68,69] and theoretically calculated data [70] were used for quantification. Inductively coupled plasma-atomic emission spectrometry (ICP-AES, ICPS-7000 ver. 2, Shimadzu Corp., Kyoto, Japan) was used to quantify Zn ion release from the Zn incorporated surface. The amounts of Zn ions released into the pooled solution were quantified each week.

2.4. Evaluation of Antibacterial Activity

The antibacterial activity was evaluated according to ISO 22196: 2007 method. To examine the antibacterial activity of the specimens, we employed *E. coli* (NBRC3972). The experiment was approved by the Pathogenic Organisms Safety Management Committee of the Tokyo Medical and Dental University (22012-025c). *E. coli* is a representative gram-negative rod-shaped bacterium commonly found in the gut. *E. coli* was cultured in Luria-Bertani (LB) broth (LB-Medium, MP Biomedicals, CA, USA) at 37 °C for 24 h. The optical density of the bacterial suspensions were measured at 600 nm using an ultraviolet-visible (UV-vis) spectrometer (V-550, JASCO, Tokyo, Japan) and diluted to obtain concentrations of 4.9×10^6 colony-forming units (CFUs) mL^{-1} . Prior to the antibacterial activity testing, all specimens were sterilized with 70% ethanol, washed with distilled water, and dried. Drops of bacterial suspension were added on all specimens, which were subsequently covered with a sterilized plastic film and incubated at 37 °C for 24 h ($n = 3$). Bacterial cells were then collected from all the incubated specimens; the obtained suspensions were diluted, 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.

2.5. Calcification by Osteogenic Cells

As described in our previous work [71], MC3T3-E1 cells (RIKEN BioResource Center, Tsukuba, Japan) were maintained in a cell culture medium: alpha modification of Eagle's minimum essential medium (α -MEM; GIBCO, Grand island, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO), 10 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, and 0.25 mg mL^{-1} amphotericin B (GIBCO). All specimens were sterilized in 70% ethanol for 20 min and thoroughly rinsed with deionized water before in vitro testing. The cells were seeded on to the sterilized specimens with an approximate initial density of 10,000 cells cm^{-2} . As a control, cells were also seeded on MAO-treated Ti without Zn. The cells were incubated at 37 °C in a fully humidified atmosphere under 5% CO_2 . For induction of osteogenic differentiation, a cell culture medium supplemented with 2 mM β -glycerophosphate (Calbiochem, Darmstadt, Germany) and 50 mg mL^{-1} L-ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan) was used when 100% confluence was reached for all the specimens. The medium used for induction was named as the differentiation-inducing medium. The differentiation-inducing medium was changed every three days.

After the seeding for the 1st, 2nd, and 3rd day, the cells attached to each specimen were harvested with trypsin/EDTA, re-suspended in a cell culture medium, and transferred to 96-well microplates. Then, the Cell Counting Kit-8 assay (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to each 96-well microplate, which contained cell suspension, 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, FL, USA). The reference wavelength was set at 630 nm. In this evaluation, the tissue culture polystyrene (TCPS) was used as a control specimen for quantification of the number of attached cells on each specimen. The cells that were attached to the TCPS were harvested by treatment with Trypsin/EDTA followed by re-suspension of cells in the cell culture medium. Cell suspensions were diluted serially. The number of cells in the cell suspension harvested from TCPS were counted by Trypan blue (Trypan Blue Stain 0.4%; Gibco) using

a hemocytometer. Then, standard curves for cell number calibration and for light absorbance were constructed and were used for quantification of the number of attached cells on each specimen.

The calcification of MC3T3-E1 cells on each specimen was evaluated by the calcified deposits by a color change reaction after alizarin red S staining. After the removal of media from all the specimens, cells were rinsed three times with phosphate-buffered saline. Cells were then fixed with 4% formalin for 1 h and rinsed three times with ultra-pure water. Each specimen was stained with 1% alizarin red S solution (adjusted to pH 4.2 with ammonium hydroxide) at room temperature for 30 min. After removing the alizarin red S solution, cells were repeatedly rinsed with ultrapure water. When the specimens were fully dry, the surface of each specimen was studied using an optical microscope (OLYMPUS SZX12, Olympus, Tokyo, Japan).

