Enhancement of antibacterial property of titanium by two-step micro arc oxidation treatment

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A customized micro arc oxidation (MAO) treatment technique was developed to obtain desirable antibacterial properties on titanium surfaces. The two-step MAO treatment was applied to fabricate a specimen containing both Ag and Zn in its surface oxide layer. Surface analyses and metal-ion release tests were performed to evaluate the presence of Ag and Zn and the ion release behavior for simulating practical usage, respectively. Additionally, the antibacterial properties of the specimens were also evaluated using gram-negative facultative anaerobic bacteria. The MAO-treated specimens containing both Ag and Zn showed excellent antibacterial properties against *Escherichia coli*, and the properties were sustained even after 28 days of immersion in physiological saline to simulate the living environment.

Keywords: Micro arc oxidation, Antibacterial property, Silver, Zinc, *Escherichia coli*

INTRODUCTION

Titanium (Ti) and its alloys are widely used in dental and medical devices owing to their excellent mechanical properties and biocompatibility. These implant devices have been associated with accelerated and strong adhesion between the implant surface and the surrounding bone to achieve a shorter healing period and immediate loading¹⁻⁴. However, in recent years, biofilm formation due to bacterial adhesion, and the subsequent colonization on biomaterials, have been recognized as major causes of failure in dental and orthopedic implant surgeries⁵⁻⁹. Once a biofilm is formed and firmly adhered to the implanted material, bacterial secretion plays a pestiferous role as a barrier against the defense mechanism of the host. Thus, pathogens are difficult to eliminate upon formation of a biofilm on a device implanted in a living body. In serious cases, there is no way to remove the contaminated devices from the patient and to prevent subsequent undesirable biological reactions such as infections. The easiest strategy to prevent the formation of biofilms on metallic devices is polishing because a roughened surface is known to enhance bacterial adhesion. It has been reported that the increase in the surface area and the formation of pockets enhanced the presence of bacteria^{10,11}. On the other hand, for dental implants and orthodontic fixators used in contact with bone, a roughened surface is always preferred to ensure hard-tissue compatibility. Therefore, another appropriate way to prevent biofilm formation is the application of antibacterial agents.

Silver (Ag) ions are known as one of the most effective

agents because they exhibit superior antibacterial properties¹²⁻¹⁵. Surface modification enables the formation of a biofunctional layer supporting Ag because a source of Ag ions can overcome the problems caused by biofilm formation on metallic biomaterials. In addition to the efficacy of Ag against bacteria, Zn has also been attracting attention as an antibacterial element owing to its antibacterial activity¹⁶⁻²².

Micro arc oxidation (MAO) is a conventional wet-process surface treatment, based on electrochemical reactions in a specific electrolyte under high voltage. The MAO treatment has already been utilized as a surface treatment in dental implants to enable immediate loading after surgeries²³⁻²⁶. MAO can incorporate calcium (Ca) and phosphorus (P) into the resulting oxide layer^{27,28}. For the treatment, the mentioned elements were originally present in the electrolyte in their ionic states. In other words, incorporation of the selected element into the oxide layer formed by MAO treatment is possible only when the element is dissolved in the electrolyte. This technique can be utilized for incorporating the desired elements into the resulting oxide layer.

Therefore, our study focused on incorporating antibacterial elements into a Ti surface to develop novel antibacterial implants without any harmful effects on osteogenic cells by MAO treatment. We have reported that Ag, Cu, and Zn were effective in inhibiting the proliferation of *Escherichia coli* (*E. coli*) and/or *Staphylococcus aureus* (*S. aureus*)²⁹⁻³². In addition, the inhibition mechanisms and their efficacy were found to differ: Ag showed a strong antibacterial effect, especially during the initial period, Cu showed specific effectiveness

Table 1 Features of antibacterial properties incorporated into Ti oxide layers by MAO treatment against *E. coli* and *S. aureus*