2.6. Statistical Analysis

Data obtained by surface characterization were calculated from three independent specimens. The results of biological tests are shown from at least two independent tests. Each test was performed using at least three independent specimens. All values are presented as means $\pm$ SD, and commercial statistical software KaleidaGraph (Version 4.1.1, Synergy Software, Reading, PA) was used for statistical analysis. One-way analysis of variance (ANOVA) was used following multiple comparisons with Student–Newman–Keuls method to assess the data, and $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Surface Characterization and Evaluation of Zinc Ion Release

Figure 1 shows SEM images of the MAO-treated Ti surface treated in the electrolyte with 2.5 mM Zn before (A) and after (B) incubation in saline. Typical connective porous morphology after MAO treatment was observed. There was no difference in the surface morphology among the specimens before and after incubation in saline for 28 days according to our SEM data under the indicated magnification. In addition, the pore size on the specimens before incubation was $5.3 \pm 2.3 \mu\text{m}$ and on the specimens after incubation during 28 days was $4.8 \pm 2.1 \mu\text{m}$. There were no significant differences in the pore size among the specimens before and after incubation.

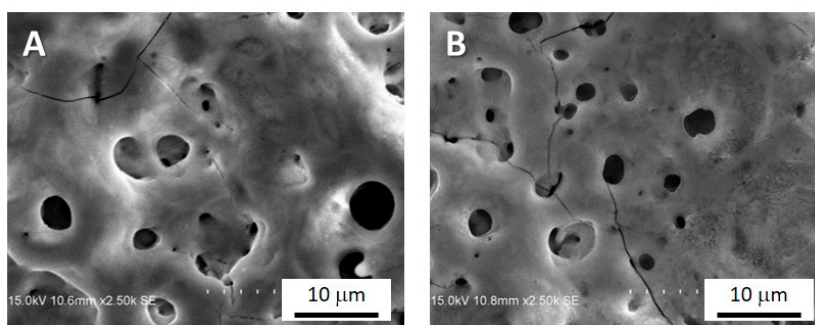


Figure 1. Scanning electron microscopy (SEM) images of specimens before (A) and after (B) incubation in saline during 28 days. These images show the connective porous oxide layer on a Titanium (Ti) surface formed by micro-arc oxidation (MAO) treatment using Zinc-containing electrolyte.

XRD spectra obtained from the MAO-treated Ti surface treated in the electrolyte with 2.5-mM Zn before and after incubation has been shown in Figure 2. The peaks originating from α -Ti and anatase TiO_2 were detected from each specimen. Moreover, the peaks originating from Zn were undetected. There was no difference in the crystal structure among the specimens before and after incubation in saline for 28 days.

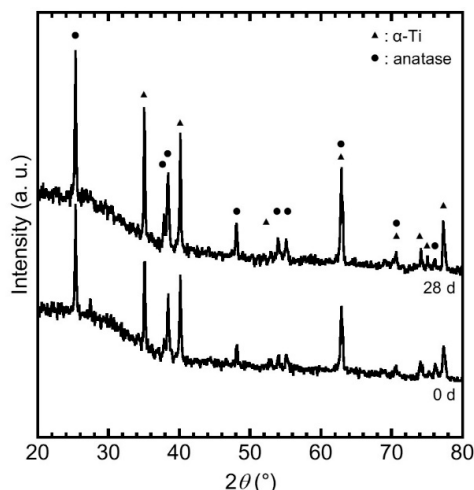


Figure 2. X-ray diffraction (XRD) spectra of the Zn-incorporated specimens before and after incubation in saline during 28 days.

XPS survey spectra of the MAO-treated Ti surface in the electrolyte with 2.5 mM Zn before and after incubation in saline has been shown in Figure 3A. In addition to peaks originating from C, O, P, Ca, and Ti, those originating from Zn and Na were detected. Narrow scan spectra around Zn 2p electron energy region is shown in Figure 3B. The binding energies of Zn 2p_{3/2} peaks obtained from all specimens were 1022.2–1022.6 eV, indicating that Zn existed as Zn²⁺. Phosphorus was found to exist as phosphate species and calcium existed as a divalent ion, because binding energies of the corresponding peaks of P 2p and Ca 2p_{3/2} were 134.1 ± 0.2 eV and 347.8 ± 0.2 eV, respectively. The binding energy of Ti 2p_{3/2} peak was 459.0 ± 0.1 eV, indicating that Ti existed as TiO₂.

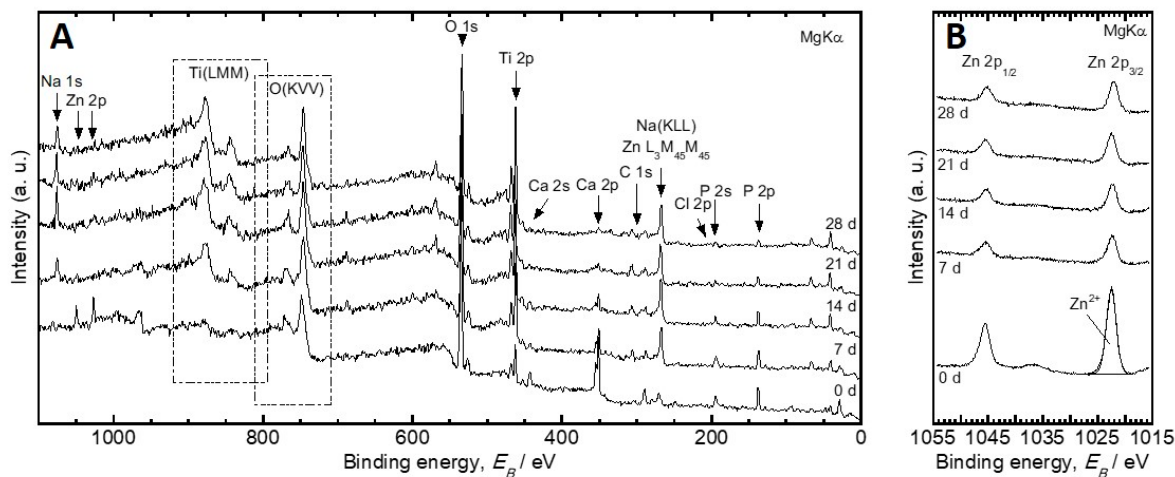


Figure 3. X-ray photoelectron spectroscopy (XPS) spectra of survey (A) and Zn 2p photoelectron energy region (B) from specimens immersed in saline for 0–28 days.

Figure 4 depicts the Wagner plot of Zn in the oxide layer incubated for 0–28 days based on Zn 2p_{3/2} peaks and Zn L₃M₄₅M₄₅ Auger peaks and reference plots from Zn²⁺ compounds as reported by previous studies [72–75]. The chemical state of an element was determined by comparison of its binding energy with modified Auger parameter values on the Wagner plot. According to the Wagner plot of Zn, the binding energy decreased, and the Auger parameter increased with incubation time. Moreover, since the value of the Auger parameter finally converged to 2010.2 eV, it became clear that the chemical state of Zn incorporated in titanium dioxide by MAO approached to that of ZnO with increasing incubation times.

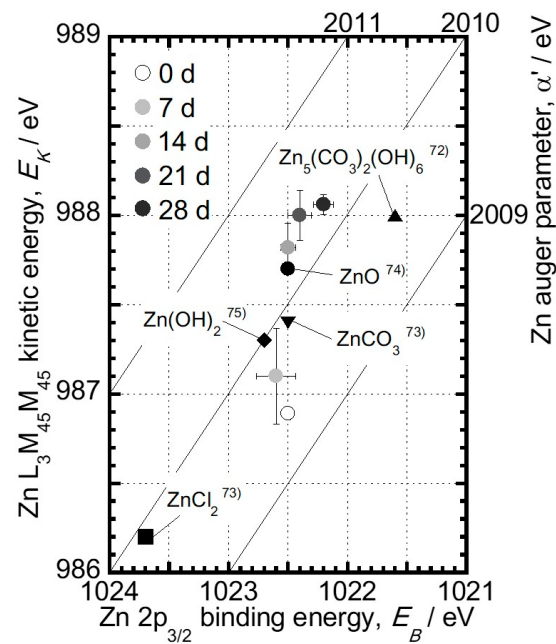


Figure 4. Wagner plot of Zn in the oxide layer incubated for 0–28 days based on Zn 2p_{3/2} photoelectron peaks and Zn L₃M₄₅M₄₅ Auger peaks ($n = 3$). Each parameter of Zn²⁺ compounds were plotted according to previous studies [72–75].