Element	Antibacterial effect		Remarks	Ref.
	Fresh: As-prepared	Aged: After immersion in physiological saline for 28 days		
Ag	Strong	Moderate	Release killing by Ag ions	29)
Cu	Moderate	Moderate	Contact killing by Cu/Cu oxides Release killing by Cu ions (Induced by bacterial attachment)	30,31)
Zn	Weak	Moderate	Contact killing by Zn oxides (Matured from initial Zn products)	32)

on *S. aureus*, and Zn showed a heightened antibacterial effect over time. The features of antibacterial elements are summarized in Table 1. From the viewpoint of solving the late-onset infection problem^{33–35} that occurs around three to eight weeks after surgery, long-term antibacterial activity of the implant material is strongly desired. An ideal biomaterial surface with antibacterial activity can prevent the initial stages of infection such as bacterial adhesion and inhibit bacterial growth at later stages.

Therefore, the purpose of this study was to fabricate a novel implant surface that realizes long-term antibacterial effects for the prevention of late-onset infections. The development of a customized MAO treatment technique that enables the incorporation of multiple antibacterial elements into the resulting oxide layer on Ti was studied. In the primary phase of this study, a two-step MAO treatment was investigated for the fabrication of a specimen containing both Ag and Zn in its surface oxide layer. Surface analyses and metal-ion release tests were performed to investigate the presence of Ag and Zn in the oxide layer. The antibacterial properties of the specimens were also evaluated using gram-negative facultative anaerobic bacteria.

MATERIALS AND METHODS

Specimen preparation

Two types of specimen disks with diameters of 8 and 25 mm were fabricated by mechanically cutting rods of commercially pure grade 2 Ti. The 8 mm Ti disks were used for the surface characterization and the 25 mm Ti disks were used for the metal-ion release and the antibacterial property evaluations.

The surfaces of the disks were mechanically ground using #150, #320, #600, and #800 grid SiC abrasive papers. This was followed by ultrasonication using acetone and isopropanol. The disks were then kept in an auto-dry desiccator until further use. The Ti disk was 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 are the same as described in a previous study³⁶. A type 304 stainless

steel plate was used as the counter electrode. The base composition of the electrolyte for the MAO treatment was 100 mM calcium glycerophosphate and 150 mM calcium acetate.

In this study, either 0–10 mM silver nitrate (AgNO₃) or 2 mM zinc chloride (ZnCl₂) was 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. Thus, a major part of the Ti disk was MAO-treated, with an annular untreated area of 0.5 mm from the edge.

The two-step MAO treatment was performed by following manner. In the first step, the MAO treatment was started in the first electrolyte until a voltage of 340/360/380 V was obtained. Then, the current application was stopped and the electrolyte was replaced immediately with the second electrolyte. Current was applied once again with an upper limit of 400 V for a total treatment time of 10 min. The definition of specimen's name was "First electrolyte"/"Electrolyte replacing voltage"+"Second electrolyte"/"Final voltage" (For example, "2Zn/380V+10Ag/400V"). Surface characterization and metal-ion release/microbial measurements were performed on the MAO-treated area and the whole specimen area, respectively.

Surface characterization and metal-ion release evaluation

A scanning electron microscope (SEM) with an energy dispersive X-ray spectrometer (EDS; S-3400NX, Hitachi High-Technologies, Tokyo, Japan) was used to observe the surface morphology and perform elemental analysis of each specimen. An inductively coupled plasma atomic emission spectrometer (ICP-AES; ICPS-7000 ver. 2, Shimadzu, Kyoto, Japan) was used to investigate the amounts of released Ag and Zn ions. The MAO-treated specimen in the electrolyte with/without Ag and Zn under various combination treatment conditions was incubated in 5 mL of physiological saline (0.9% NaCl). They were sealed in a polyethylene container to allow the release of Ag and Zn ions from the surface of the specimen. These were maintained in a thermostatic chamber at 37°C under moderate shaking (80–100 rpm).