Figure 5 shows the change in the Zn concentration in the oxide layer at each incubation time, as determined by XPS. The concentration of Zn was relatively small even before the incubation; thus, the amount of Zn incorporated during MAO treatment was small. The amount of Zn dramatically decreased to about 0.8% by the incubation in saline from 0 to 7 days and was maintained for 28 days.

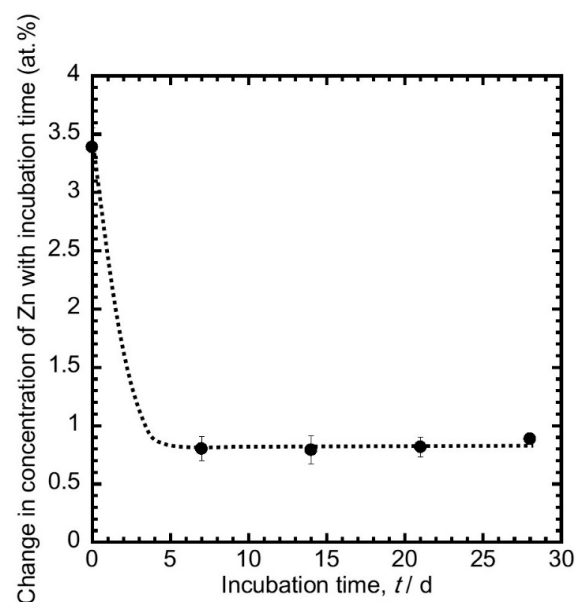


Figure 5. Change in the concentration of Zn in the oxide layer with incubation time determined by XPS ($n = 3$).

Figure 6 shows the amount of Zn ions released from the oxide layer into saline, as determined by ICP-AES. The amount of Zn ions released within the first 7 days was the highest and thereafter Zn was not detected. In summary, there was no observed release of Zn ions after 7 days.

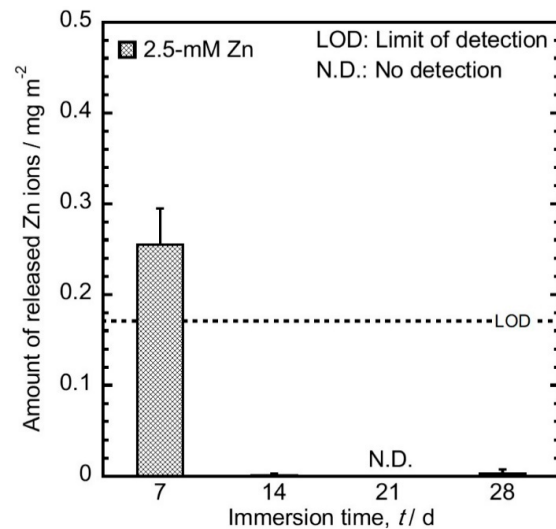


Figure 6. The amount of Zn ions released into saline every week from Ti MAO-treated in the electrolyte containing 2.5-mM Zn ($n = 3$).

3.2. Evaluation of Antibacterial Activity

Figure 7 shows the normalized CFU count for *E. coli* or antibacterial effects of MAO-treated Ti in the electrolyte containing various concentrations of Zn. The vertical axis represents the normalized bacterial number, defined as the bacterial number on each of the specimens divided by that of the control, MAO-treated Ti in the electrolyte without Zn. The normalized bacterial number that was below 1 (shown as dashed line in the figure) implied that the tested specimens had antibacterial activity. Before incubation, there was no significant difference in *E. coli* counts among the specimens. On the other hand, after incubation for 28 days, antibacterial effects against *E. coli* were observed. This effect was independent of the concentration of Zn in the electrolyte for MAO treatment, indicating that antibacterial activity of Zn developed after incubation in saline.

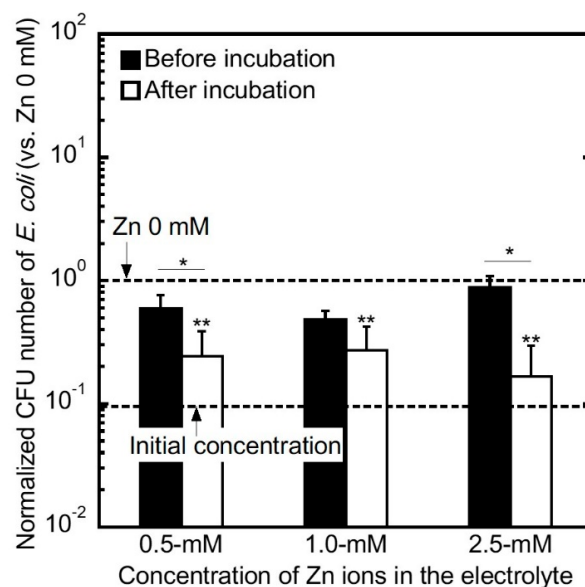


Figure 7. Antibacterial effects of Ti MAO-treated in the electrolyte containing various concentrations of Zn. Data are shown as the mean $\pm$ SD. *: Significant difference between specimens before and after incubation, **: Significant difference against the specimen without Zn ($n \geq 3$, $P < 0.05$).

3.3. Calcification by Osteogenic Cells

Figure 8 shows the number of MC3T3-E1 cells on MAO-treated Ti in the electrolyte containing various concentrations of Zn with varying culture times. The number of viable cells on all specimens increased with culture time. The Zn concentrations in the electrolyte did not differ significantly among specimens.

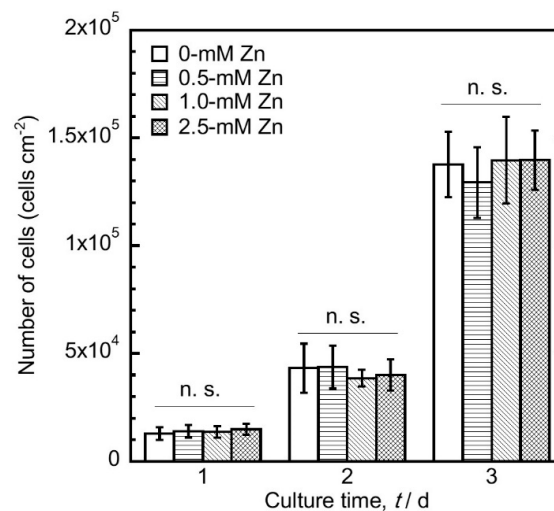


Figure 8. Number of MC3T3-E1 cells on Ti MAO-treated in the electrolyte containing various concentrations of Zn with culture time. Data are shown as the mean $\pm$ SD. n.s.: No significant difference ($n \geq 5$, $P < 0.05$).

Figure 9 shows photographs of alizarin red S staining of calcified deposits by MC3T3-E1 cells on each specimen cultured for 14 and 28 days. In case of MAO-treated specimens, we observed that only the central part (7 mm in diameter) was covered with the oxide layer formed by MAO treatment while the margins (0.5 mm) were not treated. Calcified cells were observed in all the specimens. However, untreated Ti showed bare metal-colored parts that indicated the presence of incompletely calcified cells. On the other hand, all MAO-treated specimens showed completely stained surfaces. There was no segregation seen for calcified and non-calcified areas on the MAO-treated surface. In addition, there were no visual differences among the MAO-treated specimens with and without Zn.