Every seventh day, the pooled solution was transferred into fresh physiological saline. The concentrations of Ag and Zn ions in the tested solutions collected at the first (0–7 days), second (8–14 days), third (15–21 days), and fourth (22–28 days) periods were measured by ICP-AES. After the evaluation of metal-ion release in the specimens that had been immersed in physiological saline for 28 days, the specimens were used as “aged specimen” to compare the antibacterial properties with those of the as-prepared fresh specimens.

Evaluation of antibacterial properties

The antibacterial property tests were conducted in accordance with domestic and international standard methods (JIS Z2801 and ISO 22196:2007). The proliferation of anaerobic gram-negative bacteria (*E. coli*, NBRC3972, NITE, Tokyo, Japan) on the specimens was evaluated. The suspension medium was prepared by five-hundred-fold dilution of the nutrient broth containing 3 gL⁻¹ meat extract, 10 gL⁻¹ peptone, and 5 gL⁻¹ sodium chloride. The pH of the suspension medium was adjusted using sodium hydroxide or hydrochloric acid to be between 6.8 and 7.2. The bacteria were added to the suspension medium to obtain 3.1×10⁶ colony forming units (CFU) mL⁻¹. The bacterial suspension (0.1 mL) was dropped onto a specimen and a cover film was placed immediately. The specimens and the cover films were incubated at 35°C for 24 h. Thereafter, they were washed using 9.9 mL of sterile physiological saline. The CFU of the living bacteria dispersed into the saline was determined using the culture medium sheet for *E. coli* (JNC, Tokyo, Japan).

RESULTS AND DISCUSSION

Development of a two-step MAO treatment

To consider the multiple layers containing both Ag and Zn on Ti, the problem of the upper limit of AgNO₃ concentration in the electrolyte for MAO treatment should first be solved because it limits the amount of incorporated Ag in the resulting oxide layer. In our previous study²⁹⁾, a porous oxide layer could not be formed using the electrolyte with 10 mM AgNO₃ because the applied voltage during the treatment was inadequate. Figure 1 shows the change in voltage during the MAO treatment with various AgNO₃ concentrations. When the MAO treatments were performed using electrolytes containing low to zero concentration of 2.5 mM AgNO₃, the voltage continued to increase and finally reached a maximum value of 400 V, which was set for this study. On the other hand, the voltage suddenly dropped during treatment in the case in which electrolytes with higher AgNO₃ concentrations were used. In such cases, no porous oxide layer was formed on the Ti surface. It was considered that the electrical resistance of the resultant oxide layer reduced when the Ag concentration was too high²⁹⁾. Therefore, the growth of the oxide layer, which is a typical phenomenon in valve metals, was inhibited. It was found that the upper limit of AgNO₃ concentration in the electrolyte was less than 5 mM for the original

Fig. 1 Change in voltage during MAO treatment with various AgNO₃ concentrations.

Fig. 2 Change in voltage during the two-step MAO treatment under the aforementioned conditions (0/380V+10Ag/400V).

single-step MAO treatment condition.

Therefore, the two-step MAO treatment was considered to solve the aforementioned upper limit problem of the antibacterial source in the electrolyte. In the first step, the MAO treatment was started in the base composition electrolyte without AgNO₃ until a voltage of 380 V (0/380V) was obtained. The current application was stopped, and the electrolyte was replaced immediately with the new electrolyte containing 10 mA of AgNO₃. Current was applied once again with an upper limit of 400 V (10Ag/400V) for a total treatment time of 10 min. Figure 2 shows the change in the voltage during the two-step MAO treatment under the above conditions (0/380V+10Ag/400V). The two-step MAO treatment finished without any voltage drop problem even though a high concentration of Ag ions was contained

in the electrolyte for the second step. It was confirmed that when the oxide layer with high-enough electric resistance was properly formed in the first step, larger concentration of Ag ions in the second electrolyte and the newly-formed oxide layer did not affect the breakdown process during the treatment. In other words, the MAO treatment can be used with the condition of using higher Ag-ion concentration if the base oxide layer was formed in advance.