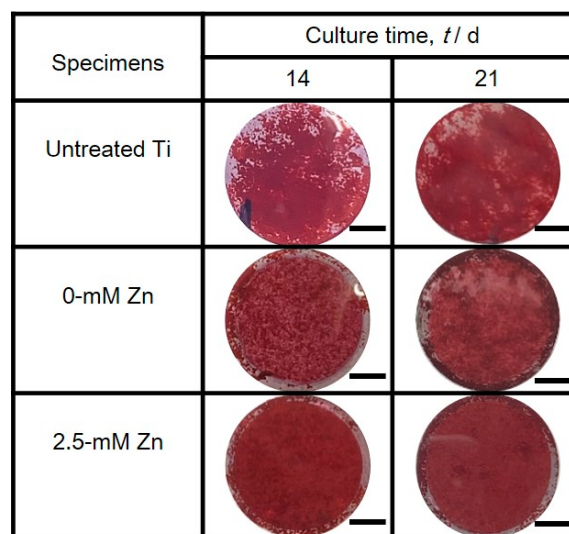


Figure 9. Color scale stained by alizarin red S of calcified deposits by MC3T3-E1 cells on each specimen cultured for 14 and 28 days. Scale bar represents 2 mm.

4. Discussion

The morphology and crystal structure of the porous oxide layer formed by MAO treatment did not show any changes during incubation in saline (Figures 1 and 2); this property is highly advantageous for implant surfaces.

The peaks originating from Zn were not detected from the specimens by XRD. The amount of Zn incorporated during MAO treatment was 3.5%, and decreased with incubation (Figure 5). Therefore, we believe that XRD peaks originating from Zn were relatively small compared with Ti, which was not detected by XRD.

The chemical state and concentration of Zn in the oxide layer changed during incubation in saline (Figures 3–5). In addition, the Zn ions were released into saline during the 7-day incubation period; thereafter, no Zn ions were eluted (Figure 6). These phenomena indicate that Zn incorporated into specimens would be released only during the initial stages of implantation in vivo. The concentration of Zn in the oxide layer decreased with the release of Zn ions during the first 7 days and was maintained at 0.8 at % in the subsequent period. In other words, despite the presence of Zn in the oxide layer, no Zn ions were released from the surface from 7 to 28 days. This indicated that the released Zn ion was adsorbed on the surface of the oxide layer to form zinc oxide as an insoluble corrosion product of Zn. The insoluble corrosion product of Zn was converted to ZnO to achieve chemical stabilization of Zn in saline. In other words, Zn incorporated into the oxide layer by MAO treatment changed the chemical state by interaction with saline and was finally stabilized by conversion into ZnO. Moreover, ZnO was already generated on the surface of oxide layer in the 14-day incubation (Figure 4). Therefore, it was considered that Zn ions did not elute during the 14–28 days period due to both the conversion into ZnO and stabilization in saline.

The specimens incubated in saline exhibited antibacterial activity against *E. coli*, while the specimens before incubation did not (Figure 7). Ti incorporated with Zn in the surface oxide layer formed by MAO treatment exhibited late-onset antibacterial activity against *E. coli*. The release of Zn ions were observed only during the initial stages of the incubation process (0–7 days). This indicated that Zn ions itself did not have any antibacterial activity. Du et al. [76] reported that the minimum inhibitory concentration (MIC) for Zn ions against *E. coli* was 768 $\mu\text{g}\cdot\text{mL}^{-1}$. The maximum concentration of Zn ions released from the specimens in this study was smaller than the previously reported MIC of Zn ions. For this reason, development of antibacterial activity by other factors needs to be considered. Prasanna et al. [77] reported that ZnO could produce reactive oxygen species (ROS) in the dark, owing to singly ionized oxygen vacancy in the crystal lattice of ZnO, and that generation of ROS contributed to its antibacterial properties. This study clearly revealed that Zn was finally converted to ZnO and was stabilized by interactions in saline. Therefore, the development of the late-onset antibacterial activity of Zn was due to the generation of ZnO in saline. In other words, this study proved that the chemical state of Zn incorporated in the oxide layer played a key role in the development of its antibacterial activity. Therefore, we believed that antibacterial activity was developed when ZnO was generated. According to Figure 4, we considered that the specimens incubated in saline during 14–21 days could exhibit the antibacterial activity, while the specimens incubated in saline during 0–7 days may not affect to bacteria. On the other hand, it is believed that bacteria interact with titanium dioxide through surface siderophores, and the oxides provide a template for biofilm formation [78,79]. For this reason, it is expected that bacteria can be captured by the interaction between the porous titanium dioxide and the siderophore on the cell surface, and killed by ZnO. We believe that Zn-incorporation for a Ti surface by MAO treatment may be suitable for realizing both bacterial capture ability and antibacterial activity, namely a ‘trap-killing’ system [80].

The MAO-treated specimens containing Zn showed no toxic effects on proliferation of osteogenic cells (Figure 8). Moreover, the calcification of cells on MAO-treated specimens with Zn were at the same level as that on the specimen without Zn. Therefore, the existence of a slight amount of Zn did not affect the osteogenic property of cells and proliferation was normal. The porous oxide layer consisted not only of titanium oxide but also incorporated Ca, P, and Zn (Figure 3). Relatively large

amounts of Ca and P were contained in the oxide layer of MAO-treated Ti-based alloy at 7.6 and 10.0 at %, respectively [81]. Ca and P are the main mineral components in bone tissues and are present 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 [47]. In our previous study, MAO-treated specimens containing a small amount of Ag showed osteogenic cell compatibility, which accelerated the calcification process by MC3T3-E1 cells without any cytotoxicity [22]. Therefore, we predicted that Zn-containing specimens in this study might have the same calcification ability.

In addition, a specific antibacterial activity of Zn, which was incorporated in the oxide layer was observed. Zn-incorporated oxide layer formed by MAO exhibited late-onset antibacterial activity and osteogenic cell compatibility, namely dual function. The amount of Zn ions released during 7 days incubation (0.02 ppm) was much less than IC_{50} for MC3T3-E1 cells reported by Yamamoto et al. [82]. Moreover, our results indicated that the small amount of ZnO generation did not affect osteogenic cells. In other words, the time transient effect of a suitable amount of Zn was effective for only bacteria. Therefore, MAO treatment of Ti using Zn made it possible to exhibit both late-onset antibacterial activity and osteogenic cell compatibility on Zn-containing TiO_2 layer. In addition, our study proposed the importance of the chemical state of Zn in the oxide layer for the development of antibacterial properties of Zn. Our results have suggested that due to the time transient effect of Zn in TiO_2 , its chemical and biological properties could be easily changed and this property could be adapted for achieving changes in simulated body fluids (Figure 10). In particular, we suggest the importance of the chemical state changes of Zn in the various environments to control its chemical and biological properties. We hope that in future, that the outcome of the present study would be useful in the design of bio-functional implants related to Zn and contribute to the development of antibacterial biomaterials.

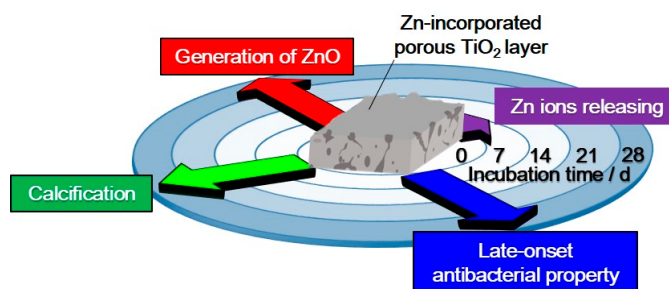


Figure 10. The schematic illustration of time transient effect of Zn in the porous titanium dioxide layer on chemical and biological properties.

5. Conclusions

This study investigated the time transient effect of Zn in titanium dioxide with incubation in saline. Although the morphology of the porous oxide layer formed by MAO treatment did not change, the chemical state of Zn in the oxide layer changed to ZnO during incubation in saline. Moreover, the antibacterial activity of the specimen with Zn developed in the late stages after the incubation process in saline for 28 days. We propose that the generation of ZnO in the oxide layer played a key role in development of antibacterial activity. In addition, the Zn-incorporated specimen did not exhibit any cytotoxicity in MC3T3-E1 cells and did not hinder the proliferation and calcification of cells. Therefore, MAO treatment of Ti using Zn is suitable for realizing both late-onset antibacterial activity and osteogenic cell compatibility.

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