EDS measurements were performed to evaluate the amounts of Ag incorporated into the resulting oxide layers by single- and two-step MAO treatments under various conditions. The chemical compositions of the oxide layers were almost same irrelevant to the treatment conditions: Ti: 12 at%, O: 67 at%, C: 3 at%, Ca: 9 at%, and P: 9 at%. However, slight amount of both Ag and Zn were detected from the specimens treated with the electrolytes containing these antibacterial elements. Figure 3 shows the relationship between the AgNO_3 concentrations in the treatment electrolyte for the second step of treatment and the amount of Ag incorporated into the resulting oxide layers. The electrolyte replacement was performed at 340, 360, and 380 V. A higher electrolyte replacing voltage resulted in a shorter treatment time because the total treatment time was limited to 10 min. From the results shown in Fig. 3, the amount of Ag incorporated in the oxide layer effectively increased with an increase in the concentration of AgNO_3 in the second-step electrolyte. On the other hand, the replacing voltage had a relatively negligible effect on Ag incorporation. The amount of Ag incorporated into the oxide layer on the specimen 0/380V+10Ag/400V was much higher than that of 2.5Ag/400V, which is the best condition for single-step MAO treatment. Ag incorporation might have occurred mainly at the later stage of treatment with higher applied voltage. Thus, it was confirmed that the two-

Fig. 3 Relationship between the AgNO_3 concentrations in the treatment electrolyte for step two of the treatment and the amount of incorporated Ag into the resulting oxide layers.

step MAO treatment is suitable to prepare specimens with higher concentration of Ag.

From the experimental results of the fundamental two-step MAO treatment, we attempted to incorporate both Zn and Ag into the oxide layer on Ti. The electrolyte containing 2.0 mM ZnCl_2 with basic components was used in the first step until 380 V; then, it was replaced with an electrolyte containing 10 mM AgNO_3 (2Zn/380V+10Ag/400V). The change in voltage during the treatment is shown in Fig. 4. The two-step MAO treatment using both Zn and Ag species was successful without any voltage drop problem. The amounts of Ag and Zn in the oxide layer were measured by EDS, and the experimental results are shown in Fig. 5. The Figure also shows experimental results of the reference specimens that were prepared by single-step MAO (2Zn/400V and 2.5Ag/400V) and two-step MAO with alternative antibacterial elements (2Zn/380V+0/400V and 0/380V+10Ag/400V). Both Ag and Zn were detected

Fig. 4 Change in voltage during the two-step MAO treatment under the aforementioned conditions (2Zn/380V+10Ag/400V).

Fig. 5 The amounts of Ag and Zn in the oxide layer measured by EDS analysis.

in 2Zn/380V+10Ag/400V; therefore, it was confirmed that a novel oxide layer containing multiple antibacterial agents was realized by the proposed two-step MAO treatment. The amount of incorporated Zn decreased when the electrolyte was replaced with a zinc-free electrolyte at 380 V (2Zn/400V *vs.* 2Zn/380V+0/400V). The amount of incorporated Zn showed the same trend as that in the results of Ag discussed above. Furthermore, the amount of incorporated Zn also decreased when the electrolyte for the second step contained 10 mM AgNO₃ (2Zn/380V+0/400V *vs.* 2Zn/380V+10Ag/400V). In the same manner, the amount of incorporated Ag slightly decreased when the electrolyte for the first step contained 2 mM ZnCl₂ (0/380V+10Ag/400V *vs.* 2Zn/380V+10Ag/400V). These results may be attributed to the conflicting effects of Ag and Zn. However, in our previous study, the antibacterial effect of the specimen prepared by single-step MAO with 0.5 mM ZnCl₂ (1/4 concentration of 2Zn/400V in this study) was enhanced as well as that of the specimen with 2.5 mM ZnCl₂ (2.5Zn/400V) after aging through a 28 days incubation in physiological saline³². Therefore, the antibacterial effect of Zn in the 2Zn/380V+10Ag/400V specimen is also expected to be adequate.

Figure 6 shows the surface morphology of the MAO-treated specimen. There were many interconnected pores on the treated specimens. The number of pores and the pore size showed almost no apparent difference among the specimens under various treatment conditions. Therefore, it could be concluded that the effect of both the two-step method and the presence of Ag and/or Zn on the oxide layer morphology is insignificant.

Figure 7 shows the amount of Ag ions released from the oxide layer into the physiological saline, as determined by ICP-AES. The highest amount of Ag ions was released within the first 7 days, and thereafter it gradually diminished. The amount of Ag ions released

from the 2Zn/380V+10Ag/400V specimen was smaller than that from the 0/380V+10Ag/400V specimen, in accordance with the Ag content (Fig. 5). Nevertheless, the release of Ag ions continued for at least 28 days. The volume of Zn ions released from the oxide layer in all tested specimens was below the ICP-AES detection limit. This may be due to the strong Zn ions adsorption effect followed by the simultaneous formation of zinc complexes on its surface³⁷. This also corresponds with the results from our previous study, which reported that Zn ions detected by ICP-AES from Zn-incorporated MAO specimens were vanishingly small, even though they showed significant antibacterial effects against *E. coli*³².

Therefore, the antibacterial properties of MAO-treated specimens containing both Ag and Zn were evaluated using *E. coli* (Fig. 8). *E. coli* could survive on the surface of the reference specimens untreated Ti

Fig. 7 The amount of Ag ions released from the oxide layer into physiological saline.

Fig. 6 Surface morphology of the MAO-treated specimen by SEM.

Fig. 8 Antibacterial property of MAO-treated specimen containing both Ag and Zn.

and MAO-treated Ti without antibacterial elements. In contrast, there was no bacterial colony (<1 CFU mL⁻¹) on the tested nutrient agar plates after incubation for 24 h. Thus, it was found that *E. coli* was completely killed within 24 h on the specimen containing both Zn and Ag. This strong antibacterial property against freshly prepared specimens of *E. coli* might be mainly derived from Ag ions released from the porous oxide layer, as shown in Fig. 7. However, the amount of Ag-ion released continued to decrease during the period of immersion in physiological saline, and it dropped by about one-third after 28 days. It was expected that the antibacterial property of the MAO-treated specimen would weaken because of the diminishing Ag-ion release in accordance with the immersion period, simulating practical usage. However, the experimental results using aged 2Zn/380V+10Ag/400V specimen (after 28 days immersion in physiological saline at 37°C) also results in no bacteria. It could be considered that the formation and the maturation of Zn products³²⁾ on the specimen surface occurred gradually during the immersion period. Therefore, this prolonged antibacterial efficacy could be attributed to the presence of Zn. It was confirmed that the excellent antibacterial property of Ti could be maintained even after a 28 days-aging period by the proposed two-step MAO treatment.

Some limitations exist in this study. First, the efficacy of Zn after an incubation period of 28 days was not fully distinguished from that of Ag. We plan to optimize the treatment conditions, such as using an increased aging period to discern the effort of Zn, and adjusting the amount of incorporated Ag and Zn because excess Ag-ion release can cause a cytotoxic effect³⁸⁻⁴³⁾. Realizing both antibacterial and hard-tissue compatibility is the ideal goal of this study²⁹⁾. The second limitation is the effectiveness of various bacterial species. *E. coli* is one of the standard testing bacteria; however, the effect of Ag and Zn on other bacteria is still unknown, even though Ag shows a broad antibacterial spectrum^{13-15,44-46)}. Gram-positive facultative anaerobic bacteria (such as *S. aureus*) and primal bacteria that play important roles in biofilm formation in practical environments should be investigated in more detail.

CONCLUSION

Both Ag and Zn were successfully incorporated on the Ti surface using the proposed two-step MAO treatment. Moreover, a higher concentration of Ag could be incorporated by the two-step MAO than that by the traditional single-step MAO. *E. coli* was effectively killed on the MAO-treated specimen containing both Ag and Zn. Excellent antibacterial property was sustained even after 28 days of immersion in physiological saline simulating the living environment. Optimization of the treatment conditions for incorporation of Ag and Zn can realize desirable bactericidal properties for a long time after implant surgery.

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